Exploring the role of GATA2 in normal and malignant haematopoiesis

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Summary

Haematopoietic stem cells (HSCs) are multipotent stem cells that sustain long-life haematopoiesis. External signals delivered from the bone marrow (BM) niche are crucial for HSC homeostasis, alongside a cell intrinsic transcriptional program driven by transcription factors (TFs). Translocations, epigenetic and genetic mutations altering TF activity, subvert HSC homeostasis leading to the development of pre-leukaemic stem cells (pre-LSCs) and LSCs that drive the development and maintenance of acute myeloid leukaemia (AML). *Gata2*, a zinc finger TF expressed in the haematopoietic system, is essential for the generation and survival of HSCs in development, however the requirement for *Gata2* in adult HSCs is less clear. Perturbation of *GATA2* function by loss of function mutations of the *GATA2* gene, gives rise to immunodeficiency syndromes with a high risk of transformation to myelodysplastic syndrome and AML. Conversely, overexpression of *GATA2* is observed in approximately 40% of AML patients and has been linked with poor prognosis. However, the precise biological impact of *GATA2* expression on AML cell fate, including LSCs, has yet to be interrogated.

In this thesis, *Gata2* is established as a critical, dose dependent regulator of HSC and AML cell fate. Conditional, acute deletion of *Gata2 in vivo* leads to a rapid loss of HSCs in a cell autonomous fashion, suggesting a survival defect in *GATA2* deficient HSCs and defining a crucial requirement for *Gata2* in HSC maintenance. Employing a step-wise mouse model of AML it was found that acute deletion of *Gata2* delays the *in vivo* development and maintenance of LSCs driven by *Meis1a/Hoxa9* oncogene expression, but was dispensable for *Mll-af9*-mediated AML. In addition, *RNAi* or small molecule inhibition of *GATA2* via K-7174 triggers apoptosis of human AML cells and murine pre-LSC cell lines. K-7174 also targets the leukaemia initiating population in pre-LSC cell lines and enhances the killing activity of standard chemotherapeutics. Therefore, *GATA2* blockade or targeting of *GATA2* related pathways may open new therapeutic options for AML. The work described here establishes the basis for future work aiming to interrogate the mechanisms mediated by *Gata2* in HSCs and LSCs in AML, and to examine the therapeutic potential for *GATA2* mediated pharmacological inhibition in AML.

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Abbreviations

2-HG = 2-Hydroxygluterate

5-FU = 5'-fluorouracil

6-OHDA = 6-Hydroxydopamine

' = Minutes

AGM = Aorta-gonad mesonephros

ALL = Acute lymphobastic leukaemia

AML = Acute Myeloid Leukeamia

Ang-1 = Angiopoietin-1

APL = Acute Promyelocytic Leukeamia

Ara-c = Cytarabine

BFU-E = Burst Forming Unit-Erythrocyte

BM = Bone marrow

BMP = Bone Morphogenic Protein

BMT = Bone marrow transplant

bp = base pair

CAR = Cxcl12-abundant reticular cells

CB = Cord blood

cDNA = Complementary DNA

CEBPA = CCAAT/enhancer binding protein alpha

CFC = Colony Forming Cell

CFU = Colony Forming Unit

CFU-E = Colony Forming Unit- Erythrocyte

CFU-G = Colony Forming Unit- Granulocyte

CFU-GEMM = Colony Forming Unit- Granulocyte/Erythrocyte/Macrophage/Megakaryocyte

CFU-GM = Colony Forming Unit- Granulocyte/Macrophage

CFU-M = Colony Forming Unit- Macrophage

CFU-Meg/Mk = Colony Forming Unit- Megakaryocyte

CFU-S = Colony Forming Unit-Spleen

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

CLP = Common lymphoid progenitor

CML = Chronic myeloid leukaemia

CMP = Common myeloid Progenitor

Cre = Cre recombinase

CSC = Cancer Stem Cell

CT = Cycle threshold

DMSO = Dimethyl sulfoxide

DNA = Deoxyribonucleic Acid

DNase = Deoxyribonuclease

DNMT = DNA Methyltransferase

dNTP = Deoxynucleotide triphosphate

EDTA = Ethylenediaminetetraacetic acid

EGF = Epidermal Growth Factor

EGFR = Epidermal Growth Factor Receptor

EpoR = Erythropoietin receptor

Evi1 = Ecotropic proviral integration site 1

FAB = French-American-British classification

FACS = Fluorescence Activated Cell Sorting

FBS = Foetal Bovine Serum

Flt3 = FMS-like tyrosine kinase 3

g = Gram

GATA = GATA binding protein

G-CSF = Granulocyte-colony Stimulating Factor

Gfi1 = Growth Factor independent 1

GFP = Green Fluorescent Protein

GMP = Granulocyte Macrophage Progenitor

GVHD = Graft versus Host Disease

Hif = Hypoxia inducible factor

HLA = Human Leukocyte Antigen

Hox = Homeobox

HPC = Haematopoietic Progenitor Cell

HSC = Haematopoietic Stem Cell

HSPC = Haematopoietic Stem and Progenitor Cell

IDH1/2 = Isocitrate dehydrogenase1/2

IL = Interleukin

IRES = Internal-ribosome-entry-site

kg = Kilogram

L = Litre

Lin = Lineage

LMPP = Lymphoid-primed multipotent progenitors

LoxP = Locus of crossover of Bacteriophage P1

LSC = Leukaemia Stem Cell

LSK = Lineage-Sca-1+c-Kit+

LT-HSC = Long Term- HSC

LTC-IC = Long term culture initiating cell

MDS = Myelodysplastic syndrome

Meis = Myeloid ectropic insertion site

MEP = Megakaryocyte Erythrocyte Progenitor

mg = Milligram

mL = Millilitre

MLL = Mixed Lineage Leukemia

mM = Millimolar

MPN = Myeloproliferative neoplasm

MPP = Multipotent Progenitor

mRNA = Messenger Ribonucleic Acid

MSC = Mesenchymal Stem Cell

MSCV = Murine stem cell virus

n = Number of biological replicates

N = Number of independent experiments

NOD/SCID = Non-obese diabetic/severe combined immune deficiency

OB = Osteoblasts

pIpC = polyinosinic:polycytidylic acid

PBS = Phosphate Buffered Saline

PCR = Polymerase Chain Reaction

qRT-PCR = Quantitative Reverse Transcription Polymerase Chain Reaction

RBC = Red blood cells

RNA = Ribonucleic Acid

RNase = Ribonuclease

ROS = Reactive oxygen species

rpm = Revolutions per minute

RT = Room Temperature

Runx1 = Runt-related transcription factor 1

SA = Streptavidin

Scf = Stem cell factor

Scl/Tal1 = Stem cell leukaemia gene

SEM = Standard error of mean

ST-HSC = Short term-HSCs

TBI = Total Body Irradiation

TET2 = Ten-eleven-translocation gene 2

TF = Transcription factor

TGF-β = Transforming Growth Factor-β

TPO = Thrombopoietin

UV = Ultra Violet

VEGF = Vascular Endothelial Growth Factor

VEGFR = Vascular Endothelial Growth Factor Receptor

WBM = Whole Bone Marrow

WHO = World health organisation

Wnt = Wingless

WT = Wild Type

YFP = Yellow Fluorescent Protein

Chapter 1

Introduction

1.1. Normal haematopoiesis

1.1.1. Current view of the haematopoietic system

Haematopoiesis is a dynamic process that guarantees the supply of all blood cell types throughout the lifetime of an individual (Orkin and Zon 2008; Jagannathan-Bogdan *et al.* 2013; Ogawa 1994; Kent and Eaves 2016) In adults, haematopoiesis takes place predominantly in the bone marrow (BM), although it also occurs in extramedullary sites – the spleen and liver - in some situations such as severe infection, pregnancy and dramatic blood loss (Orkin and Zon 2008). Haematopoiesis is initiated in the embryo in three distinct waves; two primitive waves and the definitive wave (Dzierzak and Medvinsky 1995). In the mouse embryo, the first wave takes place in the yolk sac (YS) at embryonic day 7 (E7) and produces primitive erythroid, megakaryocytes, and macrophages (Dzierzak and Medvinsky 1995). The second wave appears at E8.5 in the YS and gives rise to bipotential erythroid-myeloid progenitors (EMP) (Dzierzak and Medvinsky 1995). The definitive wave occurs in the aorta-gonad-mesonephros (AGM) at E10.5 (Dzierzak and Medvinsky 1995; Ciau-Uitz *et al.* 2014; Costa *et al.* 2012; Yoder 2004) by means of the endothelial-to-haematopoietic transition (EHT) process, in which specialised endothelial cells – named hemogenic endothelial cells (HEC) – are capable of giving rise to haematopoietic stem cells (HSCs) (Dzierzak and Medvinsky 1995; Ciau-Uitz *et al.* 2014; Costa *et al.* 2012; Yoder 2004). Multipotent HSCs are detected in the foetal liver (FL) by E14, and finally migrate to the BM shortly-before birth (Orkin and Zon 2008).

In adults, HSCs sit atop of the haematopoietic hierarchy in the BM, are able to self-renew, and give rise to multipotent and lineage-restricted progenitors that progressively lose proliferative potential and differentiate into the mature blood cell types (Orkin and Zon 2008). Pioneering studies between 1961 and 1969 from Till and McCullough, demonstrated the potential existence of HSC with many properties; the ability to engraft an irradiated recipient (McCulloch and Till 1960), self-renewal - demonstrated by the ability of transplanted HSCs to reconstitute an irradiated host after serial transplantation (BECKER *et al.* 1963), formation of myeloid colonies (CFU-S) in the spleen after BM transplantation (Siminovitch *et al.* 1963), multilineage blood-forming capacity (Wu *et al.* 1968), and physical purification (Worton *et al.* 1969). Since

the 1980s, the development and advancement of fluorescence activated cell sorting (FACS) technology and the parallel development of fluorescence-labelled antibodies recognising cell-surface markers has facilitated the identification of prospectively isolated classes of HSC and progenitor subsets (Herzenberg *et al.* 2006; Quirke 1992; De Rosa *et al.* 2001). The use of transplantation assays in mice has been a very useful tool to assess the short term (8-12 weeks) and long term (16 weeks) engraftment and reconstitution ability, of specific subsets of FACS isolated HSC and progenitor cells, whilst also measuring the self-renewal ability of stem cells to give rise to itself and reconstitute the blood system of secondary recipients (Purton and Scadden 2007; Ema *et al.* 2007). Therefore, FACS and transplantation assays, together with the ability to engineer deletion of specific genes in mouse models, have defined our knowledge of highly purified HSCs and progenitor cell subsets. Based on these experiments, a classical hierarchical model of mouse and human haematopoiesis has been formulated as detailed in **Figure 1.1**. Briefly, HSCs ($Lin^-Sca1^+c\text{-}kit^+CD150^+CD48^-$) give rise to transient multipotent progenitor (MPP) ($Lin^-Sca1^+c\text{-}kit^+CD150^-CD48^-$), haematopoietic progenitors (HPC)-1 ($Lin^-Sca1^+c\text{-}kit^+CD150^-CD48^+$), and HPC-2 ($Lin^-Sca1^+c\text{-}kit^+CD150^+CD48^+$), that will further commit into myeloid restricted common myeloid progenitor (CMP) ($Lin^-Sca1^-c\text{-}kit^+CD16/32^-CD34^+$) producing lineage-restricted progenitors, megakaryocyte-erythrocyte progenitor (MEP) ($Lin^-Sca1^-c\text{-}kit^+CD16/32^-CD34^-$) and granulocyte-macrophage progenitor (GMP) ($Lin^-Sca1^-c\text{-}kit^+CD16/32^+CD34^-$) which will inturn go through precursor stages and terminal differentiation of megakaryocytes, erythroid and myeloid cells (Ogawa 1993; Kitajima *et al.* 2006; Ema *et al.* 2014; Doulatov *et al.* 2012; Rieger and Schroeder 2012)ma. Alternatively, lymphoid-primed multipotent progenitor (LMPP) ($Lin^-Sca1^-c\text{-}kit^+Flt3^+CD34^+$) arise from MPP and give rise to common lymphoid progenitor (CLP) ($Lin^-Sca1^{low}c\text{-}kit^{low}CD127^+$) that will generate T and B precursor cells that will terminally differentiate into mature T and B lymphocytes (Miranda-Saavedra and Göttgens 2008; Brown and Ceredig 2009; Jagannathan-Bogdan *et al.* 2013; Kondo *et al.* 1997).

In humans, the identification and study of purified HSCs has been limited by access to appropriate *in vitro* and *in vivo* models to test human cells (Doulatov *et al.* 2012; Kent and Eaves 2016; Majeti *et al.* 2007; Ng and Alexander 2017; Cuellar-Rodriguez *et al.* 2012; Notta *et al.* 2011; Laurenti and Dick 2012). The development of more

specialised *in vitro* cultures with an array of cytokines, allowed for the development of long term culture-initiating cell (LTC-IC) (Ramsfjell *et al.* 1999; Sutherland *et al.* 1993). However, the most substantial development is that of immunodeficient mice which can accept transplantation and engraftment of human BM cells without rejection due to xenogeneic boundaries (C. Y. Park *et al.* 2008). Since then, improved immunodeficient mice have been engineered, NOD/SCID/IL-2R$^{\gamma null}$ (NSG), which have become the most routinely used in human studies (Brehm *et al.* 2012; Beyer and Muench 2017), although new engineered mice expressing human cytokines or harbouring human stromal cells (currently in development) will provide further insights for future human haematopoiesis research (Goyama *et al.* 2015; Tsukada *et al.* 2017; Saito *et al.* 2016). Through LTC-IC assays and transplantation experiments, human HSCs were first identified within the CD34^{+} CD38^{-} compartment (Larochelle *et al.* 1996; Bhatia *et al.* 1997), which has been further refined to a CD34^{+} CD38^{-} CD90^{+}CD45RA^{-}CD49f^{+} population (Wisniewski *et al.* 2011; Notta *et al.* 2011).

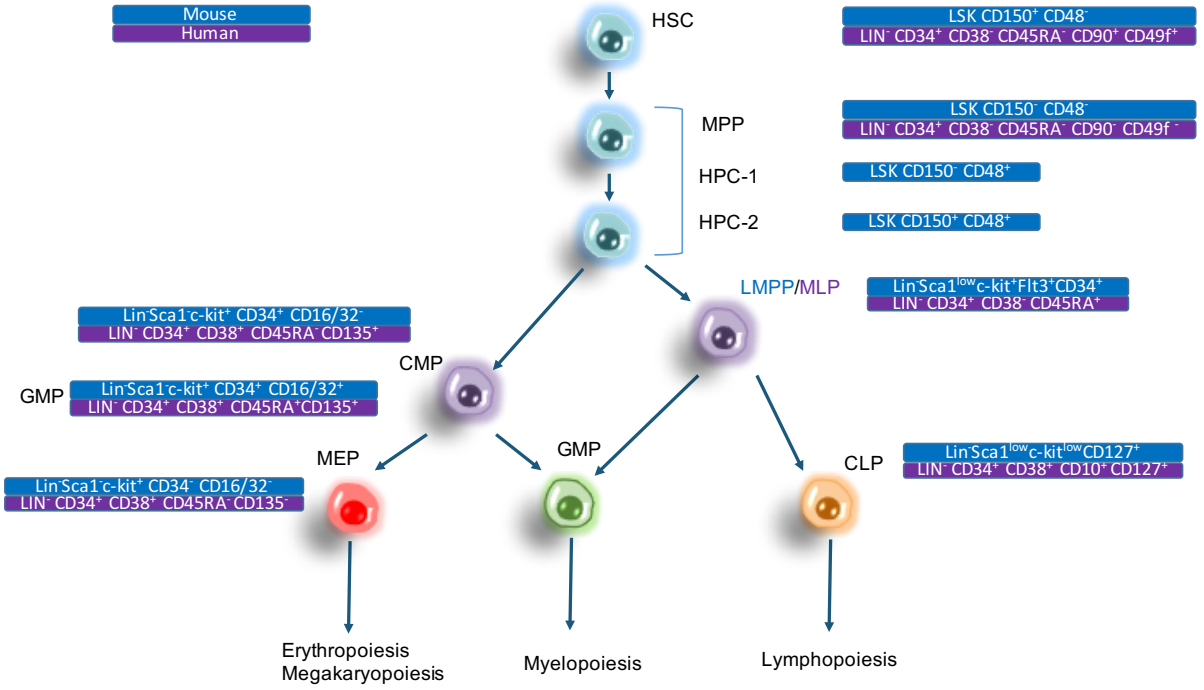


Figure 1.1. Haematopoietic hierarchy. HSCs reside at the top of the haematopoietic hierarchy where they sustain lifetime haematopoiesis. HSCs give rise to multipotent progenitor cells that progressively proliferative potential while differentiating into the main blood cell types. Mouse (blue) and human (purple) CD markers that define each population are indicated.

The traditional linear view of the haematopoietic hierarchy has recently been challenged both in the mouse and human systems. In humans, a study from John Dick's laboratory has redefined the human haematopoietic hierarchy eliminating the classical CMP oligopotent progenitor (Notta *et al.* 2016). Instead, in adult BM, HSC give rise to transient MPP or MLP that directly produce unipotent progenitors that differentiate into erythrocytes, granulocytes, monocytes and lymphocytes. Interestingly, their data indicate that lymphocytes emanate from a common progenitor for monocytes, but not granulocytes. In this model, it is posited that MEP arises directly from HSCs, in agreement with murine studies that show that megakaryocytes localise closely to HSCs and secrete critical factors such as *Pf4* and *Tgf-*β that regulate HSC quiescence (Olson *et al.* 2013; Bruns *et al.* 2014; Yoshihara *et al.* 2007). In the mouse, a similar dynamic model of haematopoiesis has been proposed by Passegué's group, where LT-HSC and SH-HSC (or MPP1) simultaneously produce two myeloid-biased MPP2 and MPP3 populations, and a lymphoid-biased MPP4 under steady-state conditions (Pietras *et al.* 2015). These data highlight the plasticity of HSCs, which in the first weeks after transplantation produces all the MPP subsets (MPP2-4) focused on myeloid cell production, while thereafter the MPP4 subset is able to restore their homeostatic function and give rise to the pool of lymphoid cells in an irradiated host (Pietras *et al.* 2015).

We are now entering a new era of single-cell studies that have shed light on the heterogeneity of HSCs at the clonal level and their contribution to haematopoiesis under steady-state conditions. *In vivo* barcoding of HSCs in mice showed that thousands of lineage-restricted and multipotent progenitors clones, but not HSCs, sustain steady-state haematopoiesis (Sun *et al.* 2014), which was also supported by another group that reported only a small fraction of HSCs contribute to native haematopoiesis in mice (Busch *et al.* 2015). However, a growing number of publications have disputed these findings (Verovskaya *et al.* 2014; Yu *et al.* 2016; Biasco *et al.* 2015; Sawai *et al.* 2016; Ito and Frenette 2016; Jaenisch *et al.* 2010; Jagannathan-Bogdan *et al.* 2013; van der Meer *et al.* 2010). For instance, a recent study employed a multi-fluorescent transgenic mouse that allows for *in vivo* tracking and molecular profiling of individual HSC clones (Yu *et al.* 2016). In agreement with contemporary findings in mouse and human, but contrary to the work from Sun *et al*.,

this study highlights that native haematopoiesis is sustained predominantly by a few HSCs clones. Discrepancies between these studies are due to the lower sensitivity of the fluorescence-based methods and the inability to measure clone size in the barcode study from Sun *et al*. Thus, it remains unclear whether HSC or progenitor cells, their combination, or cell-type and context, give rise to haematopoietic cells during steady-state conditions. The theory behind the study of steady-state haematopoiesis is based by the fact that HSCs are highly activated during transplantation, and therefore may not reflect endogenous haematopoiesis. Nevertheless, it is important to note that the steady-state studies were performed under pathogen-free conditions, whilst haematopoiesis in humans develop under more physiologically challenging conditions (i.e. pathogens, environmental stress, severe infections or blood loss), that may actually be more reflective of mouse transplantation assays.

1.1.2. Adult haematopoietic stem cells (HSCs)

HSCs possess critical features to sustain the haematopoietic system. They are able to self-renew, which is the faculty to produce other HSCs by either asymmetric or symmetric division; they are multipotent, which is the ability to produce all blood cell lineages; they undergo apoptosis to protect themselves from genotoxic stress and to regulate the number of HSCs; and they are largely quiescent, to prevent HSC exhaustion and accumulation of mutations (Kent and Eaves 2016). Understanding cell fate decisions underlying HSC biology has been aided by development of genetic tools such as tissue-specific conditional mouse models, in which a specific part of a gene is flanked by LoxP sites (floxed region) that can be recognised by Cre recombinase and, via homologous recombinase, generate deletion of the floxed region (Sauer 1998). Recently, CRISPR/Cas9 technology has also permitted the study and function of genes and noncoding regions in the haematopoietic system. **Table 1.1** summarises the haematopoietic Cre-Lox mice used to study HSC cell fate.

Table 1.1. Haematopoietic Cre-Lox mouse models to study HSCs

Mouse Strain	Inducible	Specificity	Germline Deletion?	Mating strategy	Issues
Mx1-Cre	pIpC (IFN) inducible	HSCs BM microenvironment Extramedullary tissues	No	+/- X -/- no preference	Leaky promoter – basal levels of IFN IFN activates HSCs – it is resolved by day 30 after pIpC
Vav-Cre	No	HSCs ECs ovaries	Yes	+/- male X -/- female	None
Vav-iCre	No	HSCs ECs (some strains) testes	Yes	+/- female X -/- male	None
Tie2-Cre	No	HSCs ECs	Yes	+/- male X -/-female	None

1.1.2.1. HSC self-renewal and quiescence

Self-renewal is a crucial characteristic of stem cells, and is required to maintain the rare number of HSCs throughout life. In adult HSCs, approximately 90-95% of HSCs remain quiescence (Passegué and Wagers 2006) in steady-state haematopoiesis and in times of physiological demand enter the cell-cycle in order to self-renew or differentiate.

The cell-cycle is promoted by cyclin-dependent kinases (*Cdk4/6*) that phosphorylate and inactivate *Retinoblastoma* (*Rb*) in late G1 in order to enter into S phase (Laurenti *et al.* 2015; Viatour *et al.* 2008). The main *Cdk* inhibitors are *p21, p27* and *p57,* known as *Cip/Kip* family that preferentially target *Cdk2* (Cheng *et al.* 2000; Matsumoto *et al.* 2011; Yuan *et al.* 2004), and *Ink4/arf* (*p16/p19*), *Ink4b* (*p15*), and *Ink4c* (p18) that repress *Cdk4/6* (Yuan *et al.* 2004). Before entering into cell division, *Rb* is in a hypophosphorylated state and is capable to bind and repress the function of *E2f* transcription factors (Weinberg 1995). In late G1, *Cdk4/6* phosphorylate *Rb* and thus releasing its association with *E2f* which then are able to activate the cell-cycle machinery (Weinberg 1995). To circumvent the embryonic lethality of *Rb* null mice, the role of *Rb* in HSCs was studied employing the *Mx1-Cre* system. To avoid effects from *Rb* deletion in the BM microenvironment, BM from untreated $Rb^{fl/fl}$*;Mx1-cre* mice were transplanted into *wild-type* recipients prior to pIpC induction, thus gene deletion occurs solely in HSCs (Walkley *et al*. 2006). Despite a mild but stable anaemia, no short or long-term effects in multilineage or self-renewal abilities of HSCs were observed in *Rb* deficient HSCs (Walkley *et al*. 2006). To assess whether *Rb* could regulate HSCs in an extrinsic-manner through interactions with BM niche cells, $Rb^{fl/fl}$*;Mx1-cre* mice treated with pIpC developed myeloproliferative syndrome (MPN)

and has lower levels of HSCs in the BM due to increase differentiation and mobilization to extramedullary sites (Walkley *et al*. 2007). However, *Rb*$^{fl/fl}$*;Mx1-cre* mice transplanted with *wild-type* BM and treated with pIpC are unable to induce MPN. Transplant of BM cells from *Rb*$^{fl/fl}$*;LyzM-cre*, which constitutively delete *Rb* in myeloid cells, into *Rb*$^{fl/fl}$*;Mx1-cre* mice develop MPN after pIpC deletion indicating that a reciprocal communication between niche and BM myeloid cells is required for *Rb*-mediated MPN (Walkley *et al*. 2007). *p21* controls HSC quiescence and self-renewal. Mice deficient in *p21* have less HSC in a quiescent state (G0), and are unable to reconstitute an irradiated host and sustain long-term haematopoiesis in serial transplantation assays (Cheng *et al.* 2000) indicating a self-renewal defect. Both the *Transforming growth factor-β (Tgf-β)* (Ijichi *et al.* 2004) and the *Notch* (Catelain *et al.* 2014) pathway have shown to activate *p21* expression and hence growth arrest of HSCs. However, other studies employing different mouse background indicate that *p21*-mediated HSC cell cycle regulation is not required in steady-state, but only during stress conditions (i.e. transplantation, myeloablative treatment) (van Os *et al.* 2007). Deletion of *p27* has minimal effect on HSCs but rather seems to impact progenitor cell function (Cheng *et al.* 2000). Redundancies between the *Cip/Kip* members were described in *p57*$^{-/-}$ mice with decreased quiescent cells and self-renewal defects *in vitro* CFC assays and *in vivo* transplants (Matsumoto *et al.* 2011). Codeletion of *p21* and *p27* exacerbated the phenotype observed in *p57* KO mice, whereas the phenotype could be rescued by overexpressing *p27* (Matsumoto *et al.* 2011). *TGF-β/Smad* signalling pathways also regulate HSC quiescence. Signalling through the *Smad* proteins leads to activation of cell cycle inhibitors such as *p15, p21*, *p27, p57* and other regulators that promote exit from the cell cycle (G0 state) (Ijichi *et al.* 2004; Larsson *et al.* 2003; Watabe and Miyazono 2009). Special interest has been raised in quiescence-induction mediated by *TGF-β-Smad-p57* in both mouse and human HSC fractions (Scandura *et al.* 2004). Interestingly, *Smad4* activates *Gata2* that in turn binds and activates *p57* expression to induce proliferation arrest and dormancy of murine HSCs (Billing *et al.* 2016). Plenty of evidence supports a quiescent role of *TGF-β*, however opposite and additional functions of the *TGF-β* pathway have been described. These include immune system regulation, and haematopoietic aging, which is understood to be a reflection of the diversity on the ligands, receptors, and canonical and non-canonical intracellular pathways that regulate HSC and progenitor

cells in a dose and context dependent (Meulmeester and Ten Dijke 2011; Fang *et al.* 2012; Larsson and Karlsson 2005).

Deletion of *p16/p19* has no consequences in HSC functionality (Stepanova and Sorrentino 2005), in agreement with its low expression in young adult HSCs, as a result of *Bmi1*-mediated repression (Park *et al.* 2003). However, *p16* is a critical regulator of senescence and its expression rises in aged HSCs alongside a decrease in the self-renewal abilities (Attema *et al.* 2009). Indeed, conditional deletion of *p16* leads to enhanced repopulating capacity and apoptosis resistance in aged HSCs (Attema *et al.* 2009). Conversely, *p18* modulates cell-cycle entry in young HSCs as shown by the increase in self-renewal capacity of $p18^{-/-}$ HSCs (Yuan *et al.* 2004). Both *p18* and *p21* regulate cell-cycle entry, but unlike *p21 KO* HSCs, *p18 KO* HSCs are not exhausted after serial transplantation (Yuan *et al.* 2004). *Bmi1* is a polycomb complex protein of TFs that regulate HSC self-renewal (Park *et al.* 2003). BM, but not foetal liver (FL) cells of $Bmi1^{-/-}$ showed fewer numbers of HSCs that are functionally compromised in serial transplantation BMT, indicating a self-renewal defect (Park *et al.* 2003). Gene expression analysis showed that *Bmi1* regulates HSC self-renewal by repressing *p16*, and to some extent HSC survival by repressing *p19* (Park *et al.* 2003). Enforced expression of *p16* or *p19* reduces the proliferative capacity of HSCs (Park *et al.* 2003). Similarly to *Bmi1*, BM analysis of $Gfi1^{-/-}$ mice showed reduced frequency of HSCs with higher proportion of cycling HSCs that performed poorly in serial BMT assays (Zeng *et al.* 2004), suggesting that *Gfi1* deletion leads to HSC exhaustion. In contrast, conditional deletion of *C-myc* using the Mx1-cre system leads to enhanced self-renewal and differentiation block of HSCs (Wilson *et al.* 2004). Mechanistic studies indicate that *C-myc* repress *N-cadherin* transcription thus releasing HSCs from the HSC niche in the BM to start the differentiation program (Wilson *et al.* 2004). Proliferation and expansion of murine and human HSCs *in vitro* and *in vivo* is accomplished by *Hoxb4/HOXB4* overexpression (Antonchuk *et al.* 2002; Beslu *et al.* 2004; Krosl *et al.* 2003), and in human CB HSCs, the expansion phenotype is, at least in part, mediated by *C-KIT, GATA2* and *GFIB* (Fujiwara *et al.* 2012).

The tumour suppressor *p53,* also known as the “guardian of the genome”, is a key regulator of HSC quiescence and self-renewal. In homeostasis, *p53* is bound and ubiquitinated by the E3-ubiquitin ligase *MDM2 (*Pant *et al.* 2012*). P53* activation can

be triggered by endogenous or exogenous stress signals leading to the phosphorylation of amino acids disrupting the binding of *MDM2* and the consequent formation of a *p53* tetramer which then triggers cell-cycle arrest or senescence to fix the damage produced in the DNA, or if the damage cannot be repaired, *p53* activates the expression of pro-apoptotic members of the *Bcl2* family to induce apoptosis and avoid the transmission of mutations to progenitor cells (Pant *et al*. 2012). *P53* is able to induce cell-cycle arrests through *p21* activation to stop the progression in the G1 phase (Pant *et al*. 2012). Alternatively, *p53* disrupts the G2 phase complex *cyclin B1/cdc2* complex (Pant *et al*. 2012). *P53*$^{-/-}$ mice showed a 2-to-3-fold expansion in the number of LSK cells (Chen *et al*. 2006). When bone marrow cells were transplanted, recipients injected with *p53* null BM cells had increased repopulation activity compared to control mice (Tekippe *et al.* 2003), however when HSC where transplanted, *p53* null HSCs showed a repopulation defect (Chen *et al.* 2006), similar to that observed with *p21* null HSCs (Akala *et al*. 2008).

The *phosphatidylinositol 3-kinase (Pi3k)* pathway also regulates the quiescence of HSCs. Specifically, inhibition of *Pi3k/Akt/mTor* signalling by the phosphatase *Pten*, the main negative regulator of this pathway, is essential to preserve the HSC pool (Zhang *et al.* 2006). Indeed, *Pten*$^{-/-}$ mice have hyperactivated *Pi3k/Akt/mTor* signalling, driving HSC proliferation within 5 days and the loss of self-renewal and HSC exhaustion in transplantation assays (Zhang *et al.* 2006). The exacerbated proliferation is facilitated by *Akt*-mediated inhibition of *FoxO*, leading to *p21* and *p27* inhibition, and therefore entry into the cell-cycle (Zhang *et al.* 2006). Longer term monitoring of *Pten*$^{-/-}$ mice leads to a myeloproliferative neoplasm (MPN) phenotype that can progress to acute leukaemia (Yilmaz *et al.* 2006).

The *Wnt/β-catenin* pathway has been associated with self-renewal of HSC, although contradictory results have been reported. Briefly, the lack of *Wnt* ligands leads to the creation of a destruction complex formed by *Axin, Apc, Gsk3β* and *β-catenin* in which *β-catenin* is phosphorylated and ubiquitinated for degradation by the proteasome. Upon *Wnt* ligand-receptor binding, *Gsk3β* phosphorylation by *Dvl* dissembles the destruction complex allowing *β-catenin to* accumulate in the cytoplasm and translocate into the nucleus where it activates/represses gene expression together

with *Tcf* and other co-activators/repressors (Clevers and Nusse 2012). Co-culture of human $CD34^+$ BM cells with *WNT2B, WNT5A* or *WNT10B* ligands can expand HSPCs while preserving the HSC phenotype (Murdoch *et al.* 2003). Introduction in murine HSCs of a constitutive active form of *β-catenin* can expand HSCs and efficiently reconstitute an irradiated mouse, whereas overexpression of the repressor *Axin* stops HSC proliferation (Baba *et al.* 2006). However, an engineered mouse model expressing stable *β-catenin,* expanded HSCs which then performed poorly in transplantation assays, indicating a functional HSC defect (Kirstetter *et al.* 2006). Loss of function experiments using Cre-Lox deletion of *β-catenin* in mice are also controversial. Deletion using the haematopoietic-specific *Vav-Cre* line leads to self-renewal defects in serial transplantation experiments, while deletion with the conditional *Mx1-Cre* promoter showed no defects in self-renewal of HSCs (Zhao *et al.* 2007; Jeannet *et al.* 2008). These differences are thought to be due compensatory effects from environmental or extramedullary deletion of *β-catenin* in the *Mx1-cre* model, or by dependency of *β-catenin* in foetal HSCs that cannot be compensated in adult HSCs in the *Vav-Cre* model.

1.1.2.2. HSC survival

The fine balance in the number of the HSC pool is regulated by apoptosis. HSCs under genotoxic or stress conditions undergo apoptosis to avoid accumulation of mutations that lead to aplastic, dysplastic and neoplastic haematopoiesis. Elucidating the molecular drivers that confer HSC survival is critical to tackling BM failure syndromes, MDS and AML where apoptosis is dysregulated (Lowe and Lin 2000; Alenzi *et al.* 2009).

To date, a select group of TFs are known to act as critical regulators of adult HSC survival, these include *Etv6*, *Cited2,* and *Nkap* (Hock *et al.* 2004; Kranc *et al.* 2009; Opferman 2005). To conditionally delete a specific gene in an acute-fashion manner in HSCs, studies have employed the *Mx1-Cre* mouse model, where Cre expression is under the regulation of the IFNα pathway that is experimentally activated by the administration of a double-stranded RNA named poly(I);poly(C) (pIpC) (Kühn *et al.* 1995). Deletion of these TFs in adult mice leads to a cell-autonomous loss of HSPCs

and BM failure (Hock *et al.* 2004; Kranc *et al.* 2009; Opferman 2005). *Nkap* is a repressor of *Notch* signalling (Pajerowski *et al.* 2009), and interestingly another negative regulator of *Notch* at the protein level, *Notchless*, induces HSPCs loss after condition deletion with the Mx1-Cre promoter (Le Bouteiller *et al.* 2013). Curiously these results are reminiscent of the phenotype observed after acute deletion of *Mcl1* (Opferman 2005), an anti-apoptotic member of the *Bcl-2* family (Michels *et al.* 2005), in HSCs using the same Cre-lox system. Excluding *Etv6* and *Mcl1,* where the mechanism was not investigated , these critical regulators protect HSC from apoptosis cell dead, at least in part, mediated by the *p16/p19/p53* axis (Le Bouteiller *et al.* 2013)(Kranc *et al.* 2009; Pajerowski *et al.* 2010).

1.1.2.3. HSC differentiation

In response to external and internal stimuli, approximately 100 billion blood cells are formed every day to replenish the turnover of blood cells in the haematopoietic system (Reya *et al.* 2001). Hence, strict regulation of the molecular machinery that orchestrates the differentiation program of HSCs is vital to fight infections and avoid cytopenia, autoimmune diseases or the onset of malignancies. Lineage-specific ligand-receptor pairing transmits signals through signalling pathways that activate and/or repress TFs that drive the molecular and consequently phenotypic differentiation of HSPCs into mature haematopoietic cells (Sarrazin and Sieweke 2011).

Differentiation towards lymphoid lineage is driven by *Flt3-ligand* signalling in MPPs, that induces differentiation to the CLP, and together with *Pu.1,* CLPs start expressing *IL7r* that is bound by *IL7,* produced in the BM niche. At this point, activation of the *Notch* signalling pathway induces *Gata3* expression to produce $CD3^+$ T cells that travel to the thymus to complete the differentiation process (Masopust and Schenkel 2013). In contrast, B cell differentiation is supported by TFs, *E2a* and *Early B cell factor* (*Ebf*) (Sigvardsson *et al.* 2002; Dias *et al.* 2005). This process is similar in humans, where *FLT3L-CD135* interaction in MPPs activates *GATA2* expression that gives rise to MLPs, a common precursor to lymphoid cells and monocytes and DCs (Laurenti *et al.* 2013).

Erythrocyte production is regulated by the *Epo-EpoR* interaction in MEP progenitors (Kuhrt and Wojchowski 2015), which internalise the signal mediating the *Jak/Stat* pathway to upregulate erythroid specific genes such as *Gata1* (Wierenga *et al.* 2010) and activate the expression of erythroid markers such as CD71 and TER119 as they go through the different S1-S5 precursor stages until they release their nuclei to progress from reticulocyte to mature erythrocyte (Koulnis *et al.* 2011). The *Gata1/Gata2* switch plays an important role in the development of red blood cells (see **1.3. Gata2**). Upon *Tpo* stimulation, megakaryocyte progenitors expressing the *Tpo* receptor, *Mpl*, differentiate to megakaryocytes which release platelets, a portion of the megakaryocyte cytoplasm (Ng *et al.* 2012; Deutsch and Tomer 2006).

In addition to its role in the regulation of HSPCs, the *Scf/c-kit/Pi3k* pathway also regulates mast cell differentiation (Chen *et al.* 2005). In this context, *Gata2* also appears as an essential regulator of mast cells, as it binds and activates the transcription of the *C-kit* promoter and reinforces mast cell differentiation (Ohmori *et al.* 2015; Maeda *et al.* 2010).

Myeloid differentiation commences with cytokines such as GM-CSF, G-CSF, M-CSF, IL-6 and IL-3 (Mossadegh-Keller *et al.* 2013; Sarrazin and Sieweke 2011; Umetani *et al.* 2000). This activates *Pu.1* expression that interacts with other TFs such as *Gata1* to induce myeloid commitment to the CMP progenitor (Nerlov and Graf 1998; Nerlov *et al.* 2000). *Gata1* expression is reduced from the CMP to GMP transition (Huang *et al.* 2009), which is driven by *Cebp*α (Wang *et al.* 2006) and *Runx1* (Ng *et al.* 2013). Activation of *Irf8* drives monocyte or DC differentiation (Sichien *et al.* 2016) while *Gfi1* and *Lef1* predominantly give rise to granulocytes (van der Meer *et al.* 2010; Doulatov *et al.* 2012).

1.1.3. Bone marrow niche

The idea that the BM microenvironment or niche influences haematopoiesis was first hypothesised in 1978 by Schofield (Schofield 1978). However, it was not until early 2000s that it was possible to identify and study the cells that form the BM niche. Nevertheless, in the last 5 years the understanding about the niche cell types and niche factors that regulate HSCs has increased massively due the advances in imaging and the development of niche specific mouse models.

The BM niche comprises heterogeneous cell populations, including endothelial cells, perisinusoidal cells, periarteriolar cells, adipocytes, osteoblasts, megakaryocytes, monocytes, macrophages, nerve fibres and associated Schwann cells, and mesenchymal stem cells (MSC, also known as skeletal stem cells (SSC)) (**Figure 1.2**). The advent of new genetic tools to dissect the BM niche has provided different and overlapping nomenclature for niche cells types. **Table 1.2** summarise the genetic mouse models, cell-type specificity and overlapping niche cells that have been employed so far.

BM mesenchymal stem cells (BM-MSCs) are a rare type of multipotent progenitor that reside in the BM niche and have the capacity to give rise to adipocytes, chondrocytes and osteoblasts *in vitro* and *in vivo* (Park *et al.* 2012). To date, there is still a need for more defined markers of MSCs to better purify these BM stromal cells with multilineage capacity. For instance, *Nes-GFP*$^{+}$ and *Lepr*$^{+}$ cells are highly enriched in MSCs ability *in vitro*, based on CFU-F formation and multilineage differentiation (Xie *et al.* 2015; Bo O Zhou *et al.* 2014). **Table 1.2** shows the main markers used to identify MSC in the mouse system.

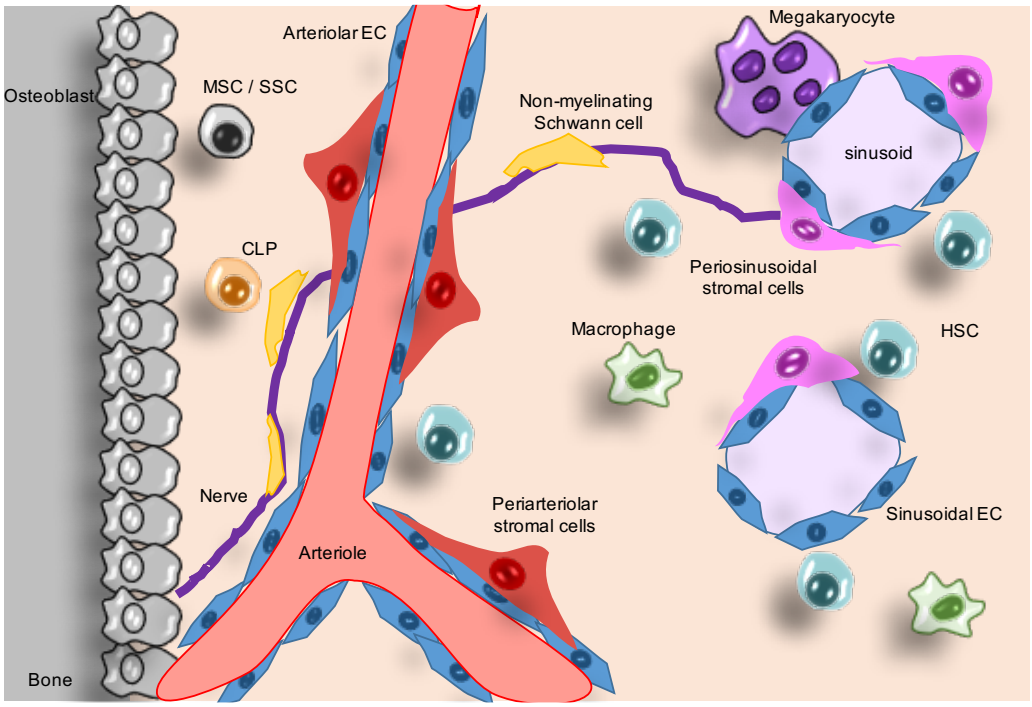


Figure 1.2. Bone marrow niche. HSCs reside close to the perivascular niches. The perisinusoidal niche is formed by fenestrated sinusoids, sinusoidal ECs, and perisinusoidal stromal cells (Lepr^{+}, CXCL12-abundant recitular CAR cells, NestinGFP^{dim}). The periarteriolar niche includes a central arteriole, arteriolar ECs, and periarteriolar stromal cells (NG2^{+}, NestinGFP^{high}). Other components of the BM niche include megakaryocytes, macrophages, nerve fibres and associated non-myelinating Schwann cells, MSC/SSC, and osteoblasts.

Table 1.2. Haematopoietic Cre-Lox mouse models to study the BM niche

Mouse Strain	Inducible	Specificity	Germline Deletion?	Mating strategy	Issues
VE-Cadherin-CreERT2	Tamoxifen-inducible	Adult: ECs Embryo: ECs and HSCs	no	+/- X -/- no preference	Adult induction does not target all ECs
Prx1-Cre	No	MSCs and perivascular cells	N.D	+/- male X -/- female	None
Nestin-Cre	No	MSCs and perivascular cells (GFP^{hi} periarteriolar, GFP^{dim} perisinusoidal) Other organs (depends on strain)	Yes	+/- X -/- no preference	Different strains available have different cell-specificity
LepR-Cre	No	MSCs and perisinusoidal cells Brain cells	No	+/- X -/- no preference	None
Osx1-GFP::Cre	Doxycycline mediate inhibition	Osteoprogenitors	No	+/- male X -/- female	growth retardation and malocclusion occur in mice carrying Cre
Mouse Col2.3-Cre	No	Mature osteoblasts	No	+/- X -/- no preference	None
Adipoq-Cre	Tamoxifen-inducible	Adipocytes	No	+/- X -/- no preference	None
NG2-CreERT2	Tamoxifen-inducible	Perioarteriolar cells in adults Broader range of BM MSCs in embryo and 3-weeks old	No	+/- X -/- no preference	Different cell specificity young and adult mice

N.D. – Not determined

Despite first evidence suggesting that HSCs are close to the inner interface of bone, the endosteum or osteoblastic niche, (Lévesque *et al.* 2010; Arai and Suda 2007), the current view of the BM niche no longer supports this theory. Rather, only a subset of lymphoid progenitors seems to be in close association with the osteoblastic niche in the endosteum (Ding and Morrison 2013). HSCs locate in the perivascular niche, that comprise endothelial and stromal cells in contact with sinusoids – perisinusoidal - or arterioles – periarteriolar, that also includes nerve fibres and non-myelinating Schwann cells (Chen *et al.* 2016). It appears that most of HSCs (80%) reside near the perisinusoidal niche, 10% in transitional zones between arterioles and sinusoids, and 10% in the periarteriolar niche, located in the centre of the BM niche and run parallel to the bone, while sinusoids are found perpendicularly orientated and distributed across the BM niche (Kunisaki *et al.* 2013; Acar *et al.* 2015). The different location of HSCs in different niches might underscore different HSCs subsets with distinct self-renewal and differentiation faculties within the BM. The perisinusoidal niche is the most hypoxic region (Nombela-Arrieta *et al.* 2013), and while the main mediators of hypoxia signalling, *Hif1*α and *Hif2*α are dispensable for HSC maintenance and self-renewal in a cell-autonomous manner (Vukovic *et al.* 2016; Guitart *et al.* 2013), previous studies that deleted *Hif1*α in HSCs and the BM niche (i.e. using the Mx1-Cre promoter) found some HSCs defects, indicating that hypoxia signalling regulates HSC function indirectly by modulating BM niche cell activity (Takubo *et al.* 2010). Perisinusoidal HSCs are in close proximity to blood constituents that passage fenestrated sinusoids (Itkin *et al.* 2016). Radical oxygen species (ROS), a byproduct of metabolism, negatively impact HSC quiescence and self-renewal while positively regulating homing and migration of HSPCs after transplantation (C. Chen *et al.* 2008; Porto *et al.* 2015; Shao *et al.* 2011). In fact, a third of perisinusoidal HSCs ($CD150^{+}CD48^{-}$) stain for ROS, while no periarteriolar HSCs stain negative for ROS (Itkin *et al.* 2016). However, it can be argued that also a third of HSCs stain positively for CD41 (Oguro *et al.* 2013), which marks megakaryocyte progenitors that associate with sinusoids (Tavassoli and Aoki 1989), suggesting that the difference seen in ROS levels in HSCs might be actually due CD41 “contamination”.

1.1.3.1. BM niche factors are essential for HSC maintenance

HSCs sense the external signals from extracellular growth factors, nutrients, and stress that regulate HSC self-renewal, maintenance and differentiation (Suda *et al.* 2005). HSCs express known receptors such as *Kit*, *Cxcr4* and *Mpl* that regulate HSC fate decisions, however the cell-specific origin of *Scf* and *Cxcl12* has only recently been interrogated. Knock-in reporter mice expressing *Scf* fluorescence proteins showed that *Scf* is not expressed in haematopoietic cells or osteoblasts, but is mainly expressed in perisinusoidal cells, and at very low levels in endothelial cells, and some arterioles (Ding *et al.* 2012). In agreement with this, deletion of *Scf* in haematopoietic cells (using *Vav1-Cre*), osteoblasts (using *Col2.3-Cre*), and periarteriolar cells (using *Nes-Cre, Nes-CreER, Ng2-CreER*) does not affect HSCs in young adult mice confirming that *Scf* does not have autocrine effects in HSCs (Ding *et al.* 2012). Deletion with *Ng2-Cre* depletes HSCs due a broad expression of $Ng2^{+}$ in niche cells during foetal stages (Ding *et al.* 2012; Bo O. Zhou *et al.* 2014). Conversely, deletion of *Scf* in endothelial cells (*Tie2-Cre*) and perisinusoidal cells (*Lepr-Cre*) leads to HSC depletion in steady-state conditions (Ding *et al.* 2012). Co-deletion of *Scf* in endothelial and perisinusoidal cells results in loss of quiescence and serially transplantable defects (Ding *et al.* 2012), indicating maintenance of adult HSCs by *Scf* produced from perisinusoidal and endothelial cells.

Using the same approach, it was found that *Cxcl12* is highly expressed in perisinusoidal cells, at lower levels in endothelial cells, and is even further attenuated in osteoblasts (Ding and Morrison 2013; Greenbaum *et al.* 2013; Asada *et al.* 2017). Deletion of *Cxcl12* in megakaryocytes (by *Pf4-Cre*), haematopoietic cells (by *Vav-iCre*), and periarteriolar cells (by *Nes-Cre, Nes-CreER, Ng2-CreER*) does not affect adult HSCs (Ding and Morrison 2013). In contrast, deletion of *Cxcl12* in endothelial cells (*Tie2-Cre*) leads to HSC depletion whilst deletion in perisinusoidal cells (*Lepr-Cre*) leads to HSC mobilization (Ding and Morrison 2013), indicating that different sources of *Cxcl12* play different functions in HSC regulation. Interestingly, *Cxcl12* deletion in osteoblasts (*Col2.3-Cre*) depletes lymphoid progenitors from the BM, confirming the close proximity of lymphoid progenitors to the osteoblastic niche (Visnjic *et al.* 2004; Zhu *et al.* 2007). However, a more recent study reinforced that $NG2^{+}$ cells also secrete *Cxcl12* and contribute to HSC maintenance in the BM niche

(Asada *et al.* 2017). Discrepancies observed between the studies conducted by the Morrison (Ding and Morrison 2013) and Frenette (Asada *et al.* 2017) labs could be explained by the overlapping activity of *Cre* transgenic mice used, and the differential GFP or Cre expression observed within the targeted populations, indicating that these Cre mice marks a heterogeneous cell population. This notwithstanding, the current dogma supports a model where HSCs fate decisions are modulated by the factors provided by endothelial and perivascular niche cells.

Adipocytes represent approximately a 5% of the total *Lepr*$^{+}$ cells, and have been linked with fatty marrow displacement in aplastic anaemia and other haematological disorders and are known to negatively impact HSC function (Brodsky and Jones 2005). In fact, engraftment of BM cells after irradiation is enhanced in genetically engineered mice that are unable to produce adipocytes suggesting, they are negative regulators of HSCs in this context (Naveiras *et al.* 2009). Interestingly, a recent report has highlighted a positive HSC supporting role for adipocytes in the BM niche. Conditional deletion of *Scf* in adipocytes (with *adipoq-CreER*) had no impact in HSC under steady-conditions (Zhou *et al.* 2017). Under regenerative conditions (i.e. irradiation or 5-FU treatment), sinusoids are lost and instead adipocytes proliferate and become more predominant in the BM (Zhou *et al.* 2017). Deletion of *Scf* in adipocytes (*adipoq-CreER*) under these circumstances, delayed haematopoietic regeneration and reduced survival of irradiated and 5-FU treated recipients (Zhou *et al.* 2017). Deletion of *Scf* in endothelial cells after irradiation did not impact HSC numbers (Zhou *et al.* 2017), indicating that adipocytes are the main source of *Scf* for HSCs after haematopoietic injury.

Therefore, the BM niche is highly heterogeneous and contains distinct subpopulations that are able to regulate HSCs at different stages of development. There is still controversy regarding the contribution of each niche cell type to haematopoiesis, however it seems that HSCs are dependent on specific niche cells in steady-state, BM regeneration and stress, highlighting the malleability of the haematopoietic to different physiologic stressors. Future work is required to further specify niche cell types and to discern whether specific subsets of HSC and/or progenitor cells are dependent on specific niche cells within the BM.

1.1.3.2. HSC differentiation – cell autonomous or niche dependent?

The stem cell theory from McCulloch (Till and McCulloch 1961) supports that HSC have an intrinsic program that governs its cell-fate decisions, while Schofield's niche theory (Schofield 1978) postulates that signals from the BM microenvironment direct HSC fate. Since then, researchers have provided supportive evidence for each theory, reaching a consensus with a combined theory, whereby the crosstalk between HSCs and the BM niche mediated by secreted factors (i.e. cytokines, growth factors, chemokines), regulate HSC fate decisions. However, the study from Yu *et al*., (Yu *et al.* 2016) employing a multifluorescent reporter mice that allow the clonal tracking of HSCs during differentiation, has raised some questions concerning the impact of the BM niche in lineage commitment.

The study from Yu *et al*. (Yu *et al.* 2016), supports previous data indicating that HSCs are biased towards the production of particular blood lineages, even at the clonal level (Hoppe *et al.* 2016; Dykstra *et al.* 2007). Different colour-code HSCs clones had different myeloid/lymphoid potential; in addition, HSC clones with lymphoid potential were less proliferative while myeloid-skewed clones were more proliferative, and this was phenocopied after transplantation of specific HSC clones into irradiated mice (Yu *et al.* 2016). Interestingly, this predetermined lineage-bias is not discernible at the transcriptional level, but it is marked by its epigenome, DNA methylation and chromatin accessibility, suggesting that, at the clonal level, epigenetics regulates how a HSC will behave in steady-state and stress conditions. This predetermined lineage-biased HSC model raises some questions regarding the plasticity of HSCs and its differentiation potential in response to BM niche signals. It was originally theorised that HSC are poised by expressing very low levels of TFs that drive differentiation to several lineages and that under external stimuli produced an imbalance of this TFs into the desired blood cell type (Laslo *et al.* 2006). This new evidence suggests that it exists differential TF expression at the single-cell level in progenitor cells, therefore indicating that HSCs are hardwired at the clonal level in a cell-autonomous manner to determine their differentiation fate (Baryawno *et al.* 2017). Nevertheless, these findings do not discard the idea that the niche is crucial for HSC activity; for example, it has not been ruled out experimentally that the niche could modify the epigenetic status of HSCs. With the growing evidence that subsets of HSCs can be found in association with

specific BM niches, it is clearly possible that specific niche cells can, at the very least, support the predetermined differentiation fate of each HSC clone.

1.2. Acute myeloid leukaemia

Acute myeloid leukaemia (AML) is a heterogeneous blood cancer characterised by the rapid growth of abnormal white blood cells (termed blasts) and a block in normal blood cell differentiation (Hanahan and Weinberg 2011) that presents with variable morphology, genetic and epigenetic alterations to influence the prognosis and therapeutic response to standard chemotherapeutics and novel therapies (De Kouchkovsky and Abdul-Hay 2016; Shafer and Grant 2016). AML is the most common type of leukaemia in adults (Hanahan and Weinberg 2011). The prevalence of AML in adults is of 3.8 per 100,000 individuals, increasing to 17.9 cases per 100,000 in adults older than 65 years old (De Kouchkovsky and Abdul-Hay 2016; Boissel *et al.* 2005), with the median age of adult AML being 67 years old. Morbidity in AML is not directly related to the malignancy of the leukaemia cells per se, but instead due the lack of functionally mature immune and red blood cells produced; thereby causing haemorrhaging and infections leading to mortality (Lowenberg *et al.* 1999).

Diagnosis of AML has traditionally followed the French–American–British (FAB) classification, based on the morphology and cytochemistry alongside the presence of more than 30% blasts in the BM (Papaemmanuil *et al.* 2016) (**Table 1.3**). Later, the World Health Organization (WHO) incorporated the cytogenetic groups and set out the frequency of AML blasts in the BM to higher than 20% (Arber *et al.* 2016)(**Table 1.4**). These classifications help to stratify AML patients into good, intermediate, or poor prognosis groups that ultimately inform treatment strategy. However, the most relevant factor dictating treatment and survival is patient age. The 5-year survival rate is 60% in patients aged 15 to 24, almost 40% between 25 and 64, and nearly 5% in patients 65 and above (Büchner *et al.* 2005). This is because older (or unfit) patients are susceptible to comorbidity related to intensive treatments and often present with adverse cytogenetic risk group, frequently derived from MDS or therapy-related AML (Büchner *et al.* 2005).

Table 1.3. French-American-British (FAB) AML classification

FAB subgroup	Type of leukaemia	Prognosis Frequency in AML	Morphology
M0	Acute myeloblastic leukaemia without maturation	Poor less than5%	Immature myeloblasts, lacks definite myeloid differentiation by conventional morphology or cytochemical analyses
M1	Acute myeloblastic leukaemia with minimal maturation	Intermediate 20%	Immature myeloblasts predominate, <10% promyelocytes, myelocytes or monocytes
M2	Acute myeloblastic leukaemia with maturation	Good 30%	Immature myeloblasts predominate, but more maturation than in M1
M3 and M3v (variant)	Acute promyelocytic leukaemia	Very good 10%	Promyelocytes predominate, marked granulation in more than 30% cells, granules not visible by light microscopy in M3v
M4 and M4EO	Acute myelomonocytic leukaemia	Intermediate 25%	Mixture of abnormal monocytic cells (>20%) and myeloblasts/promyelocytes (>20%), 30% eosinophils in M4EO
M5a and M5b	Acute monocytic leukaemia	Intermediate 10%	Monocytic cells predominate (>80%) in M5a, >80% nonerythoid cells are immature monoblasts, >20% are mature monocytes in M5b
M6	Acute erythroleukaemia	Poor Less than 5%	Myeloblasts and erythroblasts predominate, abnormal multinucleated erythroblasts containing PAS-positive blocks
M7	Acute megakaryoblastic leukaemia	Poor Less than 5%	Megakaryocytic cells as shown by platelet peroxidase activity on electron microscopy or by tests with platelet-specific antibodies, often myelofibrosis and increased BM reticulin
Others	Undifferentiated acute leukaemia, hypocellular AML	Unkown	

AML can develop as a primary disease, *de novo*, or can arise secondarily, from previous haematological disorders such as cytopenias (Fanconi's anaemia, congenital mono or neutropenia), immunodeficiency syndromes and myelodysplastic syndromes (MDS) –termed secondary AML (De Kouchkovsky and Abdul-Hay 2016; Boissel *et al.* 2005). Secondary AML also encompasses therapy-related AML, whereby patients from other cancers, (typically colon or breast cancer, or types of leukaemia) as a result of treatment with high-dose chemotherapy, gain mutations associated with a complex karyotype and adverse prognosis and overall survival (De Kouchkovsky and Abdul-Hay 2016; Boissel *et al.* 2005).

Table 1.4. World Health Organisation (WHO) WHO classification of myeloid neoplasms and acute leukaemia

AML Classification	AML Subtypes
AML with recurrent genetic abnormalities	t (8; 21) (q22; q22); RUNX1-RUNX1T1
	Inv (16) (p13; 1q22) or t(16; 16) (p13.1; q22); CBFB-MYH11
	APL with t (15; 17) (q22; q12); PML-RARA
	AML with t (9; 11) (p22; q23); MLLT3-MLL
	AML with t (6; 9) (p23; q34); DEK-NUP214
	AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
	AML (megakaryoblastic) with t (1; 22) (p13; q13); RBM15-MKL1
	Provisional entity: AML with BCR-ABL1
	Provisional entity: AML with mutated NPM1
	Provisional entity: AML with mutated CEBPA
	Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
AML - not otherwise specified (NOS)	AML with minimal differentiation
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukaemia
	Acute monoblastic/monocytic leukaemia
	Pure erythroid leukaemia
	Acute megakaryoblastic leukaemia
	Acute basophilic leukaemia
	Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferations related to Down's syndrome	Transient abnormal myelopoiesis (TAM)
	Myeloid leukaemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm	
8. Acute leukaemia of ambiguous lineage	Acute undifferentiated leukaemia
	Mixed phenotype acute leukaemia with t (9; 22) (q34; q11.2); BCR-ABL
	Mixed phenotype acute leukaemia with t (v; 11q23); KMT2A rearranged
	Mixed phenotype acute leukaemia, B/myeloid, NOS
	Mixed phenotype acute leukaemia, T/myeloid, NOS
	Provisional entity: Natural killer (NK)–cell lymphoblastic leukaemia/lymphoma

Gain of function mutations of signalling pathway receptors (*FLT3*, *KIT, FGFR, NOTCH*) or its mediators (*RAS, MAP,*) leading to constitutive activation are frequently observed in AML (Martelli *et al.* 2013; Link 2012). *FLT3* activating mutations as a result of internal tandem duplication (*FLT3-ITD)* or tyrosine kinase domain (*FLT3*-TKD) that confer ligand-independent activation, are very frequent in AML and correlate with adverse prognosis (Meshinchi *et al.* 2006). Specific FLT3 inhibitors compete with ATP binding in the intracellular domain and have been used in AML clinical trials (Weisberg *et al.* 2010). Although the first generation of inhibitors had limited success, a second

generation of *FLT3* inhibitors such AC220 show promising results in clinical trials (Zarrinkar *et al.* 2009; Small 2006). Resistance to *FLT3* inhibitors are mediated by acquired mutations that reactivate *FLT3* downstream signalling pathways, including *PI3K/AKT/MTOR*, *RAS/MAPK,* and *JAK/STAT5* also in *FLT3-ITD* cases (Weisberg *et al.* 2010; Kindler *et al.* 2010). Despite that, no mutations have been identified in members of the *PI3K/AKT/MTOR* pathway in AML, aberrant activation mediated by tyrosine kinase receptor (TKR) or crosstalk between signalling pathways, has been described to be responsible for therapy resistance in many cases (Bertacchini *et al.* 2015; Porta *et al.* 2014; Yap *et al.* 2008). Specific inhibitors of this pathway showed modest efficacy in AML clinical trials, although dual and multiple targeting of different components of the pathway in combination with chemotherapy show promising results (Bertacchini *et al.* 2015; S. Park *et al.* 2008; Vachhani *et al.* 2014; Sandhöfer *et al.* 2015). The *JAK/STAT* pathway is frequently activated by upstream mutations in TKR (Furqan *et al.* 2013; Li 2008), and the main mediator in AML is *STAT5*, which is able to activate the expression of genes driving proliferation and apoptosis resistance (Harrison 2012). Activating mutations in *RAS* are found in approximate 30% of AML patients and it has been reported that chemotherapy often activates this pathway (Schlenk *et al.* 2008; Kadia *et al.* 2012), therefore contributing to drug resistance. Recently, mutations in epigenetic regulators (*TET2, IDH1/2, DNMT3A*) have also been implicated in the pathogenesis of AML (discussed in Section 1.2.1 and 1.2.2) (Melnick 2010).

This thesis will focus on the mutations, (epi)genetic dysregulation, and chromosomal inversions or translocations involving TFs leads to aberrant miss-expression of their transcriptional targets, which favours leukaemia development. The most common and studied TFs in AML include *MLL*, *PML, EVI1, PU.1, GATA1/2, RUNX1, CEBPα,* and *HOX* genes. For instance, hereditary AML is caused by mutations in TFs essential for haematopoiesis such as *RUNX1, CEPBα* and *GATA2* (Owen *et al.* 2008). *RUNX1* mutations predispose to familial platelet disorder (FPD), an autosomal dominant disease characterised by thrombocytopenia and lower platelet count, with high risk for progression of MDS and AML (Harada and Harada 2009). Sporadic mutations in the *RUNX1* gene have also been described in *de novo* AML, and in both hereditary and sporadic these mutations are linked to adverse outcome (Osato 2004; Schnittger *et*

al. 2011; Gaidzik *et al.* 2011). Unlike other hereditary AML predisposing genes, *CEPBα* germline mutations cause AML without previous haematological conditions, and are normally associated with FAB subtypes of undifferentiated phenotype (Smith *et al.* 2004), in agreement with the role of *CEPBα* in myeloid differentiation (Wang *et al.* 2006). Patients can present with mono or biallelic *CEPBα* mutations, and generally are included in the good prognosis AML group (Tawana *et al.* 2015; van Waalwijk van Doorn-Khosrovani *et al.* 2003).

Specific chromosomal translocations can also lead to AML, as exemplified by acute promyelocytic leukaemia (APL), FAB M3 AML subtype, characterised by the differentiation block at the promeylocytic stage (Ryningen *et al.* 2008). Initially, APL had a grave prognostic (Wang and Chen 2008), however the discovery that APL originates with the chromosomal translocation *t(15;17)(q22;q21)/PML-RARA* present in all APL cells, prompted researchers to test the ability of all trans retinoic acid (ATRA) to interact, dissociate and provoke the degradation of *PML-RARA*, thereby inducing the terminal differentiation of APL cells into granulocytes (Wang and Chen 2008). Currently, differentiation therapy employing ATRA and arsenic trioxide (ATO) is a successful therapy able to achieve cure rates up to 80% in APL patients (Cull and Altman 2014). Attempts to reproduce this induction of differentiation in other AML FAB subtypes have not been successful (Johnson and Redner 2015). For instance, addition of ATRA to induction therapies of non-APL AML has failed (Delva *et al.* 1993). This is due to a lack of expression or RAR signalling in non-APL AML, where it has been found that many oncogenes in non-APL AML bind and repress RAR (Delva *et al.* 1993). Thus, novel approaches are required to circumvent the lack of expression of RAR in non-APL AML in order to release the block in differentiation of AML blasts cells. Other chromosomal translocations like *t(8;21)(q22;q22)/RUNX1-RUNX1T1* or *inv(16)(p13q22)/t(16;16)(p13;q22)/CBFB-MYH11* are also included in the good prognosis group, and can be treated with relatively high success using intensive regimens of chemotherapy (Corbacioglu *et al.* 2010). In striking contrast, translocations implicating EVI1 (3q translocation or inversion), monosomy of chromosome 5 or 7, and MLL fusion proteins have generally poor prognosis (Lugthart *et al.* 2008; Krivtsov and Armstrong 2007).

One of the alterations typically associated with the FAB-M4 or M5 subtypes of human AML is the translocation t(9;11)(p22;q23) producing the fusion protein *MLL-AF9* (*MLLT3*) (Alonso *et al.* 2008). Mixed lineage leukaemia (*MLL*) has over 60 different fusion partners in AML, although as implied by the name, the MLL gene could be rearranged in both AML and acute lymphoid leukaemia (ALL), or present with mixed myeloid/lymphoid leukaemia (Super *et al.* 1997; Krivtsov and Armstrong 2007). The wild-type MLL gene encodes a SET domain containing H3K4 histone methyltransferase activity that interacts with many other proteins to regulate the epigenetic status of the cell (Bernt *et al.* 2011; Krivtsov and Armstrong 2007). Indeed, retroviral insertion of *MLL-AF9* in murine HSPCs initiates the leukaemogenic program *in vitro* and is able to induce AML *in vivo* (Krivtsov *et al.* 2006; Somervaille and Cleary 2006). One of the direct targets and main drivers of *MLL-AF9* leukaemia is the *HOX* family of TFs, especially *HOXA9* (Faber *et al.* 2009). Overexpression of *HOXA9* is found in human and mouse *MLL-AF9* AMLs, and deletion of *HOXA9* abrogates AML development (Faber *et al.* 2009). In addition, co-expression of *Meis1a,* a *HOX* transcriptional cofactor with *Hoxa9* in HSPCs confers aberrant self-renewal and promotes leukaemia formation (Kroon *et al.* 1998). *HOXA9* and *MEIS1A* are not only overexpressed in *MLL-AF9* AML, but also in 40% of adult AML patients and are linked with adverse outcome (Lawrence *et al.* 1993).

Gene dosage is a critical factor for AML development, exemplified by *PU.1*, an Ezb transformation-specific sequence (Ets) TF indispensable for myelomonocytic differentiation during normal haematopoiesis (Scott *et al.* 1994; McKercher *et al.* 1996). Mutations or down-regulation of *PU.1* have been reported to play a central role in AML (Bonadies *et al.* 2010). One of the regulatory mechanisms of *PU.1* expression is mediated by an upstream regulatory element (URE) (Mak *et al.* 2011; Li *et al.* 2001). Whereas *Pu.1* knockout (KO) mice die at birth (Scott *et al.* 1994), the KO of URE induces a decrease of the PU.1 gene expression by 80%, leading to AML and B-CLL-(B-CLL) like disease (Rosenbauer *et al.* 2004).

Understanding the molecular basis of TFs in malignant haematopoiesis is essential to develop new and targeted therapies for specific AML molecularly defined subtypes. Such TF perturbations specifically occurring in HSCs or progenitor cells, lead to the emergence of rare leukaemia stem cells (LSCs), which sustain and nurture the bulk of

leukaemia cells in AML. Evidence for the existence of LSCs in AML has been an area of intense investigation over the last 20 years.

1.2.1. Acute myeloid leukaemia stem cells

LSCs, like their normal HSC counterparts, comprise a small fraction of the total leukaemic population, and there is evidence of a hierarchical cellular organization of human AML consistent with the cancer stem cell (CSC) model (**Figure 1.3**) (Dick 2005). In AML, the first conclusive evidence of LSCs was published in 1997 (Bonnet and Dick 1997). Bonnet and Dick fractionated the AML cells from patients using cell surface markers and tested the leukaemia initiating capacity (LIC) of each subset of AML cells *in vivo* in transplantation assays (Bonnet and Dick 1997). They obtained the highest LIC in a small $CD34^+CD38^-$ population, which also contains HSCs in healthy patients, whilst detecting a lower LIC activity in the $CD34^+CD38^+$ subset, containing progenitor cells (Bonnet and Dick 1997). During the past 2 decades, distinct surface markers have been identified to define LSCs. In the majority of AML patients, 75%, are $CD34^+$, defined by more than 10% of CD34 positive cells, while the remaining 25% have less than 10% of CD34 positive cells, named $CD34^-$ AML, where the highest LSC activity resides in the $CD34^-$ fraction (Thomas and Majeti 2017). Therefore, it appears that different populations with LSC activity are found in both the CD34 positive or negative fraction, within the same patient. This in turn led to a study analysing the immunophenotype of 100 $CD34^+$ samples, and together with xenotransplantation assays, confirmed the coexistence of two distinct LSC subsets in 80% of the samples; a fraction resembling lymphoid-primed multipotent progenitors (LMPP-like LSCs) defined by $Lin^-CD34^+CD38^-CD90^-CD45RA^+$, and a GMP-like LSC subset, $Lin^-CD34^+CD38^+CD123^+CD45RA^+$ (Goardon *et al.* 2011). The LMPP-like population was able to give rise to the GMP-like subset, though this was not a reciprocal event (Goardon *et al.* 2011). In 15% of $CD34^+$ AML, LSCs are believed to reside in a dominant MPP-like population ($CD34^+CD38^-CD90^-CD45RA^-$). The highest LSC capacity resides in the $CD34^+CD38^-$ (MPP-like and LMPP-like fractions), and these LSCs are molecularly distinct from the GMP-like LSCs (Goardon *et al.* 2011). Other surface markers have been investigated in order to identify LSCs, such CD123, CD44, CD47, TIM3, CD99 (Bonardi *et al.* 2013; Han *et al.* 2015; Schubert *et al.* 2011; Jan *et al.* 2011), reflecting both the heterogeneity of the disease itself and LSC populations.

Thus, further research is required to define consistent markers of LSCs, to understand the underlying biology, in order to track residual disease and stratify patient treatment during therapy.

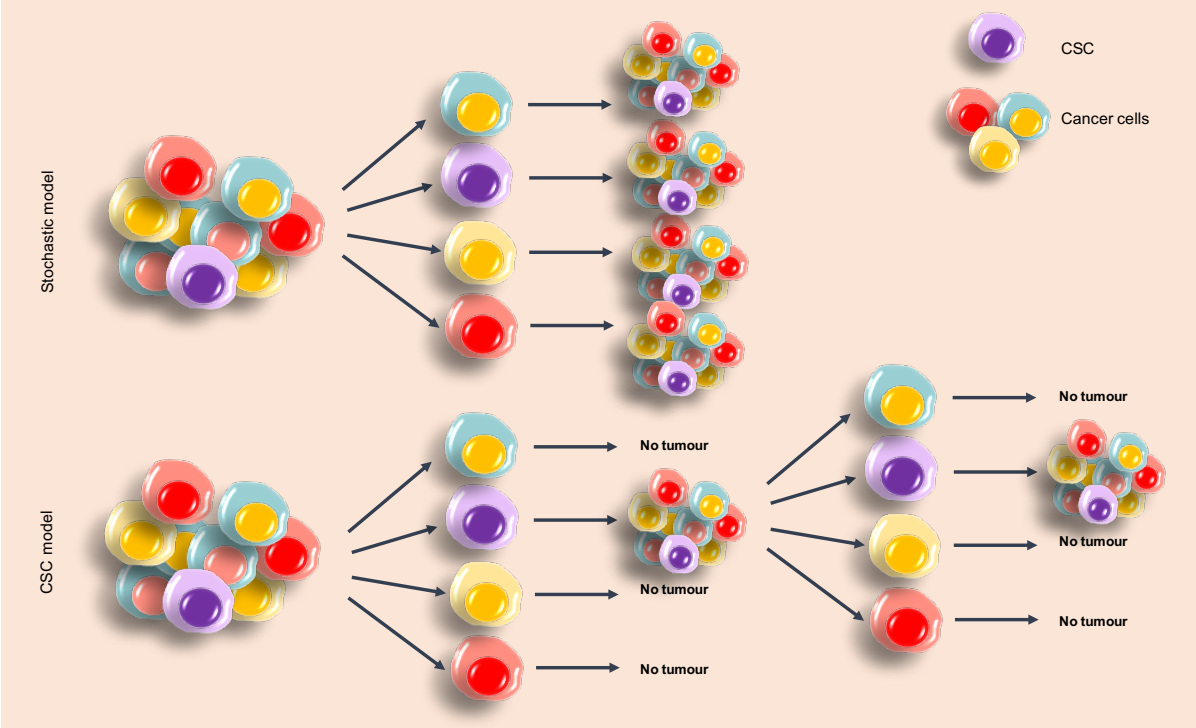


Figure 1.3. Stochastic versus cancer stem cell (CSC) model. According to the stochastic model, any cancer cell within a tumour is able to initiate a tumour. In contrast, the CSC model postulates the existence of biologically distinct classes of cells with different features. Only a subset of cells, CSCs, can initiate tumour growth; CSCs possess self-renewal and produce non tumour initiating cells that make up the bulk of the tumour.

Efforts in recapitulating human AML biology using transgenic or retroviral mouse models, have led to the development and characterisation of mouse models that harbour specific chromosomal translocation or mutations. One of the most studied translocations in these mouse models is *MLL-AF9*. Using a retroviral system that overexpresses the human *MLL-AF9* transgene, two studies led by Krivtsov *et al*., and Somervaille and Cleary, transformed and initiated leukaemogenesis from GMP or HSPCs, and were able to induce and propagate AML upon transplantation in irradiated mice (Krivtsov *et al.* 2006; Somervaille and Cleary 2006). They found a leukaemia hierarchy, in which LSCs are as frequent as 25-30%, are positive for the mature myeloid-markers *Mac1* and *Gr1*, but also express the stem cell marker *C-kit* (Somervaille and Cleary 2006). *Krivtsov et al*. defined these LSCs as GMP-like since they immunophenotypically resembled murine GMP ($Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}CD34^{low}CD16/32^{+}$) (Krivtsov *et al.* 2006). Interestingly, GMP-like LSCs resemble the gene expression profile of their *wild-type* counterparts, and the reactivation of a

HSC self-renewal signature, including *Hox* genes and *Evi1* (Krivtsov *et al.* 2006; Krivtsov *et al.* 2013). Another epigenetic modifier, *Tet2,* that is frequently mutated in AML (Young *et al.* 2016), give rise to AML when *Tet2*$^{-/-}$ mice are crossed with *Flt3*^{ITD} mice. In these animals, the LSC population resides in the MPP-like population (LSK CD48^{+}CD150^{-}), while GMP are not able to induce or propagate the disease in this model (Shih *et al.* 2015). Therefore, heterogeneity in AML LSCs is not only observed in patients, but also in AML mouse models, which offer a tractable model to develop specific inhibitors of LSCs. Indeed, several epigenetic inhibitors, such as BET inhibitors, that are currently used in AML, were first tested using mouse LSC models that target the bromodomain and extra terminal (BET) family of proteins (BRD2, BRD3, BRD4 and BRDT), which bind to histones and are crucial for the chromatin remodelling of *MLL* oncogenic translocations (Fong *et al.* 2015; Shafer and Grant 2016). Another component of the *MLL* epigenetic complex is the methyltransferase *Dotl1,* whose deletion abrogates *MLL-AF9* AML, has led to the development of small molecule inhibitors of *Dotl1* (Spurr *et al.* 2016), currently in clinical trials of AML (Nguyen *et al.* 2011; Daigle *et al.* 2013; Daigle *et al.* 2011). Isocitrate dehydrogenase 1 or 2 (IDH1/2), commonly known as Krebs cycle enzyme (Grassian *et al.* 2014), is also able to regulate the epigenetic status by histone modification (Figueroa *et al.* 2010). Mutations on both IDH1 and IDH2 have been found in around 10% of AML patients (Patel *et al.* 2011), provoking a differentiation block and DNA and histone hypermethylation, at least in part, by disrupting *Tet2* activity (Figueroa *et al.* 2010) and accumulation of the oncometabolite 2-hydroxygulatarate (Lin *et al.* 2015). IDH inhibitors are currently showing promising results in AML clinical trials in patients with IDH1 or 2 mutations (Levis 2013; Fujii *et al.* 2016; Shafer and Grant 2016).

In agreement with the CSCs model, LSCs are thought to drive leukaemogenesis by nurturing the bulk of blasts cells, and are responsible for AML relapse in response to standard chemotherapy (Dick 2005). These properties are endowed in LSCs due to the acquisition of an aberrant self-renewal capacity, apoptosis resistance, while also being able to maintain a quiescent and hypoxic state (Dick 2005).

Several TFs such as *HOXA9* confer self-renewal of LSCs (Kroon *et al.* 1998). *Bmi1* confers self-renewal of LSCs in *MLL-AF9* as *Bmi1* deficient LSCs are unable to propagate AML into secondary recipients, and these self-renewal effects are mediated

in part by *Hoxa9* (Smith *et al.* 2011). The Wnt/β-catenin and hedgehog signalling pathway have also been found to be responsible for LSC self-renewal (Wang *et al.* 2010; Heidel *et al.* 2011). miRNA-126 has been recently identified to be highly expressed in AML patient and to act as critical regulator of LSCs quiescence (Lechman *et al.* 2016). miRNA-126 overexpression in LSCs induces quiescence and thus promotes chemotherapy resistance by negatively impacting the PI3K/AKT/MTOR pathway (Lechman *et al.* 2016), a known regulator of LSC self-renewal (Sykes *et al.* 2011).

Quiescence is characterised by a low-level metabolic rate (Laporte *et al.* 2011; Signer *et al.* 2014), hence understanding what fuels LSCs could be used therapeutically to awaken quiescent LSCs that are resistant to apoptosis. Like in HSCs, there is controversy about the hypoxia requirements in LSCs. Dominique Bonnet's group found that *HIF2* is expressed in AML, and *HIF2α* knockdown using short hairpin RNA (shRNA) resulted in impaired AML growth *in vitro* and *in vivo,* highlighting the potential use of HIF inhibitors in AML (Rouault-Pierre *et al.* 2013). *HIF2α* deficient AML cells produce ROS that provoke endoplasmic reticulum (ER) stress, which in turn leads to apoptosis by activation of the unfolded protein response (UPR) pathway (Rouault-Pierre *et al.* 2013). However, in contrast to the use of shRNA knockdown in cells cultured in normoxia (Rouault-Pierre *et al.* 2013), recent findings using haematopoiesis-specific gene knockout mouse models, demonstrate that deletion of *Hif2α* accelerated LSC development in *Meis1a/Hoxa9* mouse models and in a *Mll-af9* transgenic mouse models (Vukovic *et al.* 2015). Interestingly, co-deletion of both *Hif1α* and *Hif2α* synergise to accelerate the development of AML (Vukovic *et al.* 2015), suggesting that *Hif1α* and *Hif2α* are tumour suppressors in AML driven by *Mll-af9* and *Meis1a/Hoxa9* oncogenes, consistent with a previous report showing that *Hif1α* acts as a tumour suppressor in *Meis1a/Hoxa9* and *Mll-enl* mouse models (Velasco-Hernandez *et al.* 2014). In addition, it was demonstrated that neither pharmacological inhibition or CRISPR/Cas9 deletion of *HIF2α* affected growth of AML cell lines harbouring *MLL-AF9* translocation (Vukovic *et al.* 2015), highlighting the need for stratification and assessment of HIF targeting in individuals AML molecular subtypes. A role for *Hif2α* as a tumour suppressor has also gained support from other cancer types, such as glioblastoma (Acker *et al.* 2005).

Despite the exhaustive research in understanding LSC biology in AML, and with the exception of APL, a meaningful improvement in the survival of AML patients over the last 40 years has not been forthcoming (De Kouchkovsky and Abdul-Hay 2016). Advances in understanding epigenetic regulation of LSCs and in LSC-BM niche interactions have afforded the development of new and specific inhibitors of LSCs with clinical potential. Yet, it seems unlikely to find a drug that targets all types of AML LSCs, but rather to design specific inhibitors that abrogate the main drivers underlying LSC resistance in each cytogenetic and molecular subtype.

1.2.2. AML Pre-LSCs and cell of origin

In mice, several studies aimed to decipher the cell of origin in AML, and the impact of gene deletion depending of the cell of origin. *MLL-AF9* is able to initiate clonal leukaemic colony growth from HSCs, CMP and GMP with similar cloning efficiency *in vitro*, yet *in vivo* AML development is faster in AML that originates from HSCs compared to GMP (Krivtsov *et al.* 2013). This was underpinned by expression of HSC specific genes that confer self-renewal, such as *Evi1*, which are expressed in L-GMP derived from HSCs but not from L-GMP derived from GMP (Krivtsov *et al.* 2013). This signature is capable of stratifying *MLL-AF9* AML patients; as a result, L-GMP-HSC signature is linked with poor prognosis and shorter overall survival (Krivtsov *et al.* 2013). Krivtsov *et al*., also demonstrated that the L-GMP-HSC signature conferred chemotherapy resistance (Krivtsov *et al.* 2013)(**Figure 1.4**). Another study employed a *Mll-af9* knock-in mouse model, in which the *MLL-AF9* translocation is under the control of the endogenous Mll promoter, develop aggressive AML within 5-6 months after birth and closely recapitulate multiple features of human leukaemia (Corral *et al.* 1996). This research outlined that HSC, but not CMP or GMP, from preleukaemic *Mll-af9*^{KI} mice were able to recapitulate AML in an irradiated host (W. Chen *et al.* 2008). They compared the *Mll-af9*^{KI} mice with the same retroviral *MLL-AF9* model used by Armstrong's lab, and found that the dose of *MLL-AF9* in GMPs was much higher in the retroviral model compared to the knock-in mice, suggesting that *MLL-AF9* can reprogram and activate the HSC signature in GMP only at supraphysiological dose levels (W. Chen *et al.* 2008). Finally, since the latency of AML development after transplantation was similar to the latency required in the starting *Mll-af9*^{KI} cohort, the authors concluded that HSCs in this model harbour the cell of origin (pre-LSCs), but

not LSCs, or that they were not LSCs at the time-frame used for their experiments (8-week old mice) (W. Chen *et al.* 2008). Interestingly, when Vukovic *et al*. (Vukovic *et al.* 2015) transplanted LSK cells from leukaemic *Mll-af9*^{KI} mice, the recipients also developed AML with long latency (30 weeks), however, when they re-transplanted LK cells, containing GMP and CMP, into recipients, the latency of AML development was shorter (20 weeks), suggesting that LSCs in this model might reside in the progenitor compartment (Vukovic *et al.* 2015). Of relevance, the function of specific genes in these models is dispensable or required depending on the cell of origin. For instance, genetic deletion of *β-catenin* showed that LSK but not CMP from *Meis1a/Hoxa9* require *β-catenin* for LSC development, whereas GMP but not LSK nor CMP require *β-catenin* for LSC development driven by *Mll-enl* oncogene (Siriboonpiputtana *et al.* 2017; Wang *et al.* 2010; Yeung *et al.* 2010).

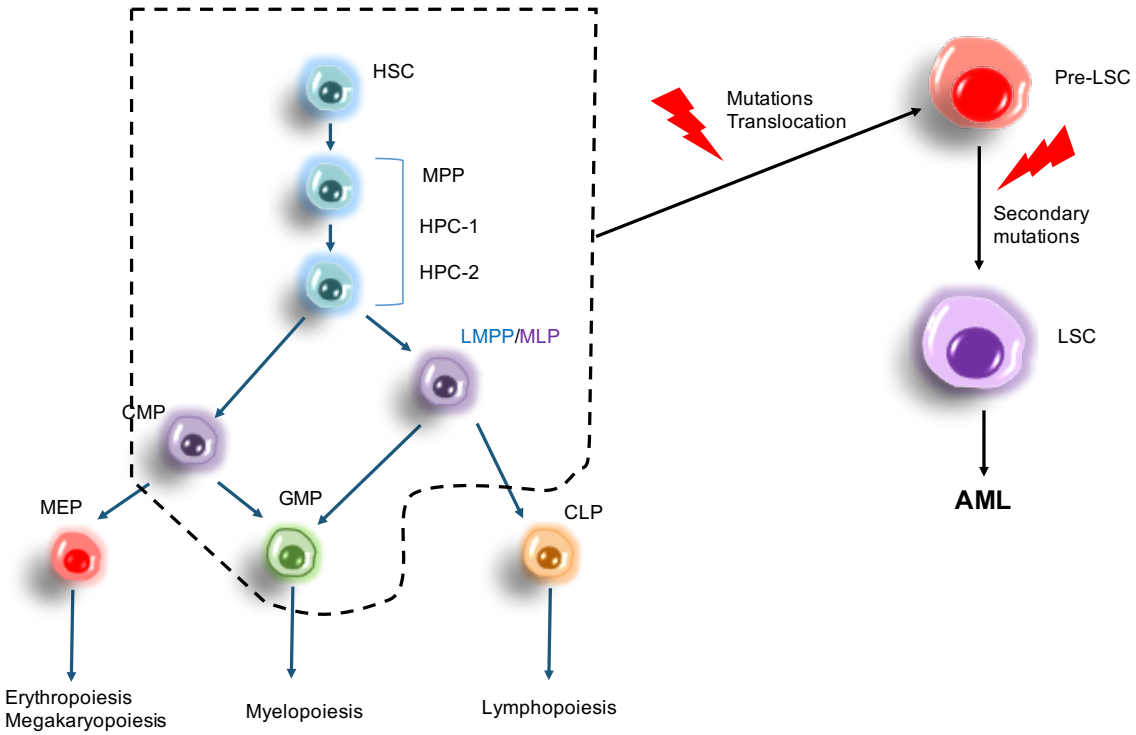


Figure 1.4. Cell of origin in AML. The cell of origin in AML could be a HSC or a downstream myeloid progenitor. Mutations or translocations in HSC or myeloid progenitors (black dotted lines) leads to the generation of Pre-LSC. Pre-LSCs that acquire secondary mutations become LSCs leading to AML development.

The dogma of LSC biology and cell of origin in AML changed when attempting to identify residual HSCs from LSCs in patients. With the combination of the cell surface markers TIM3 and CD99, researchers were able to sort HSCs from LSCs, and surprisingly found that most of the patients harboured founder mutations in normal HSCs, but not secondary mutations present in LSCs, and these HSCs were termed pre-LSCs (Jan *et al.* 2011; Shlush *et al.* 2014). Molecular analysis of these pre-LSCs showed that they mainly harbour mutations in epigenetic modifiers (*TET2, DNMT3A*, and *IDH1/2*) but not or few proliferative mutations (Sykes *et al.* 2015), suggesting that the pre-leukaemic status is guaranteed by aberrant epigenetic regulation, while acquisition of proliferative mutations may drive the evolution from pre-LSCs to LSCs. Xenograft and mouse model studies of these epigenetic modifiers showed that loss of *TET2* or *DNMT3A* and gain of function of *IDH1/2* leads to enhanced self-renewal activity of HSCs (Sykes *et al.* 2015; Shlush *et al.* 2014). HSC pre-LSCs from patients are able to give rise to multilineage haematopoiesis in NSG mice, whilst also retaining the same mutation. Single-cell analysis of pre-LSCs at diagnosis and follow-up analysis post treatment at remission and relapse, showed that the frequency of pre-LSCs at relapse is an indicator of prognosis (Shlush *et al.* 2017). This suggests that pre-LSCs and not exclusively LSCs, are responsible for relapse. Clonal analysis of LSCs in AML patients confirmed that in some patients, the LSC clones had different secondary mutations at relapse when compared to at first diagnosis, whereas the founder mutation was present at all stages (Sykes *et al.* 2015). Importantly, Pre-LSC from patients exhibit chemotherapy resistance; given that LSCs are formed from pre-LSCs, they thus provide an additional cellular reservoir that may cause relapse and leukaemia progression after initial treatment.

Clonal, pre-leukaemic haematopoiesis therefore occurs when HSCs present with initiating mutations, especially in epigenetic modifiers, that are a characteristic prelude to AML. Efforts are now focusing on elucidating the prognostic value of these mutations, and whether preventive treatment would benefit these individuals (Sperling *et al.* 2016; Steensma *et al.* 2015). Therefore, the identification of pre-LSCs has allowed the prospect for preventive treatment; however, the lack of distinct cell-surface markers to detect pre-LSCs remains problematic, as they remain indistinguishable from normal HSCs.

1.2.3. Malignant niche in the initiation and pathogenesis of AML

Harmony between HSCs and the signals that they receive from their surrounding BM microenvironment or extramedullary tissues preserves life-long production of blood cells. Disruption of these interactions can lead to malignant haematopoiesis, since it is thought that malignant cells (i.e. pre-LSCs, LSCs, MDS-CSCs) require a specialised niche that support their growth and expansion, whilst are unfavourable for normal HSCs, aiding the expulsion or depletion of residual normal haematopoiesis during malignant transformation and progression. Whether primed malignant cells secrete factors and modify the niche to create a malignant environment, or mutated niche cells favour transformation of malignant cells from normal HSCs, are current theories that have been proposed.

Fibrosis, neuropathy, and pro-inflammatory cytokines are common features present in the BM of leukaemic mice and patients (Scott *et al.* 2007; Tabe and Konopleva 2015; Hanoun *et al.* 2014). The osteoblastic niche has gained special relevance in haematological malignancies. Preferential osteoblastic differentiation of MSCs has been shown using mouse models of MPN (Schepers *et al.* 2013), CML (Bowers *et al.* 2015), and AML (Wei *et al.* 2008). In contrast with normal HSCs, in these settings, LSCs preferentially locate near the endosteum (Lane *et al.* 2011; Zhou *et al.* 2016), are able to recruit *Nestin*$^{+}$ cells that undergo osteoblastic differentiation (Hanoun *et al.* 2014; Arai and Suda 2007) and release pro-inflammatory cytokines and growth factors such as *IL-6, G-CSF, GM-CSF, IL-1*β that support LSC growth (Ishikawa *et al.* 2007). Human primary AML cells lodged within the osteoblastic-niche in NSG mice and hard to engraft primary MDS cells are able to induce MDS-like disease in NSG mice when they are co-transplanted with patient derived MSCs (Ishikawa *et al.* 2007). This highlights that mouse and human LSCs are highly niche-dependent, and especially osteoblastic-dependent. The *Wnt* pathway plays an important role in the osteoblastic niche in disease initiation. Constitutive expression of stable *β-catenin* in osteoblasts in mice leads to upregulation of Jagged1 and consequent activation of Notch signalling pathways in HSPCs, leading to AML development (Kode *et al.* 2014). In addition, 38% of MDS/AML patients show nuclear accumulation of *β-catenin* in osteoblastic cells in

conjunction with upregulation of *Notch* effectors *Hes1, Hes5 and Hey1* in $CD34^+$ AML cells (Kode *et al.* 2014).

The sympathetic nervous system (SNS) is also an important regulator of the BM niche. In mouse models of MPN driven by the *Jak2 V617F* mutation in HSCs, remodelling of the BM niche with osteoblastic lineage skewing and loss of nerve fibres accelerates MPN development (Arranz *et al.* 2014), features also seen in patients (Mascarenhas *et al.* 2012). Treatment with β3 agonists reduced disease progression by indirectly promoting the formation of *Nestin*$^+$ MSCs that in turn affected MPN cells (Arranz *et al.* 2014). In murine models of AML driven by the *Mll-af9* oncoprotein, osteoblastic remodelling is also observed in the BM niche, and disruption of β2 adrenergic signalling in malignant stromal cells favours AML development (Hanoun *et al.* 2014). Therefore, adrenergic agonists used in the clinic, especially those who target β2/3 adrenergic receptors, should be considered in clinical trials for haematology malignancies. Development of MPN disease seems to be extrinsically initiated by the BM niche. A well-known cell-cycle regulator, *Retinoblastoma* (*Rb*), is dispensable for HSC maintenance in a cell-autonomous manner (Walkley and Orkin 2006), however niche-dependent deletion of *Rb* induces overproliferation and extramedullary haematopoiesis of HSCs, key features of a MPN phenotype (Walkley *et al.* 2007). Activating mutations of *Ptpn11,* that encodes *Shp2* phosphatase, a positive regulator of the *Ras* pathway, leads to a MPN in mice (Dong *et al.* 2016). Cell-autonomous activation of *Ptpn11* with *Vav-Cre* produce MPN, but with a weaker phenotype than when activated also in the BM niche with *Mx1-Cre* (Dong *et al.* 2016). Activation of *Ptpn11* in differentiated osteoblasts or endothelial cells did not induce MPN, though activation in MSCs, perivascular cells and osteoprogenitors leads to HSC hyperactivation and secretion of *CCL3* with progression to MPN-like disease. In addition, pharmacological targeting of *CCL3* reversed the development of MPN initiated by the BM niche (Dong *et al.* 2016).

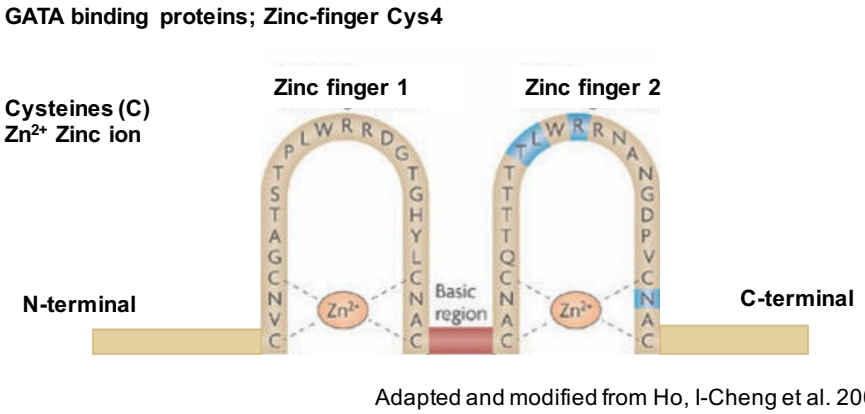
The interactions between the niche and leukaemic cells have other implications for therapy and relapse after treatment. Malignant haematopoietic and niche cells can build together an environment that protects leukaemic cells, providing sanctuary from standard chemotherapy (Jacamo *et al.* 2014; Duan *et al.* 2014; Pallasch *et al.* 2014).

For example, mouse and human ALL cells were found surrounded by osteoblasts, and *Nestin*$^{+}$ with characteristics resembling that of α-smooth muscle cells, thus generating a physical and protective niche that attenuates the level of chemotherapeutics that reaches leukaemic cells (Duan *et al.* 2014).

1.3. GATA family of transcription factors

Transcription factors (TFs) are proteins that are able to sense signals provided from signalling pathways, integrate the signal and give an appropriate response in form of activation or repression of specific genes. The characteristic feature of a TF is the ability to bind the DNA at the promoter or enhancer regions. As a general rule, TFs co-operate with other TFs and form multiprotein complexes with co-activator/repressors that will define the target specificity and refine the type of transcriptional regulation (Vaquerizas *et al.* 2009).

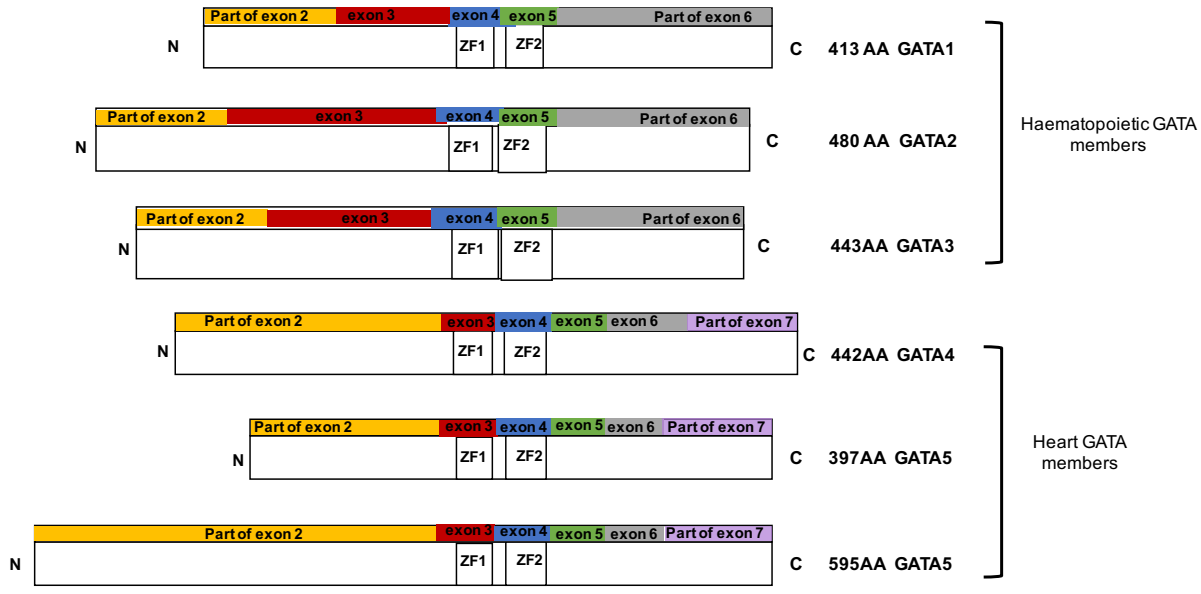
GATA binding proteins are a family of zinc finger (ZF) transcription factors (TFs) that bind to "(A/T)GATA(A/G)" sequences in the promoter region of the DNA, to activate or repress the transcriptional activity of a target gene (Merika and Orkin 1993; Ko and Engel 1993). *GATA* belong to the subfamily Cys2Cys2 ZF-TFs, in which a zinc ion stabilizes the ZF fold structure by interacting with the four cysteines (Merika and Orkin 1993; Ko and Engel 1993). *GATA* TFs have two zinc-finger domains that allow binding to DNA. Each zinc-finger domain has four cysteine residues that chelate zinc in order bind to the DNA or to interact with other TFs (Merika and Orkin 1993; Ko and Engel 1993). Each zinc-finger domain has different functions, whereby the zinc-finger domain 1 or the N-terminal increases the DNA-protein or protein-protein interactions, and the zinc-finger domain 2 or C-terminal has ability to bind DNA (Merika and Orkin 1993; Ko and Engel 1993) (**Figure 1.5**).



Adapted and modified from Ho, I-Cheng et al. 2009

Figure 1.5. GATA binding proteins family of Zinc Finger TFs. AA and functional ZF domain of *GATA* factors. Four cysteines interacts with a zinc ion which provide structural stability and is required for proper DNA-protein or protein-protein bindings. Specific AA represented here belong to *GATA3*.

There are 6 members in the *GATA* family (*GATA1-6*) (Merika and Orkin 1993; Ko and Engel 1993). They are each temporally expressed and within specific tissues (Patient and McGhee 2002; Lowry and Atchley 2000; Charron and Nemer 1999), although due to the strong homology between them, in some biological processes, their expression overlaps as they co-regulate each other (Merika and Orkin 1993; Ko and Engel 1993). They play major roles in the development and adult maintenance of the haematopoietic and cardiovascular system, and breast and prostate tissues (Patient and McGhee 2002; Lowry and Atchley 2000; Charron and Nemer 1999). *GATA* family protein activity has also been found in the nervous system, lung, pancreas, gut, endothelium, and fat tissue (Patient and McGhee 2002; Lowry and Atchley 2000; Charron and Nemer 1999). In broad terms, *GATA1-3* is primarily found in the haematopoietic system, whereas *GATA4-6* is expressed in the heart, liver, lung, gonad and gut (**Figure 1.6**).



Adapted and modified from Marjolein HFM Lentjes et al., 2016

Figure 1.6. GATA members. Gene structure showing the ZF domain and surrounding exons from all the GATA members.

The first *GATA* member to be identified, *GATA1*, was described in 1988 in chickens (Evans *et al.* 1988). It plays an essential role in haematopoiesis, specifically in primitive and definitive erythropoiesis (Pevny *et al.* 1991). In fact, *Gata*1 activates globin gene expression, essential for red blood cell function (Miccio *et al.* 2011). Stuart Orkin's laboratory found that *Gata1 KO* embryonic stem (ES) cells failed to contribute to

primitive haematopoiesis in chimera experiments (Pevny *et al.* 1991). *Gata1* is also expressed in mast cells and megakaryocytes, but not in HSPCs (Pevny *et al.* 1991). Specifically, higher expression of *GATA1* is found in MDS patients (Hopfer *et al.* 2012), while *GATA1* mutations are associated with anaemia/thrombocytopenia, and with transient myeloproliferative disorder (TMD) and acute megakaryoblastic leukaemia (AMKL) in Downs syndrome patients (Ahmed *et al.* 2004).

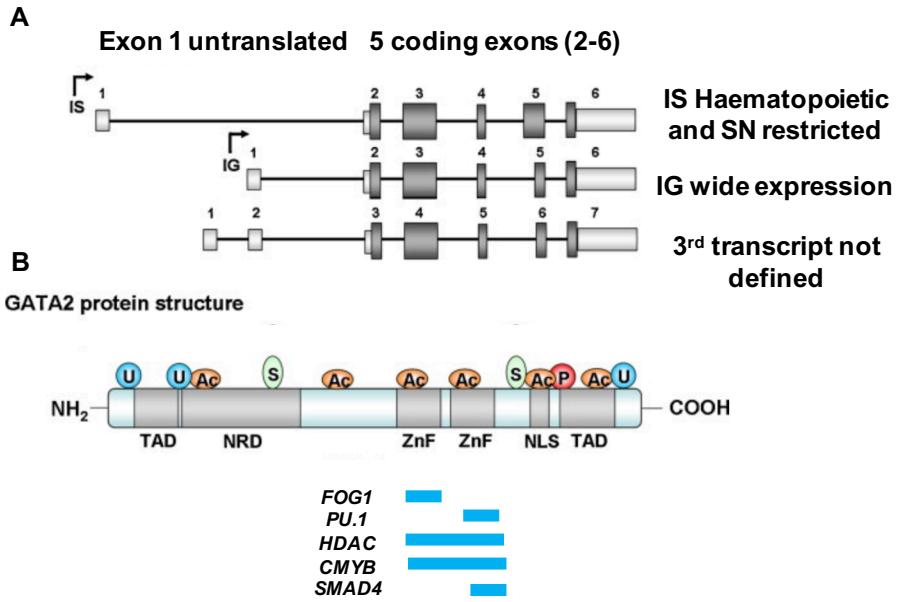
GATA3 and *GATA2* were identified in the chicken (Lilleväli *et al.* 2007), where in the first instance, *Gata3* expression was detected in T cells (Ho *et al.* 2009). In line with this observation, *Gata3* plays a role in T cell development by activating the T cell receptor (TCR) (Ho *et al.* 2009) whilst gene knockout studies found that *Gata3* also contributes to the formation of T cells in the thymus (Hamburg *et al.* 2009). In addition to its role in T cells, and given its high expression in LT-HSC, a role for *Gata3* has been postulated in murine LT-HSC. Indeed, in gene knockout studies it has been described that *Gata3* is required for regulating the cell-cycle entry of LT-HSC (Frelin *et al.* 2013), a finding that has largely been corroborated by other groups (Ku *et al.* 2012).

GATA2 was first described in human endothelial cells, where it was required for the expression of preproendothelin-1 (Dorfman *et al.* 1992). In contrast to *Gata1*, *Gata2* was found to be essential for multiple haematopoietic lineages, and not just restricted to the erythroid lineage (Tsai *et al.* 1994). *Gata2* expression is more widespread outside of the haematopoietic system; in the brain, kidney, endothelial cells, placenta, pituitary gland, prostate, adipocytes and lungs (Sheng and Stern 1999; Dorfman *et al.* 1992; Ma *et al.* 1997; Charles *et al.* 2006; Xiao *et al.* 2016; Tsai *et al.* 2005). In addition, dysregulation of *Gata2* levels (mutation or altered expression) have been implicated in haematological malignancies, and more recently in solid cancers.

1.3.1. *GATA2* gene

The murine *Gata2* gene is located on chromosome 6 spanning 8.5 kb, while the human *GATA2* gene, of approximate 14 kb, is located on chromosome 3 (Grass *et al.* 2006). In both systems, the *Gata2* gene encodes five exons, and two or three (human) mRNA transcripts give rise to different splicing isoforms (Gao *et al.* 2015). The longest,

termed *Gata2-IS (I-specific, distal first exon),* is preferentially expressed in haematopoietic and neuronal cells, while the shorter isoform, named *Gata2-IG (I-general, proximal first exon*), is expressed in all the other tissue types (Minegishi *et al.* 1998). The distal IS exon contains an untranslated region and therefore both isoforms produce the same 480 amino acid protein (Minegishi *et al.* 1998). To date, the function of the IS exon is unclear. For instance, a knock-in mouse driving GFP expression in the *Gata2* IS promoter, thus abolishing IS promoter activity, displays normal numbers of HSPCs (Minegishi *et al.* 1998; Minegishi *et al.* 2003), suggesting that the *Gata2-IG* isoform can compensate for the lack of IS promoter activity (**Figure 1.7**).



Adapted and modified from Vicente C et al., 2012

Figure 1.7. *GATA2* isoform and protein regulation. (A) Human *GATA2* gene structure showing IS, IG and a 3rd transcript that not yet been defined. (B) *GATA2* protein structure showing post-translational modifications (PTM) including ubiquitination, acetylation, sumoylation, and phosphorylation. Protein-protein interactions with *FOG1, PU.1, HDAC, CMYB,* are also illustrated (Zn finger domain participating in the interactions is highlighted). (C) *GATA2* cis regulatory elements named according to their relative position to IS promoter: -77 kb, -3.9 kb, -2.8, -1.8, +9.5.

Five different conserved *Gata2* cis-acting sites have been described to regulate *Gata2* expression in a temporal and cell-dependent fashion. The regions, named according to their relative position to the IS promoter, are the -77 kb, -3.9 kb, -2.8 kb,-1.8 kb and +9.5 kb (Katsumura and Bresnick 2017). Interestingly, *Gata2* is able to bind to these binding sites and activate its own transcription (Katsumura and Bresnick 2017), whilst at the same time, other activators or repressors can displace and modulate *Gata2* expression through these cis-regulatory elements (Snow *et al.* 2010). Genetic deletion of each of these sites has shown that in HSPCs, *Gata2* binds to the -2.8 kb site to maintain high *Gata2* levels (Johnson *et al.* 2012; Gao *et al.* 2013), while during erythroid differentiation *Gata1* displaces *Gata2* from the -1.8 kb site in order to repress *Gata2* expression, a requirement for the terminal differentiation of erythroblasts (Suzuki *et al.* 2013). No functional role in haematopoiesis has been attributed to the -3.9 kb site so far (Katsumura and Bresnick 2017). More recently, it was described that the +9.5 kb site localised in the fourth intron, acts as an enhancer in the endothelium and HSCs during HSC generation, and in fact, deletion of this site abolishes the emergence of HSCs in mice (Gao *et al.* 2016). The -77 kb site is not required for HSC generation, but instead is required for *Gata2* transcription in myeloid progenitors, and in particular in MEPs (Mehta *et al.* 2017), since -77 kb knock-out mice produce lower number of BFU-E and are unable to induce *Gata1* expression (Mehta *et al.* 2017). Finally, compound heterozygote (CH) mice revealed that *Gata2* requires both +9.5 kb and -77 kb enhancer activity in the same allele to sustain *Gata2* expression in myeloid progenitors (Mehta *et al.* 2017). Collectively, understanding the activators or repressors of *Gata2* in specific cis-regulatory elements has offered insights into the important regulatory mechanisms in HSC maintenance and generation and in malignancies where *Gata2* expression is dysregulated.

1.3.2. Gata2 function during HSCs development

Germline deficient *Gata2*$^{-/-}$ mice die with anaemia at embryonic day 10.5 (E10.5) indicating that *Gata2* is required for primitive haematopoiesis (Tsai *et al.* 1994). Chimera experiments showed that *Gata2*$^{-/-}$ ES cells were unable to contribute to the formation of myeloid and lymphoid cells at the FL stage and in 1-to-3-month-old mice, suggesting that *Gata2* is a pivotal regulator of HSCs in the haematopoietic system (Tsai *et al.* 1994). Further experiments using *Gata2*$^{-/-}$ ES cells, showed that *Gata2* is

not required for the terminal differentiation of erythroid and myeloid lineages, yet it is required for the proliferation and survival of early haematopoietic progenitor and the formation of mast cells (Tsai and Orkin 1997). Finally, the phenotype observed in the ES cell haematopoietic differentiation culture was reversed in *Gata2*$^{-/-}$;*p53*$^{-/-}$ mice, suggesting that *Gata2* and *p53* interact during early HSPC formation (Tsai and Orkin 1997). Analysis of *Gata2*$^{+/-}$ has demonstrated that *Gata2* plays functionally distinct roles in the generation of HSCs in the embryo, displaying quantitative defects in HSCs in yolk sac (YS), aorta-gonad mesonephron (AGM) and foetal liver (FL), but not in BM, and qualitative defects in HSCs in all haematopoietic generating sites throughout ontogenesis (Ling *et al.* 2004).

Table 1.5. *Gata2* haematopoietic phenotype observed in mouse models

Mouse model	Deletion and specificity	Survival	Haematopoietic phenotypes in knockout mice lacking Gata2	Haematopoietic phenotypes in recipient mice transplanted with HSC/BM cells from Gata2 KO mice
Germline Gata2 KO	Germline ESC	E10.5	Anaemia	Multilineage defects in chimera experiments (HPC defect)
Germline Gata2 HET		Alive	Reduce number of HSCs in AGM and FL Regeneration defect of BM HSCs after 5-FU treatment Reduced number and functionality of HSCs and GMPs in adult BM Increased BM HSC quiescence and apoptosis	Self-renewal defect of FL and BM HSCs
Vec-Cre	Germline E7.5-E8.5 Endothelial cells (ECs) and HSCs before HSC generation	E14	FL anaemia Reduced CFU-C	N.D.
Vav-Cre	E10.5-11.5 After HSC generation Also detected in ECs	Past E16	E11 AGM and FL CFU-C numbers decreased Decreased HSC – reduced CFU-C capacity Increased apoptosis of FL HSCs	E14 FL HSC lack of long-term reconstitution
LysM-Cre	Myeloid cells	Alive	No changes in mature blood cells	N.D.
Tie2-Cre	Germline E6.5 ECs and HSCs	Embryonic lethal	Heterozygous mice survive until adulthood with decreased myeloid and dendritic cells.	N.D.
Tamoxifen-Cre	Conditional upon tamoxifen injection	10-14 days after tamoxifen induction	Multilineage defect	Multilineage defect

N.D. – Not determined

To study the absolute requirement for *Gata2* in developing HSCs in the FL, a tamoxifen-inducible Cre mouse was employed to circumvent the lethal phenotype observed at E10.5 in *Gata2*$^{-/-}$ embryos (Lim *et al.* 2012). A decrease in the numbers and functionality of *Gata2*$^{-/-}$ FL HSCs was observed (Lim *et al.* 2012). Mirroring this study, in other genetic models, *Gata2* has been deleted in haemogenic endothelium cells before the generation of HSCs using the *Vec-Cre* system, and after HSC formation using the *Vav-Cre* system (de Pater *et al.* 2013)(**Table 1.5**). Reduced numbers and qualitative defects of HSCs in transplantation and CFU assays were

observed after deletion of *Gata2* with either the Vec or Vav system (de Pater *et al.* 2013). These results were accompanied by an increase of apoptotic HSCs, indicating that *Gata2* is essential for the generation and survival of HSCs in the mouse embryo, in a cell autonomous manner (de Pater *et al.* 2013). In agreement with this, using a *Gata2* Venus reporter mouse, it was shown that almost all HSPCs express *Gata2*, although rare *Gata2*-independent HPC population with higher expression of *Gata3* and *Gata4* were observed, indicating some redundancy of *Gata* family members in the development of the haematopoietic system (Kaimakis *et al.* 2016).

1.3.3. *Gata2* transcriptional network during HSC development

Although many studies have found interactions and downstream/upstream regulators of *Gata2* in HSC generation, endothelial-haematopoietic transition (EHT) is a highly elaborate process that involves the co-operation and formation of complex TF networks. This is exemplified by the study from Pimanda *et al.* who employed genome wide analysis to study the combinatorial interactions for ten TFs involved in HSC generation (*Scl/Tal1, Lyl1, Lmo2, Gata2, Runx1, Meis1, Pu.1, Erg, Fli1* and *Gfi1b*) (Beck *et al.* 2013). A network comprising seven TFs named “heptad” of TFs, including *Scl/Tal1, Lyl1, Lmo2, Gata2, Runx1, Erg,* and *Fli1*, was found to regulate the emergence of HSPCs in a combinatorial manner (Diffner *et al.* 2013). With the exception of *Lmo2* (McCormack *et al.* 2010) the remaining TFs have DNA binding activity, and the different combinations of this heptad regulate the transcriptional activity of target genes by binding to regulatory elements of the promoter regions (Diffner *et al.* 2013).

The first evidence of *Notch1* and *Gata2* regulation in haematopoiesis was found in relation to myeloid and erythroid differentiation using murine cell lines. Overexpression of *Notch1* in F5-5 or 32D cell lines inhibited erythroid and granulocytic differentiation, respectively (Kumano *et al.* 2001). In this setting, *Gata2* was upregulated, and repression of *Gata2* with dominant-negative *Gata3* or *Pu.1* overexpression was able to rescue the differentiation block phenotype (Kumano *et al.* 2001). *Rbpjk* null mice have been used to study the ontogeny of HSC in the mouse embryo; Rbjpk is a co-activator that together with the intracellular domain of *Notch* activates the transcription of *Notch*-target genes (Robert-Moreno 2005). *Rbpjk*$^{-/-}$ mice died at E10 with a defect

of progenitor output in CFC assays (Robert-Moreno 2005). Gene expression analysis of the AGM region showed decrease expression of *Runx1*, *Gata2* and *Scl* in *Rbpjk*$^{-/-}$ mice (Robert-Moreno 2005). In addition, *Gata2* expression in wild-type AGM cells, correspond to high *Notch1*-expressing cells. Moreover, Co-IP assays in AGM cells demonstrated that *Notch1* binds to the *Gata2* promoter, but that interaction was ablated in *Rbpjk*$^{-/-}$ mice, concluding that *Gata2* activation by *Notch1* is one of the key events in haematopoietic determination in the mouse embryo (Robert-Moreno 2005). In support of this, it has been reported that *Gata2b* expression in the zebrafish hemogenic endothelium is *Notch*-dependent (Butko *et al.* 2015).

Evi1 is a zinc-finger TF that regulates HSC in the embryo and in the adult, and is associated with poor prognosis in AML (Sato *et al.* 2008; Goyama *et al.* 2008; Lugthart *et al.* 2008). *Evi1* KO mice die at E10.5 with developmental defects in several tissues, indicating that *Evi1* is a key regulator during development (Sato *et al.* 2008). In fact, *Evi1*$^{-/-}$ embryos displayed a phenotype reminiscent of that observed in *Gata2*$^{-/-}$ embryos, including reduced FL HSC numbers and activity (Sato *et al.* 2008). *Gata2* expression was decreased in *Evi1*$^{-/-}$ embryos, and re-expression of *Gata2* rescued the proliferation defects of *Evi1*$^{-/-}$ HSCs *in vitro* (Sato *et al.* 2008). Finally, it was demonstrated that *Evi1* binds and activates the transcription of *Gata2* (Yuasa *et al.* 2005).

Etv2 (Ets variant gene 2) is a TF that regulates mesoderm specification into haematopoietic and endothelial lineages (Kataoka *et al.* 2011). *Etv2* expression is present at E7.5 in the YS, and *Etv2*$^{-/-}$ embryos are nonviable after E9.5 (Koyano-Nakagawa *et al.* 2012). An *Etv2-EYFP* transgenic mice confirmed that *Etv2* is expressed both in haematopoietic and endothelial cells (Liu *et al.* 2015). It has been postulated that *Etv2* activates the expression of several TF important for primitive and definitive haematopoiesis including *Scl*, *Lmo2* and *Fli1* (Liu *et al.* 2015). In addition, co-operation between *Etv2* and *Gata2* has also been described (Shi *et al.* 2014). Co-expression of *Etv2* and *Gata2* was present in the blood islands of the YS at E7.5, and in endothelial and haematopoietic cells at E8.5 (Shi *et al.* 2014). Interaction between *Gata2* and *Etv2* proteins was corroborated with Co-IP and GST pulldown assays, and observed that their interaction increased the transactivation activity of *Gata2* and *Etv2*

on the *Lmo2* promoter (Shi *et al.* 2014), providing genetic evidence of a critical multi-protein complex that governs primitive and definitive haematopoiesis.

Runx1 is a TF essential for HSC generation (Chen *et al.* 2009), with its deregulation being implicated in MDS and AML pathogenesis (Harada and Harada 2009; Watanabe-Okochi *et al.* 2008). In fact, *Runx1*$^{-/-}$ embryos die from a complete absence of FL HSC (Chen *et al.* 2009). *Runx1* is essential for the HSC formation from the haemogenic endothelium as demonstrated by the *Vec-cre* system, while *Runx1*$^{-/-}$*;Vav-iCre* mice survive to adulthood without HSC defects (Chen *et al.* 2009). This indicates that *Runx1* is dispensable for HSC function once they are formed in the embryo. In the same study where it was found that *Notch* signalling is required to activate *Gata2b* in the hemogenic endothelium, the authors also reported that consequently *Gata2b* activated the transcription of *Runx1,* indicating that *Gata2* is an upstream activator of *Runx1* in the hemogenic endothelium (Butko *et al.* 2015). To further corroborate the interaction of *Gata2* and *Runx1*, independent synergistic effects were found when crossing *Runx1*$^{+/-}$ and *Gata2*$^{+/-}$ heterozygous mice (Wilson *et al.* 2010). Both heterozygous mice displayed defects during HSC development, but survive to adulthood, as reported previously, however *Runx1*$^{+/-}$*;Gata2*$^{+/-}$ heterozygous mice were not viable and FL cells had reduced CFC capacity (Wilson *et al.* 2010), implicating a genetic interaction of *Runx1* and *Gata2* during the EHT process and, ergo, HSC formation.

Scl/tal1 is a bHLH TF first identified as part of the t(14;21)(q11.2;q22) chromosomal translocation in T-ALL patients (Visvader *et al.* 1998). Similarly to *Gata2*$^{-/-}$ mice, *Scl*$^{-/-}$ embryos die at embryonic day E9.5-10.5 due to anaemia (Porcher *et al.* 1996), indicating that *Scl* is a key TF during primitive haematopoiesis. Interestingly, despite the continued *Scl* expression in the haemogenic endothelium, FL and adult HSCs, conditional deletion of *Scl* with *Tie2-Cre,* which is expressed in the ECs and HSCs once they are formed, had no impact on HSC formation or maintenance (Schlaeger *et al.* 2005). This suggested that *Scl* is required for specification of the mesoderm into specialised haemogenic endothelium (HE), though is dispensable for HSC generation. Another study found that *Scl* is required for the appearance of *Vec*$^{+}$ EC cells marking the HE, confirming an early role for *Scl* in HE specification (Mead *et al.* 1998). Göttgens *et al*., reported that a protein complex formed by *Gata2, Fli1* and *Elf1* are required for activation of *Scl in vivo* (Pimanda *et al.* 2007). Additional studies showed

that enforced expression of *Gata2* in differentiating EBs induces *Scl* expression, and in addition they found regulatory elements in the *Scl* promoter occupied by *Gata2* (Lugus *et al.* 2007). Taken together, it is likely that *Gata2* is expressed before the appearance of the HE through *Notch1* and/or *BMP4* signalling, and in conjunction with *Fli1* activates *Scl* expression to specification of HE.

Lmo2 (*LIM domain only 2*) was first discovered in T-ALL (Draheim *et al.* 2011). In a similar vein to the Gata2-/- phenotype, *Lmo2*$^{-/-}$ embryos lack primitive erythropoiesis in the YS and die at E10.5 (Warren *et al.* 1994). *Lmo2*$^{-/-}$ ES cells were able to produce macrophage colonies, but failed to generate erythroid colonies *in vitro* (Warren *et al.* 1994). *Lmo2*$^{-/-}$ ES chimera experiments showed that *Lmo2* is also essential for definitive haematopoiesis and adult HSCs (Yamada *et al.* 1998). *Lmo2* is able to interact with *Gata* factors in different cell contexts; with *Gata1* for erythroid development (Wang *et al.* 2007), *Gata2* in HPC development and adult HSCs (Coma *et al.* 2013) and *Gata3* in T cells and T-ALL (Manaia *et al.* 2000).

1.3.4. *Gata2* function in adult murine HSC

As germline *Gata2*$^{-/-}$ mice failed to survive to adulthood, in order to study the role of *Gata2* in adult HSCs, Rodrigues *et al*., studied the haematopoietic system of *Gata2*$^{+/-}$ mice and found a decrease in the total numbers of HSCs in the BM, together with qualitative defects of HSCs in transplantation assays (Rodrigues *et al.* 2005). Finally, they reported an increase of HSC quiescence and apoptosis, coupled with a downregulation of the protein levels of the anti-apoptotic gene Bcl-xL in *Gata2*$^{+/-}$ mice (Rodrigues *et al.* 2005). These data support the idea that gene dosage in the context of *Gata2* levels must be tightly regulated for proper HSC functionality. A 5-fold decrease of BM HSC numbers in *Gata2*$^{+/-}$ has been confirmed in another study (Guo *et al.* 2013) that used single-cell analysis. Interestingly, the five most differentially expressed genes in *Gata2*^{high} HSC compared to *Gata2*^{int} HSC, were *Cd150*, *Cd41*, *Gata1, Nmyc* and *Gata2* itself, suggesting potential downstream target genes of *Gata2* in adult HSCs (Guo *et al.* 2013). More recently, *Gata2* was conditionally deleted in adult mice using a tamoxifen-Cre system, and upon *Gata2* deletion multilineage differentiation failure was observed in the BM, spleen and thymus of *Tx;Gata2*$^{-/-}$ mice. To overcome the caveats derived from *Gata2* deletion in extramedullary sites using a

systemic (non-tissue specific) Cre model, they also performed cell-autonomous transplantation assays to confirm the multilineage defects from *Tx;Gata2*$^{-/-}$ BM cells and found a depletion of *c-kit* expression in *Tx;Gata2*$^{-/-}$ BM cells, indicating HSPCs loss after *Gata2* conditional deletion (H. S. Li *et al.* 2016).

1.3.5. GATA2 transcriptional network in adult murine HSC

In addition to a role in HSC formation and FL HSC, the upstream regulator of Gata2, *Evi1*, is also a critical regulator of adult HSC. *Evi1* deletion in adult HSC using the *Mx1-Cre* system or lentiviral Cre, results in HSC reduction *in vitro* and *in vivo* without affecting differentiated cells (Goyama *et al.* 2008). In addition, haploinsufficient *Evi1* mice display reduced HSC numbers, and reduced recovery after 5-FU treatment (Bard-Chapeau *et al.* 2014). *Gata2* was downregulated in lentiviral Cre deleted *Evi1*$^{-/-}$ LSK cells (Goyama *et al.* 2008). Though Gata2 was not assessed, the phenotype observed in *Evi-1* haploinsufficient mice is reminiscent of that observed by Rodrigues *et al*., 2005 and Ling et al., 2004 in *Gata2* haploinsufficient mice.

Cited2 is a regulator of the hypoxia signalling pathway and is able to bind and inhibit *Hif1* (Shin *et al.* 2008). *Cited2* deletion in adult HSCs, employing the *Mx1-cre* system gave rise to a rapid loss of HSPCs in a cell-autonomous manner (Kranc *et al.* 2009). Gene expression and rescue experiments demonstrated that the *Cited2*-mediated HSC survival phenotype was mediated via *Ink4a/Arf* and *p53* (Kranc *et al.* 2009). Luciferase and ChIP assays have shown that *Cited2* could act as a positive regulator of *Gata2* (Saito *et al.* 2015). Indeed, *Cited2*$^{-/-}$ FL HSCs have reduced number and functionality, and *Cited2*$^{-/-}$ FL LK cells have 4-fold reduction in *Gata2* expression (Chen *et al.* 2007).

Tel/Etv6 is one of the first TFs found to be essential for adult HSC. Conditional deletion of *Etv6* with the *Mx1-Cre* promoter gave rise to a rapid loss of HSC and BM failure in a cell autonomous manner (Hock *et al.* 2004). Specific deletion of mature haematopoietic cells using specific lineage-Cre mice resulted in no changes in the frequency of myeloid, lymphoid and erythroid cells, indicating that *Etv6* is a critical regulator for HSPC maintenance, rather than a regulator of mature haematopoietic cell differentiation (Hock *et al.* 2004). Collaboration between *Etv6* and *Gata2* was

described in the induction of the hemogenic program from mouse fibroblasts (Pereira *et al.* 2013). Using a combination of four TF, including *Gata2, Etv6, Gfib* and *cFos*, the authors were able to transform mouse fibroblasts into cell expressing endothelial and haematopoietic gene programs and a population of LT-HSC marked by $CD150^{+}CD48^{-}$ $cKit^{+}$, suggesting the presence of definitive haematopoiesis (Pereira *et al.* 2013). Taken together, these results highlight the potential to generate functional adult HSC through a simple cocktail of four TFs.

1.3.6. *Gata2* function in myeloid progenitor cells.

While *Gata2* is expressed at relatively low levels in myeloid progenitors compared with HSCs (see **Chapter 3**), Rodrigues *et al*. have shown that *Gata2*$^{+/-}$ GMPs perform poorly in CFC *in vitro* assays and in *in vivo* transplantation experiments (Rodrigues *et al.* 2008). They found that *Gata2*$^{+/-}$ GMPs have lower levels of the Notch target *Hes1*, and that *Gata2* directly binds to the *Hes1* promoter (Rodrigues *et al.* 2008). Finally, *Hes1* overexpression in *Gata2*$^{+/-}$ GMPs rescued the functional defects, indicating the *Gata2-Hes1* axis as a regulator of GMP function (Rodrigues *et al.* 2008). These data also highlight the idea that even at low levels of expression, *Gata2* has an impact in haematopoietic progenitor cell function. Furthermore, this reinforces the idea of a cross-talk between *Gata2* and *Notch1* related pathways, in this case in myeloid progenitor regulation.

1.3.7. Exploring *Gata2* function using overexpression studies

Gata2 overexpression studies have contributed to the understanding of *Gata2* biology, and, in concert with analysis of *Gata2*$^{+/-}$ alluded to above, have specifically highlighted that *Gata2* levels need to be tightly regulated under physiological conditions. Overexpression of *Gata2* inhibits erythroid differentiation in primary erythroblasts or transformed erythroid cell lines (Ikonomi *et al.* 2000). In BM cells or leukaemia cell lines, enforced expression of *Gata2/GATA2* induced megakaryocytic while inhibiting erythroid differentiation (Ikonomi *et al.* 2000)(Huang *et al.* 2009). Enforced expression of *Gata2/GATA2* with retroviral, tamoxifen, or doxycycline-based systems, reduced proliferation, blocked differentiation, and induced quiescence in mouse and human BM HSPCs (Kitajima *et al.* 2002; Persons *et al.* 1999; Tipping *et al.* 2009; Heyworth

et al. 1999). Murine or human HSPCs expressing *Gata2/GATA2* performed poorly in CFC and long-term culture initiating cell (LTC-IC) functional assays, and were unable to contribute to haematopoiesis *in vivo,* although they did not undergo apoptosis (Persons *et al.* 1999; Tipping *et al.* 2009). Known cell cycle regulators of HSPCs, *p21, p27,* along with *Cdk4*, and *Cdk6* were modulated after *Gata2/GATA2* overexpression in HSPCs (Persons *et al.* 1999; Tipping *et al.* 2009). In agreement with the tight regulation of *Gata2* levels under normal conditions, it has been described that low-level *Gata2* overexpression can immortalise murine BM cells *in vitro* by enhancing the self-renewal ability of myeloid progenitors. When transplanted into irradiated mice, *Gata2-overexpression* BM cells induced expansion of the myeloid lineage and lymphoid lineage block, although mice never progressed to full-blown AML (Nandakumar *et al.* 2015). Taken together, overexpression studies have shown that *Gata2/GATA2* effects are dose-dependent, and that distinctive HSPC phenotypes can be observed at different *Gata2/GATA2* thresholds levels.

1.3.8. *Gata2* function and transcriptional network during blood lineage specification

Fine-tuning of *Gata2* levels are required for HSCs differentiation into lineage-restricted progenitors and consequently mature blood cells. A single TF is not able to drive HSC differentiation; instead cooperation with other TFs is required to orchestrate the molecular and cellular differentiation of HSCs into mature blood cells. In this regard, *Gata2* often liaises with *Gata1* and *Friend of Gata1* (*Fog1*) to induce erythroid differentiation, with *Gata3* for DC differentiation, and with *Pu.1* for monocyte and mast cell differentiation.

Erythroid differentiation is symbolised by the "*Gata* switch" (Suzuki *et al.* 2013; Snow *et al.* 2011). *Gata2* levels are high in HSCs, where it auto-activates its own transcription, whereas *Gata1* are very low or non-existent (Suzuki *et al.* 2013; Snow *et al.* 2011). Under *Epo* stimulation or other intrinsic factors that direct erythroid specification, *Gata2* binds and activates *Gata1* expression in MEP cells (Guo *et al.* 2016), and the *Gata1* protein accumulates and reaches the desired threshold. At this threshold Gata1 interacts with *Fog1* and other co-repressors to replace *Gata2* from its own regulatory elements, repressing *Gata2* expression and enabling terminal

differentiation (Grass *et al.* 2003). Genetic deletion of individual regulatory sites of the *Gata2* gene have shown that the *Gata* switch governing erythroid differentiation, belongs to the -1.8 kb regulatory site of the *Gata2* promoter (Fujiwara *et al.* 2009).

Pu.1 (*Spi1*) is a member of the *Ets* family of TFs. *Pu.1*$^{-/-}$ mice die at a late embryo stage (E18) or within 48 hours after birth (McKercher *et al.* 1996). Normal levels of erythrocytes and megakaryocytes were found in *Pu.1*$^{-/-}$ mice, however a lack of lymphoid and myeloid cells was observed (Scott *et al.* 1994). *Pu.1*$^{-/-}$ FL cells contributed to the erythroid and megakaryocyte lineages but not to other myeloid or lymphoid lineages, and therefore are not able to sustain long-term haematopoiesis in irradiated recipients (Scott *et al.* 1994). Administration of antibiotics to new-born *Pu.1*$^{-/-}$ mice allowed for a 2-week follow up with the formation of T cells in the thymus and granulocytes in the BM, but not B cells nor macrophages, indicating that *Pu.1* is essential for the development of B cells and macrophages (McKercher *et al.* 1996). *Pu.1* is able to antagonise the transcriptional activity of *Gata1* in erythroid development (Nerlov *et al.* 2000), *Gata2* in monocytic development (Walsh *et al.* 2002), and *Gata3* in T cell specification (T helper subtype) and DC development (Yashiro *et al.* 2015). Conversely, *Gata2* and *Pu.1* work together in mast cell development (Ohmori *et al.* 2015). In fact, *Gata2* and *Pu.1* interact and activate the *c-kit* promoter, essential for mast cells (Walsh *et al.* 2002). It is thought that *Gata2* expression in mast cells is driven at least, in part, mediated by the *Jak/stat5* signalling pathway (Li *et al.* 2015).

Patients with GATA2 mutations frequently present with clinical immunodeficiency syndromes including dendritic cell (DC) deficiency, indicating a potential role for *GATA2* in DC development (Wlodarski *et al.* 2017). An *in vitro* culture system to induce DC differentiation using BM feeder cells and *Flt3-L,* demonstrated that murine LSK, CMP and CDP (common DC precursor) cells have reduced capacity to produce *Cd11c*$^{+}$ cells after tamoxifen-mediated *Gata2* deletion (Onodera *et al.* 2016). Gene expression analysis revealed that *Gata2*$^{\Delta/\Delta}$ CMP differentiated towards the DC lineage, had higher expression of *Gata3,* and validated the *Gata2*-mediated repression of *Gata3* by ChIP assay, highlighting the *Gata*-switching phenomenon in different cell types (Onodera *et al.* 2016). The authors also found a decrease in the frequency and number of DCs after *in vivo* deletion of *Gata2* using a tamoxifen-Cre deletion, and concluded *Gata2* is important for DC differentiation (Onodera *et al.*

2016). However, the authors failed to detect decrease in DCs in *Gata2;Cd11c-cre* mice or *Gata2*$^{+/-}$ mice, suggesting that *in vivo* pan-deletion of *Gata2* produced BM multi-lineage failure instead of a *Gata2*-specific DC deficiency (Onodera *et al.* 2016). Future studies should address whether the DC deficiency observed in *GATA2*-deficient patients is human specific or if it can be recapitulated in the mouse using other DC-specific promoters.

1.3.9. *Gata2* function in the haematopoietic bone marrow niche

Gata2 is a critical regulator of adipocytes, a key negative cell regulator of the haematopoietic bone marrow niche (Brodsky and Jones 2005). *Gata2* and *Gata3* expression is high in preadipocytes of white adipose tissue, and differentiation to adipocytes is associated with reduction of *Gata2/3* levels (Tong *et al.* 2000). It has been reported that *Gata2/3*-mediated adipocyte suppression occurs through two or more different mechanism. In preadipocytes, *Gata2/3* bind to regulatory elements of the promoter and repress the transcriptional activity of the well-known adipocyte marker *PPAR*γ (Tong *et al.* 2000), and independently, *Gata2/3* physically interacts with the positive TF regulators of adipogenesis *CEBP*α and *CEBP*β (Xu *et al.* 2009a; Tong *et al.* 2005). This inhibitory interaction is mediated through the ZF2 domain of *Gata2/3* and the bZip domain of *CEBP*α/β (Xu *et al.* 2009a; Tong *et al.* 2005). A series of studies validated this mechanism for *Gata2*-mediated adipocyte regulation in mouse and human MSCs using loss of function and overexpression techniques in *in vitro* differentiation assays (Schupp *et al.* 2009). It has also been shown that *Gata2* not only regulates adipocyte differentiation and preadipocyte levels, but also that of MSCs which produce adipocytes and positive regulators of the niche, such as osteoblasts. *In vitro* deletion of *Gata2* with lentiviral Cre or *in vivo* deletion employing niche-specific Cre in MSCs, resulted in differentiation of MSC into adipocytes, but also into osteoblasts (X. Li *et al.* 2016). Gene expression analysis showed that *Gata2* could block osteoblastogenesis by inhibiting *Runx2* and *β-catenin* signalling, well-known positive regulators of osteoblast differentiation (X. Li *et al.* 2016). In addition, *Gata2* deletion in murine MSCs or *GATA2* knockdown in human MSCs reduced MSC proliferation *in vitro*, with increased G0/G1 phase (X. Li *et al.* 2016). Taken together, this evidence points to *Gata2* as a key regulator for MSC function and proliferation, and for adipocyte terminal differentiation.

The impact of *Gata2* deletion in BM MSCs in haematopoiesis has recently been evaluated (Hasegawa *et al.* 2017). *Gata2* deletion using the *Nestin-CreER*, *Prx1-Cre*, and *LepR-Cre* had no impact in HSPCs frequency or total counts in the BM of young mice. However, conditional deletion of *Gata2* using the global *ER*-Cre mice in transplantation setting, lead to a decrease of BM cellularity and the frequency of CMP progenitors. Discrepancy between the results obtained with different mouse models could be due the wider spectrum of BM microenvironment or extramedullary tissues that *ER-Cre* deletes, and can influence haematopoiesis (Hasegawa *et al.* 2017). Alternatively, experiments with niche-specific Cre mice were performed under steady-state conditions while experiments with *ER-Cre* mice were executed in (stress-related) transplantation assays. In addition, longer follow up after BM niche deletion, HSC self-renewal, and specific EC *Gata2* deletion were not explored in this study (Hasegawa *et al.* 2017). Therefore, the function that *Gata2* has, in niche-dependent haematopoiesis regulation, is still largely unclear.

1.3.10. *GATA2* function in human haematopoiesis

Expression of *GATA2* in human haematopoietic cells has been reported. Expression of the *GATA2* IS isoform is restricted to the $CD34^+$ fraction, whilst the *GATA2* IG isoform is higher in $CD34^+$ cells, but also present in the $CD34^-$ fraction (Pan *et al.* 2007). Modelling of EHT and haematopoietic differentiation using human ESCs has provided information about the role of *GATA2* during human haematopoietic development. $GATA2^{-/-}$ ESCs formed similar levels of HECs ($CD31^+$ $CD34^+$ $CD43^-$) compared to parental ESCs, however they generated a very low number of HPCs ($CD34^+$ $CD43^+$), indicating that *GATA2* is dispensable for specification of HECs but essential for EHT and HPC formation *in vitro* (Huang *et al.* 2015). The few $GATA2^{-/-}$ HPCs left were able to form CFU-E and CFU-M colonies, but lacked CFU-G, suggesting that *GATA2* is required for granulocyte specification in human HPCs (Huang *et al.* 2015). Using a GATA-2 GFP reporter ESC line, *GATA2* was observed to be present in HECs, and only *GATA2*-HECs were able to produce HPCs ($CD34^+$ $CD43^+$) (Huang *et al.* 2016). In addition, *GATA2* marks HPCs, since the ability to form CFCs was exclusive to *GATA2* expressing HPCs (Huang *et al.* 2016). In agreement

with previous work, *GATA2* expression was observed in mature CFU-G, while differentiated CFU-E and CFU-M were GFP^- (Huang *et al.* 2016).
Enforced *GATA2* expression induces quiescence of CB $CD34^+CD38^-$ HSCs *in vitro* and *in vivo* by reducing the expression of the cell-cycle regulators *CDK4, CDK6* and *CCND3* (Tipping *et al.* 2009). Knock-down of *GATA2* by two-fold in CB $CD34^+$ HSPCs reduced the gene expression of HSC regulators such *KIT, GFI1B* and *HOXB4* (Fujiwara *et al.* 2012). In addition, luciferase and ChIP assays confirmed the activation and direct *HOXB4* promoter-binding by *GATA2* (Fujiwara *et al.* 2012). Interestingly, enforced expression of *HOXB4* has been shown to expand HSC both *in vitro* and *in vivo* (Antonchuk *et al.* 2002; Schiedlmeier *et al.* 2003). In agreement with the role of *GATA2* in murine HSCs, it appears that both down and upregulation of *GATA2* reduces proliferation of human HSCs. However, to date, the requirement of *GATA2* in human HSPC is largely unexplored.

1.3.11. *GATA2* in bone marrow failure syndromes and malignant haematopoiesis

Consistent with its role in adipocyte differentiation, *GATA2* is downregulated in the BM of aplastic anaemia (AA) (Fujimaki *et al.* 2001), where patients present with a reduction of HSPCs and followed by a replacement of adipocytes (Brodsky and Jones 2005). AA patients also showed decreased *GATA2* and increased *PPARγ* levels in MSCs, a mechanism found to be responsible for terminal differentiation of adipocytes (Xu *et al.* 2009a).

Haploinsufficiency of *GATA2* has been associated with *EVI1* AML in the poor prognosis t(3;3)(q21;q26) chromosomal translocation and inv(3)(q21;q26) inversion. Patients with this chromosomal translocation/inversion display high *EVI1* levels. In this context, two studies found that a previously described *GATA2* distal haematopoietic enhancer (G2DHE) located at -77 Kb of the *GATA2* transcriptional start site, is relocated in close proximity to the *EVI1* gene in 3q21 AML (Gröschel *et al.* 2014; Yamazaki *et al.* 2014). In the first study, chromosome conformation capture technique was employed to isolate a 9 kb element from inv(3)/t(3;3) AML samples and cell lines. Analysis of p300 binding in the 9 kb region helped to characterise the G2DHE and

deletion of the enhancer decreased *EVI1* expression and impaired AML growth, similar to that of *EVI1* KD (Gröschel *et al.* 2014). *GATA2* expression was lower in a mixture of AML samples and cell lines with inv(3)/t(3;3) compared to unselected non-inv(3)/t(3;3) AML samples (Gröschel *et al.* 2014). Nevertheless, two previous reports showed that *GATA2* is overexpressed (77-83%) in patients with inv(3)/t(3;3) compared to healthy BM cells (Lahortiga *et al.* 2004; Vicente *et al.* 2012), suggesting that despite single-allele contribution to *GATA2* expression, *EVI1*-mediated activation of *GATA2* appears to compensate and even increase *GATA2* levels.

In the second study, a bacterial artificial chromosome (BAC) containing the inv(3)(q21;q26) inversion was used to generate a transgenic mouse with the inversion, with and without the G2DHE (Yamazaki *et al.* 2014). Only inv(3)(q21;q26) mice with the G2DHE developed leukaemia with a median latency of 200 days, indicating that G2DHE is required for *EVI1* upregulation in inv(3)/t(3;3)-leukaemias (Yamazaki *et al.* 2014). Curiously, inv(3)(q21;q26) mice developed three types of leukaemia, a B lymphoid leukaemia (type 1), a mixed leukaemia (type 2), and a $Gr1^{+}Mac1^{+}$ (GM) myeloid leukaemia (type 3). Of interest, *EVI1* has recently been reported to be expressed and contribute to the leukaemogenic potential in ALL, and especially in B-ALL (Hinai and Valk 2016). Immunophenotypic analysis revealed an expansion of LT-HSCs ($CD34^{-/low}Flt3^{-}$) in 12-week-old preleukaemic inv(3)(q21;q26) mice, which is considerably decreased in inv(3)(q21;q26) mice lacking the G2DHE (Yamazaki *et al.* 2014). Appearance of an abnormal $CD34^{high}Flt3^{-}$ population was seen in inv(3)(q21;q26) mice of GM myeloid or mixed type, while B lymphoid type was characterised by an expansion of LT-HSCs, supported by higher levels of $c\text{-}kit^{+}$ and $CD34^{+}$ in the PB blood of B lymphoid leukaemia inv(3)(q21;q26) mice (Yamazaki *et al.* 2014). Gene expression analysis showed that *EVI1* was higher in inv(3)(q21;q26) mice compared to inv(3)(q21;q26) mice lacking the G2DHE, supporting that the G2DHE is essential *Evi1*-driven leukaemia. *EVI1* was highly expressed in LT-HSC, while expression was minimal in the $CD34^{high}Flt3^{-}$ population, suggesting that the LSC capacity resides in LT-HSC in *Evi1*-driven leukaemia (Yamazaki *et al.* 2014). Of interest, *Gata2* was upregulated by 2.5-fold in LT-HSC of inv(3)(q21;q26) mice (Yamazaki *et al.* 2014). In this study, the two *Gata2* alleles were present, however it is not clear whether *Gata2* haploinsufficiency contributes to *Evi1*-leukaemia. However, it remains a possibility that *Evi1* activates the expression of the remaining *Gata2* allele

thus leading to normal or increased levels in inv(3)/t(3;3)-leukaemias. To answer this question, another study crossed a type II, harbouring both B and GM leukaemias, inv(3)(q21;q26) mice with a germ line *Gata2*$^{+/-}$ mouse (Katayama *et al.* 2017). Haploinsufficiency of *Gata2* accelerated the onset of leukaemia in the starting cohort, and showed prevalence for the type I (B lymphoid) leukaemia. However transplant of total BM or B220^{+}Gr1^{-}c-kit^{+} fraction, which contains the LSC activity, from type I mice showed similar survival between recipients receiving cells from *Gata2*$^{+/+}$ and *Gata2*$^{+/-}$ inv(3)(q21;q26) mice, indicating that *Gata2* haploinsufficiency accelerates LSC formation but is not absolutely required for leukaemia propagation in this model (Katayama *et al.* 2017). *Gata2* levels were found higher in the B220^{+}Gr1^{-}c-kit^{+} fraction from type III (myeloid leukaemia) mice compared to type I mice, and this negatively correlated with *Evi1* expression, concluding that pre-leukaemic cells with high *Gata2* levels induce a GMP-like phenotype that give rise to myeloid leukaemia with longer latency and Gr1^{+} differentiation, while *Gata2* haploinsufficiency leads to a B lymphoid blast-like phenotype with aggressive leukaemia development (Katayama *et al.* 2017). Several caveats arise from this study, since and most importantly, the authors showed that *Gata2* expression in the LSC fraction (B220^{+}Gr1^{-}c-kit^{+}) of the type I leukaemia from inv(3)(q21;q26) mice w identical to *Gata2* levels found in *Gata2*$^{+/-}$ inv(3)(q21;q26) mice (Katayama *et al.* 2017). In addition, no proof of *Gata2* downregulation in the *Gata2*$^{+/-}$ inv(3)(q21;q26) mice was shown in the study (Katayama *et al.* 2017). Secondly, inv(3)/t(3;3)-leukaemias have only been described in AML; that high *Gata2* levels induced a myeloid phenotype with longer latency and Gr1^{+} differentiation is at odds to the poor prognosis inv(3)/t(3;3)-leukaemias found in AML. *Evi1* expression was also lower in the myeloid type II leukaemias, and only high *Evi1* and low *Gata2* levels were correlated with B lymphoid leukaemia described in this study. Therefore, inv(3)(q21;q26) mice are not an ideal model to study the pathogenesis of *Evi1* and *Gata2* in relation of inv(3)/t(3;3)-AML leukaemia.

Epigenetic regulation of the *GATA2* locus has also been observed in AML. Mutations in *TET2*, an enzyme that converts 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC) to modulate gene expression, are linked to adverse outcome when they are found in the context of *FLT3*^{ITD} (Shih *et al.* 2015). To investigate the genetic interaction between *TET2* and *FLT3*^{ITD}, a study generated *Tet2*$^{\Delta/\Delta}$*;Vav-Cre*$^{+}$*;Flt3*^{ITD} transgenic mice (Shih *et al.* 2015). *Tet2*$^{\Delta/\Delta}$*;Vav-Cre*$^{+}$

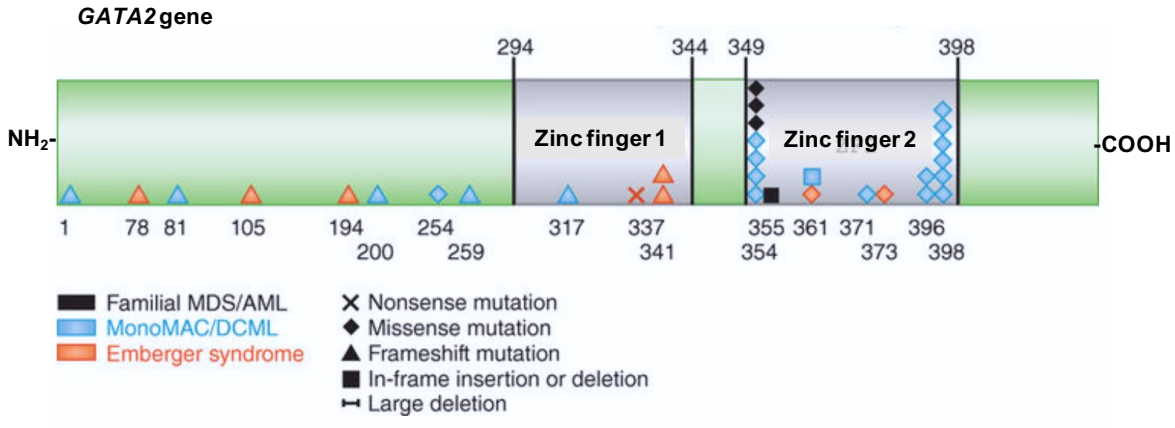
developed MPN as previously described (Li *et al.* 2011), while *Tet2*$^{\Delta/\Delta}$*;Vav-Cre*$^{+}$*;Flt3*^{ITD} mice developed frank AML with a median latency of 80 weeks. LSK and GMP from *Tet2*$^{\Delta/\Delta}$*;Vav-Cre*$^{+}$*;Flt3*^{ITD} mice were expanded, however only LSK were capable to propagate AML into secondary recipients, thus LSK cells contained the LSC in this model (Shih *et al.* 2015). RNA-seq and methylation profiling of LSK cells revealed a hypermethylated signature in genes that control self-renewal and differentiation of HSCs, including *Gata2* (Shih *et al.* 2015). Enforced expression of *Gata2* induced erythroid differentiation in colony assays and in transplantation assays, whereas no *Gata2* expressing *Tet2*$^{\Delta/\Delta}$*;Vav-Cre*$^{+}$*;Flt3*^{ITD} BM cells induced AML *in vivo* (Shih *et al.* 2015). Therefore, methylation is an important regulator of *Gata2* activity in AML. Epigenetic repression of *Gata2* in *Mll-af9* AML is mediated by *Prc2*, a chromatin regulator that methylates H3K27 sites; methylation of H3K27 is linked with inactive gene promoter activity. Genetic ablation of *Eed,* one of the *Prc2* complex components, delays *Mll-af9*-mediated AML by depressing *Gata2,* among other genes (Danis *et al.* 2015). In addition, *Gata2* overexpression impaired colony formation of *Mll-af9* LSK cells (Danis *et al.* 2015), in agreement with previous reports where enforced expression of *Gata2* halts leukaemic proliferation of *Mll-enl* cells *in vitro* (Bonadies *et al.* 2011).

1.3.12. *GATA2* mutations in MDS/AML

In 2011, a series of studies described for the first time that *GATA2* heterozygous mutations form an autosomal dominant disorder eventually leading to MDS and AML (Hyde and Liu 2011; Hahn *et al.* 2011; Hsu *et al.* 2011; Ostergaard *et al.* 2011). In addition, *GATA2* germline mutations linked together a group of genetic haematological disorders without a previous known cause. Thus, *GATA2* joined an exclusive family of germline mutations, together with *RUNX1* and *CEBPA*, that predispose patients to malignant haematopoiesis. *GATA2* mutations were found in families with DCML deficiency, that present with loss of DCs, monocytes, and B and NK cells; families with monocytopenia with Mycobacterium avium complex infection (MonoMAC) syndrome; families with lymphoedema and MDS (Emberger syndrome); and familial MDS/AML (Hyde and Liu 2011; Hahn *et al.* 2011; Hsu *et al.* 2011; Ostergaard *et al.* 2011). Patients with DCML, MonoMAC and Emberger syndromes have higher likelihood to

develop AML, although in some *GATA2* deficient families MDS and AML have been diagnosed without previous haematological abnormalities (Dickinson *et al.* 2014).

Since this discovery, approximately 100 different mutations have been described in the *GATA2* gene, in association with *GATA2* syndromes (Dickinson *et al.* 2014) (**Figure 1.8**). Frameshift and nonsense mutations, which completely abolish *GATA2* activity in the mutated allele, often present with an earlier onset of AML (Hyde and Liu 2011). Deletions, missense mutations, and point mutations in regulatory elements have also been described in *GATA2* syndromes (Hyde and Liu 2011). A missense mutation linked with rapid onset of MDS/AML is the T354M in the ZF2 domain (Hahn *et al.* 2011), and it is believed that T354M acts as a dominant negative, thus further reducing *GATA2* activity within HSPCs. Nevertheless, no other mutations have been found to act as dominant negative nor have acquired secondary mutations in the second *GATA2* allele been observed during disease progression (Dickinson *et al.* 2014). Acquired mutations of the *wild-type* allele leading to loss of heterozygosity (LOH) is a common feature of tumour suppressor genes in hereditary cancer (Hügel and Wernert 1999). Bearing in mind that in contrast to hereditary AML predisposing genes (i.e. *CEBPA, RUNX1*), *GATA2* mutations do not present with LOH, meaning that low levels of *GATA2* are still required for pre-leukaemic HSCs, and possibly for disease progression in *GATA2* hereditary syndromes.



Adapted and modified from R. Katherine Hyde et al., 2011

Figure 1.8. GATA2 mutations in familial MDS/AML. *GATA2* mutations found in hereditary *GATA2* syndromes. The number indicates the AA altered, and the colour and type of sign the type of disease and mutations involved. This is only a representation, since over 100 mutations in the *GATA2* gene have been identified to date.

Other secondary mutations have, however, been reported to be relevant for evolution to MDS and AML in *GATA2* syndromes. Monosomy of chromosome 7 was linked with *GATA2* MDS patients and adverse outcome. Indeed, a study of *GATA2* syndromes in children and young adults showed that 72% of *GATA2* MDS patients also present with monosomy 7 (Wlodarski *et al.* 2015). In addition, mutations in *ASXL1* were found in *GATA2* patients with myeloid transformation, rapid onset and poor outcome MDS/AML (West *et al.* 2014). Curiously, *ASXL1* secondary hits were almost exclusively found in females with *GATA2* mutations, however the reason for the gender bias observed remains unknown (Lübking *et al.* 2015). Thus, in terms of understanding leukaemic development, *GATA2* syndromes provide a suitable model to study disease evolution from the pre-leukaemia stage to frank AML.

In addition, it has been reported that chronic mild neutropenia or B cell deficiency precedes the development of *GATA2*-related MonoMAC syndrome, MDS and AML (Pasquet *et al.* 2013). This suggests that earlier detection and treatment of *GATA2* syndromes should be considered prior the development of MDS and AML or late stages of immunodeficiency, since both infections and malignancy are responsible for *GATA2*-related deaths.

1.3.13. *GATA2* mutations as secondary hits in *CEBPA* AML.

GATA2 loss-of-function mutations are observed in sporadic AML. In this setting, *GATA2* somatic mutations are found almost entirely with familial and somatic *CEBPA* mutations. CEBPA double mutants define a favourable AML subtype, cytogenetically normal (CN) and M1-M2 FAB subtype (Greif *et al.* 2012). *GATA2* mutations were detected in a large proportion of *CEBPA* double mutants AML (18-41%), 6-16% in *CEBPA* single mutant, and less than 1% in *CEBPA wild-type* patients (Greif *et al.* 2012; Fasan *et al.* 2012; Green *et al.* 2013). Mutant levels analysis revealed the coexistence of both mutations in the same cell, suggesting that *CEBPA* sporadic or familial AML cases have a higher tendency to acquire secondary *GATA2* mutations (Greif *et al.* 2012). In addition, some *CEBPA* double mutant patients loose or gain a *GATA2* mutation after CR or relapse, indicating that *GATA2* is a secondary hit in this AML subset (Green *et al.* 2013). While some studies reported that *GATA2* improved the OS of the already favourable *CEBPA* double mutant patients, other reports showed

that *GATA2* mutations have no impact in the OS of these patients. Thus, the biological and clinical impact of GATA2 mutations in this setting is unclear.

1.3.14. *GATA2* overexpression in AML

Evidence supporting an oncogenic role for GATA2 was discovered in CML. The L359V missense mutation in the ZF2 domain of GATA2 confers a gain of function with increased DNA binding activity (Zhang *et al.* 2008). L359V GATA2 mutation was found exclusively in CML patients during acute myeloid blast crisis (which is similar to acute myeloid leukaemia), which was linked with a more aggressive disease progression (Zhang *et al.* 2008). Downstream analysis showed that L359V GATA2 was able to bind and inhibit PU.1, a key regulator for myeloid differentiation (Zhang *et al.* 2008). Indeed, *in vitro* differentiation assays with HL60 and *in vivo* BCR/ABL-mediated CML revealed that L359V GATA2 impaired myelomonocytic differentiation, implying that increased GATA2 transcriptional activity is a key event for the differentiation blockade in BCR/ABL CML cells, a requirement for the transformation from chronic phase to acute myeloid blast crisis phase (Zhang *et al.* 2008).

To date, the L359V *GATA2* gain of function mutation has never been described in AML or any other malignancy (Zhang *et al.* 2009). However, overexpression of *GATA2* has been linked with poor prognosis in a high proportion of MDS and AML patients. *GATA2* overexpression has been linked with the $Lin^{-}CD34^{+}CD38^{-}CD123^{+}$ population in high-grade MDS; this is a CSC population also observed in AML (Li *et al.* 2014). In addition, another study showed *GATA2* overexpression correlated with *GATA1* downregulation in 84.8% (28 of 33) in the BM of patients with high-grade MDS (Fadilah *et al.* 2002). In AML, overexpression of *GATA2* was found in 65% (155 of 237) of paediatric patients, compared to healthy BM (Maaike Luesink *et al.* 2012). *GATA2* levels decreased in patients who achieved CR, while remaining high in patients who relapsed. *GATA2* is an independent poor prognostic factor in paediatric AML, with shorter OS, EFS and DFS, especially in association with *WT1* overexpression, FAB-M5, inv(16), MLL translocation, and $FLT3^{ITD}$ (Maaike Luesink *et al.* 2012). In adult AML, a study showed that *GATA2* is overexpressed in 37.4% of AML patients, and that the overexpression group correlated with the adverse outcome cytogenetics and molecular AML subgroups, including complex karyotype, −5/5q- and/or −7

monosomies, inv(3)/t(3;3), *WT1* and *EVI1* overexpression, elder patients, and secondary AML (Vicente *et al.* 2012). In agreement with this, adult AML patients with *GATA2* overexpression had worst OS, EFS and DFS (Vicente *et al.* 2012). How overexpression of GATA2 impacts leukaemic cell fate in AML, however, remains unclear.

1.4 Specific aims of thesis

Our laboratory has previously demonstrated that *Gata2* haploinsufficiency negatively impacts HSC and GMP numbers and functionality (Rodrigues *et al.* 2005; Rodrigues *et al.* 2008). However, the requirement for *Gata2* in HSCs remains poorly defined. Increasing evidence for a novel putative oncogenic role for *GATA2* in AML, has become apparent from expression analysis that link *GATA2* overexpression with adverse outcome. However, the biological role of *GATA2* in leukaemia initiation and progression has not been tested. This thesis will therefore evaluate the impact of *Gata2* in normal and leukaemia cells, including LSCs, with the following aims:

1. Evaluate the impact of conditional acute deletion of *Gata2* in murine HSCs using the *Mx1-Cre* mouse model.

2. Assess the expression and biological impact of acute deletion and pharmacological blockade of *Gata2* in established pre-LSCs and LSCs, driven by the introduction of the AML causing *Mll-af9* translocation or *Meis1a/Hoxa9* oncogenes.

3. Investigate the effect of *GATA2* reduction with *RNAi* or a small molecule inhibitor in established human AML cell lines.

Chapter 2

Material and Methods

Suppliers name and addresses are listed in **Table 2.1**

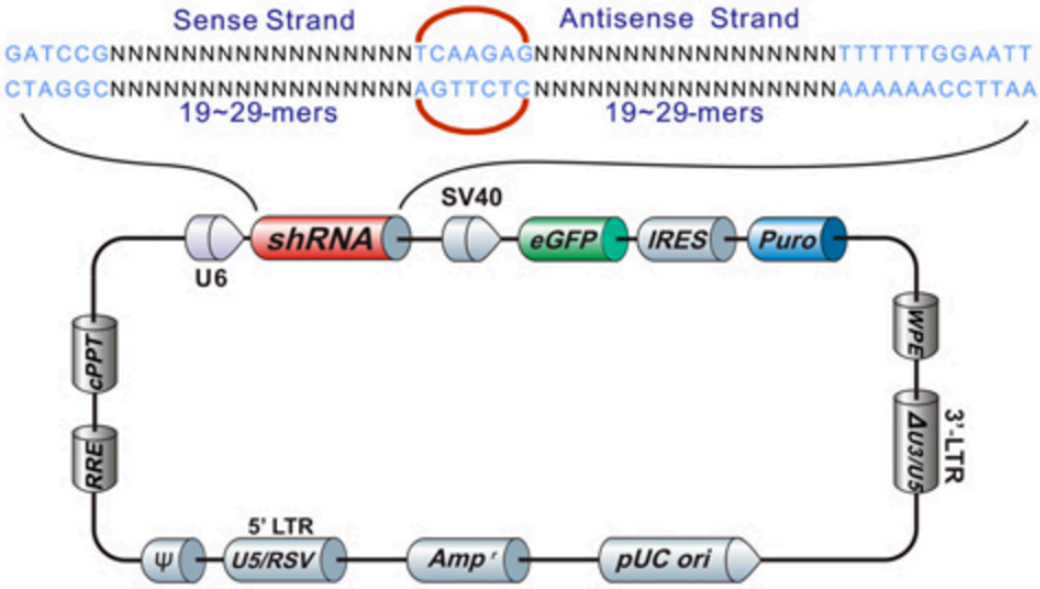
Table 2.1 Supplier addresses

Name of company	Address
Applied Biosystems, Warrington, UK	Lingley House, 120 Birchwood Boulevard, Warrington, WA3 7QH, UK
BD Biosciences, Oxford, UK	Edmund Halley Road - Oxford Science Park, OX4 4DQ Oxford, UK
BioLegend UK Ltd.	4B Highgate Business Centre, 33 Greenwood Place, London, NW5 1LB, UK
Bioquote limited	Bioquote Limited, The Raylor Centre, James Street York, YO10 3DW, UK
BioLine Reagents Ltd.	Edge Business Centre, Humber Rd, London NW2 6EW, UK
Cell Biolabs InC.	7758 Arjons Dr, San Diego, CA 92126, USA
ClonTech	Avenue du President Kennedy 78100 Saint-Germain-en-Laye, France
Eppendorf UK Ltd.	Endurance House, Vision Park, Histon, Cambridge, CB24 9ZR
Fisher Scientific UK	Bishop Meadow Road, Loughborough, Leicestershire, LE 1 5RG, UK
GE Healthcare	Nightingales Lane , Chalfont St Giles , Buckinghamshire , HP8 4SP
Genecopoeia	39 rue de Houdan – BP 15, 78612 Le Perray-en-Yvelines Cedex, France
GraphPad Software, Inc.	7825 Fay Avenue, Suite 230, La Jolla, CA 92037 USA
Invitrogen, Paisley, UK Ltd. Part of Life Technologies	Invitrogen, Paisley, UK Ltd, 3 Fountain Drive, Paisley, UK
The Jackson laboratories	600 Main Street, Bar Harbor, ME USA 04609
Leica Microsystems	Davy Avenue Knowlhill, Milton Keynes, MK5 8LB, UK
Miltenyi Biotech	Almac House, Church Lane, Bisley, Surrey, GU24 9DR, UK
New England Biolabs	75-77 Knowl Piece, Wilbury Way, Hitchin, Herts SG4 0TY, UK
PeproTech EC Ltd	PeproTech House, 29 Margravine Road, London, W6 8LL, UK
Qiagen	Fleming Way, Crawley, West Sussex, RH10 9NQ, UK
Sigma-Aldrich	The Old Brickyard, New Rd, Gillingham, Dorset, SP8 4XT, UK
STEMCELL Technologies	Building 7100, Cambridge Research Park, Beach Drive, Waterbeach, Cambridge, UK, CB25 9TL
Tree Star, Inc.	340 A Street #101 Ashland, OR 97520, USA

2.1. Plasmids and DNA preparation.

psPAX2, VSVG, MSCV-neo, MSCV-puro, MSCV-Meis1a-puro, MSCV-Hoxa9-neo, pRRL-Cre-iVenus, and pRRL-iVenus were a kind gift from Prof. Kamil Kranc (University of Edinburgh). MSCV-MLLAF9-GFP and MSCV-GFP were a kind gift from Dr. Daniela Krause (Goethe University Frankfurt). psi-LVRU6GP containing shGATA2 were purchased from Genecopoeia. shGATA2 plasmids details and sequences for knockdown can be found in **Figure 2.1** and **Table 2.2**.

All plasmids were transformed in *Stbl3* bacteria (Sigma) and cultured in LB Broth media (Sigma) with 100 µg/mL of ampicillin (Gibco). Bacteria from glycerol stock were cultured overnight in 250 mL of media in a shaker. The next day, culture was harvested and DNA plasmid extracted following manufacturer's instructions from the EndoFree Plasmid Maxi Kit (Qiagen). DNA was eluted in DNAase free water (Molecular probes) and aliquoted at >1 µg/mL concentration, with 260/280 ratio between 1.8-2 and 260/230 ratio between 1.8-2.2. Purified DNA was stored at minus 20°C.



Adapted from Genecopoeia

Figure 2.1. Vector information for psi-LVRU6GP plasmid. shGATA2 target sequences are listed in Table 2.2. U6 is the human U6 small nuclear 1 promoter (polimerase III promoter); Simian virus 40 (SV40) alloqs the transcription termination and polyadenylation of mRNA transcribed by RNA polymerase II; enhanced green fluorescent protein (eGFP) allows for the detection and isolation of cells transduced with shRNA; Encephalomyocarditis virus internal ribosome entry site (IRES) recruits ribosome to initiate translation internally on a transcript independent of its 5' end. Multiple proteins can be made from a polycistronic transcript containing multiple ORFs separated by IRES; puromycin resistance gene (puro) allows cells to be resistant to puromycin; woodchuck hepatitis virus posttranscriptional regulatory element (WPE) enhances viral RNA stability in packaging cells leading to higher titer of packaged virus; truncated HIV-1 3' long terminal repeat (3'-LTR) allows packaging of viral RNA into virus and self-inactivate the 5'LTR by a copying mechanism during viral genome integration, contains polyadenylation signal for transcription termination; pUC origin of replication (pUC ori) facilitates plasmid replication in E. coli leading to high-copy plasmid number; Ampicillin resistance gene (Amp') allows E. coli to be resistant to ampicillin; truncated HIV-1 5' long terminal repeat (5' LTR) allows transcription of viral RNA and its packaging into virus; HIV packaging signal (Ψ) allows packaging of viral RNA into virus; HIV-1 Rev response element (RRE) allows rev-dependent nuclear export of viral RNA during viral packaging; central polypurine tract (cPPT) facilitates the nuclear import of HIV-1 cDNA through a central DNA flap;

Table 2.2 GATA2 knockdown sequences. Knockdown based on accession number NM_032638.3

Clone	Target sequence	Location
scramble	5'-gcttcgcgccgtagtctta-3'	-
31	5'-gcaaattgtcagacgacaacc-3'	1382
32	5'-ggacaactttatgtagagaaa-3'	2027
33	5'-cctctctgaaatagccgaact-3'	2269
34	5'-cctttaaagtgagtactgtta-3'	3324

2.2. Mouse models and mouse husbandry.

All mice were of *C57BL/6* genetic background. Sex-matched 8 to12 week-old mice were used. Animal experiments were authorized by the UK Home Office (project license 30/3380).

2.2.1. *C57BL/6* and *C57BL/6 SJL*.

C57BL/6 and their counterparts *C57BL/6 SJL* were purchased from The Jackson Laboratory (Bar Harbor, ME USA). *C57BL/6* mice express the CD45.2 isoform in white blood cells, while *C57BL/6 SJL* mice express the CD45.1 isoform.

2.2.2. $Gata2^{tm1Sac}$.

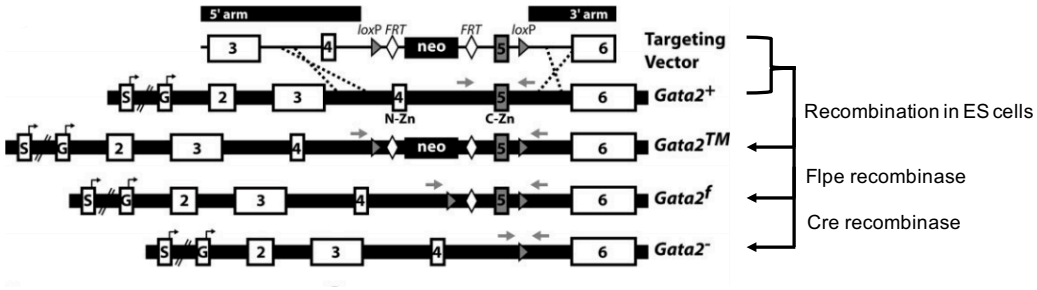
Gata2 transgenic mouse was kindly donated by Professor Julian Downward (The Francis Crick Institute, London). The mice were originally engineered in laboratory of Sally Camper (Charles *et al.* 2006). A targeting vector containing a neomycin resistant cassette and loxP sites flanking the fifth exon were inserted into the *Gata2* locus (**Figure 2.2**). The fifth exon contains the C-terminal zinc finger domain, thus abolishing the DNA binding of *Gata2* and generating a *Gata2* KO mouse after crossing with a Cre transgenic mouse. The deletion of the exon 5 gives rise to a non-functional truncated *Gata2* protein, according to Charles *et al.* 2006. Western blot showed the appearance of protein band different of the size of *wild-type Gata2* protein. In addition, they crossed the $Gata2^{fl/fl}$ mice with a generic Cre mouse (*Cmv-Cre*) to delete *Gata2* in all organs. As observed in the original papers from Stuart Orkin lab using conventional *Gata2* KO mice (Tsai et al. 1992), no *Gata2* null mice were born, and they observed that the mice died between E10.5 and E11.5 due to severe anaemia, in agreement with the conventional *Gata2* KO mice (Charles *et al.* 2006).

2.2.3. Mx1-Cre, breeding with $Gata2^{tm1Sac}$ and induction of Gata2 deletion *in vivo*.

Mx1-Cre transgenic mouse was kindly donated by Professor Kamil Kranc (Scottish Centre for Regenerative Medicine, Edinburgh). *Mx1-Cre* transgenic mouse has been

described previously (Kühn *et al.* 1995). In this strain, Cre recombinase is expressed in the entire haematopoietic system, and in the bone marrow microenvironment. *Mx1-Cre*$^{+/}$;*Gata2*$^{fl/fl}$ were bred with *Mx1-Cre*$^{-/-}$;*Gata2*$^{fl/fl}$ to obtain *Mx1-Cre*$^{+/-}$;*Gata2*$^{fl/fl}$ and control *Mx1-Cre*$^{-/-}$;*Gata2*$^{fl/fl}$ mice.

To achieve full deletion of *Gata2* alleles in *Mx1-Cre*$^{+/-}$;*Gata2*$^{fl/fl}$ and control *Mx1-Cre*$^{-/-}$;*Gata2*$^{fl/fl}$, mice were administered 6 intraperitoneal doses (250 µg/dose) every other day of polyinosinic:polycytidylic acid (pIpC) (GE Healthcare). pIpC is a double-stranded RNA that binds to *Tlr3* receptors and activates the type I IFN pathway which ultimately activates the *Mx1-Cre* promoter.



Modified from Charles et al. 2006

Figure 2.2 Gata2^{tm1sac} transgenic mouse construct. The fifth exon contains the C-terminal zinc finger domain, thus abolishing the DNA binding ability of Gata2. A targeting vector containing a neomycin resistant cassette flanked by FRT sites and loxP sites flanking the fifth exon were inserted into the Gata2 locus in ES cells. Mediating Flpe recombinase, the neomycin cassette was excised generating the Gata2^{fl} allele.

2.2.4. ID method.

Three weeks-old pups were ear notched for identification purposes according to our ID system (**Figure 2.3**). Ear punch samples were used for genotyping of mice.

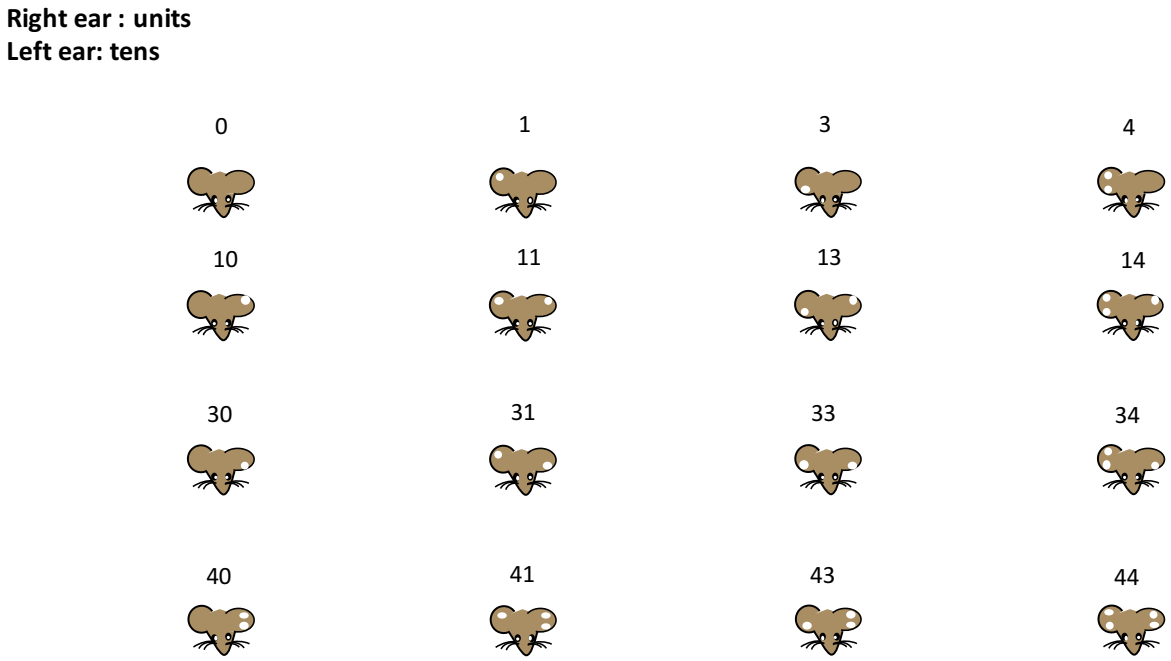


Figure 2.3 mouse ID system

2.2.5. DNA extraction and genomic PCR.

DNA extraction was carried out according to the manufacturer's protocol (ISOLATE II Genomic DNA Kit, Bioline). Ear punch samples were incubated in lysis buffer GL and proteinase K for 2-3 hours at 56°C. Next, to achieve finale lysate, lysis buffer G3 was added and the sample further incubated for 10 minutes at 70°C. Ethanol (96%) was added to adjust DNA binding conditions, and the samples were then loaded in an ISOLATE II Genomic DNA Spin Column. Samples were then centrifuged, washed with wash buffer GW1 and GW2, and eluted in 70°C preheated nuclease free water (Molecular probes). Samples were stored at -20°C until required for PCR. Genomic PCR was carried out following manufacturer's protocol (MangoMix™, Bioline). DNA primers (Sigma) and PCR settings are listed in **Table 2.3**. Electrophoresis was performed using 1% agarose (Molecular probes) gel for Cre, and 2% agarose gel for Gata2.

Table 2.3 Genotyping primers

Primer name	sequence	PCR programme
Gata2 forward	5'-GCCTGCGTCCTCCAACACCTCTAA-3'	1. 95°C 5 minutes 2. 94°C 1 minute 3. 60°C 1 minute 4. 72°C 1 minute Repeat steps 2-4 34 times 5. 72°C 10 minutes 6. 4°C hold
Gata2 reverse	5'-TCCGTGGGACCTGTTTCCTTAC-3'	
Generic Cre forward	5'-TGACCGTACACCAAAATTTG-3'	1. 95°C 3 minutes 2. 94°C 30 seconds 3. 55°C 30 seconds 4. 72°C 1 minute Repeat steps 2-4 340times 5. 72°C 5 minutes 6. 4°C hold
Generic Cre reverse	5'-ATTGGCCCTGTTTCACTATC-3'	

2.2.6. Dissection and PB extraction.

Mice were culled by cervical dislocation, and spleen, thymus, femur, tibia, and hip bone were aseptically removed and placed in PBS (Gibco) with 2% FBS. To obtain PB, a blade was used to nick the tail vein. Blood (around 30-50 µL) was collected in a EDTA-coated bleeding tube (Starstedt).

2.3. Human samples.

Human BM MNCs for gene expression analysis were purchased from STEMCELL technologies. Human AML samples were obtained from the Bristol Royal Infirmary (NHS University Hospitals Bristol). Details from human AML samples are listed in **Table 2.4**.

Table 2.4 AML samples

ID name	Age	Sex	Clinical details
HD209	6	M	2ry AML to Ewing Sarcoma
HG352	7	M	t(8;21)(q22;q22)
CP338	14	M	APML t(15;17)(q22;q12)
RD279	14	F	-
RD296	14	F	-
OD368	2	M	t(9;11)(p22;q22), t(11;21)(p23;q8)
CG336	14	F	MLL rearranged
AB312	15	F	MDS/AML
ZH340	3	M	t(8;21)(q22;q22)
RT161	6	M	AML M5
OA342	2	F	AMKL
KU250	7	F	2ry AML/relapse
DS356	-	-	Adult AML
JC125	12	M	AML
MD064	-	-	Relapse after BMT, M0/1
KA122	10	F	-
ND026	8	F	M4/M5
MS332	17	F	NK AML
BD002	7	F	Bi-lineage Del5q abnormal 21
JC110	-	-	-
SW104	18	M	Post-BMT
WW061	1	M	t(9;11)
FJ080	4	M	-
SW238	11	F	-
DD226	6	M	Relapse
RG126	10	M	-
OP067	7	M	-

2.4. Bioinformatics analysis.

AML and control patient datasets were downloaded from GEO (Edgar et al. 2002) and ArrayExpress (Kolesnikov *et al.* 2015), to yield a case/control AML cohort hybridized to the same array (Affymetrix Human Genome U133 Plus 2.0 GeneChip). AML cohort (n=2611), control cohort: (n=77) from GEO (GSE14468, GSE22845, GSE10358, GSE12417, GSE13159, GSE14062, GSE15434, GSE16015, GSE38987, GSE22056, GSE33223, GSE17855, GSE15389) and ArrayExpress (E-MTAB-3444). Raw Affymetrix data were downloaded in raw CEL format and imported into an in-house analysis pipeline written in R (version 3.1.1) using Bioconductor (Huber *et al.* 2015) packages from limma (Ritchie *et al.* 2015), affy (Gautier *et al.* 2004) and oligo (Carvalho and Irizarry 2010). Data were normalised using RMA and differentially expressed genes/transcripts were identified using limma "best practice", and p-values were corrected for multiple testing using Benjamini-Hochberg (false discovery rate). Samples were then run through 2 bespoke R scripts to enable visualization per gene, where the WGNCA package (Langfelder and Horvath 2008) was used to convert probe-level data to gene-level data. These datasets were then used to produce boxplots.

2.5. GATA2 inhibitor and chemotherapeutics.

GATA2 inhibitor K-7174 (Bioquote limited) was dissolved in DMSO with a stock concentration of 10 mM and stored at minus 20°C protected from the light. Each working aliquot was used and stored at 4°C for up to one week. Etoposide (VP16; Sigma) was dissolved in DMSO and stored protected from the light at room temperature with a stock concentration of 10 mM. Ara-C (Cytosine β-D-arabinofuranoside; Sigma) was dissolved in PBS with a stock concentration of 1 mM and stored protected from the light at minus 20°C.

2.6. Tissue culture.

All cell culture experiments were performed in a Maxisafe 2020 Class II Biological Safety Cabinet (Thermofisher) and incubated at 37°C, 20% O_2 and 5% CO_2.

2.6.1. Human cell lines.

All cell lines used in this study tested negative for mycoplasma.

HEK293T (Cell Biolabs) cell line was derived from HEK293 by stably transfecting the SV40 large T antigen. Upon transfection of a viral plasmids encoding the SV40 origin, the plasmid will transiently replicate and produce a high copy number, increasing the viral production. HEK293T cells were maintained in DMEM (Gibco, UK) with 10% heath inactivated FBS (Gibco) and 2mM L-glutamine (Gibco). Doubling time of approximate 24 hours. Cells were passaged every 2-3 days when confluency reached 70-90%.

Platinum E (Cell Biolabs) cell line was derived from HEK293T by introducing the packaging plasmids gag, pol and env under the expression of the strong promoter EF1α. Internal ribosome entry site (IRES) were added in order to select the packaging plasmid with puromycin (env) and blasticydin (gag-pol). Platinum E cells were maintained in DMEM with 10% heath inactivated FBS and 2mM L-glutamine. Puromycin (1 μg/mL; Gibco) and blasticydin (10 μg/mL; Gibco) were added to the Platinum E cell line to maintain the packaging plasmids, but withdrawn prior transfection with calcium phosphate. Doubling time of approximate 24 hours. Cells were passaged every 2-3 days when confluency reached 70-90%.

THP1 cell line was a kind gift from Professor Dipak Ramji (Cardiff University). THP1 is a human monocytic cell line derived from a 1-year-old male with AML FAB M5. THP1 cells were maintained in RPMI 1640 (Gibco) with 10% FBS and 2mM L-glutamine. Doubling time of approximate 30 hours. Cells were passaged every 2-3 days at a density of 200,000-400,000 cells/mL.

K562 cell line was a kind gift from Professor Richard Darley (Cardiff University). K562 is a human erythroleukaemia cell line derived from a 53-year-old female with CML in blast crisis. K562 cells were maintained in RPMI 1640 with 10% FBS and 2mM L-glutamine. Doubling time of approximate 20 hours. Cells were passaged every 2-3 days at a density of 100,000-200,000 cells/mL.

KG1 cell line was a kind gift from Professor Richard Darley (Cardiff University). KG1 is a human erythroleukaemia derived from the parental KG1 cell line, derived from a 59-year-old male with erythroleukaemia that transformed into AML at relapse. KG1a cells were maintained in IMDM (Gibco) with 20% FBS and 2mM L-glutamine. Doubling time of approximate 20 hours. Cells were passaged every 2-3 days at a density of 100,000-200,000 cells/mL.

NOMO1 cell line was a kind gift from Professor Richard Darley (Cardiff University). NOMO1 is a human monocytic cell line derived from a 31-year-old female with AML FAB M5a. NOMO1 cells were maintained in RPMI 1640 with 10% FBS and 2mM L-glutamine. Doubling time of approximate 35 hours. Cells were passaged every 2-3 days at a density of 300,000-500,000 cells/mL.

2.6.2. Murine AML cell lines.

Cell lines from murine *Meis1a/Hoxa9* and *Mll-af9* Pre-LSCs at CFC3 and LSCs from moribund mice were established liquid culture in IMDM 10% FBS, 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 20 ng/mL SCF (Peprotech), 10 ng/mL IL-3 (Peprotech), and 20 ng/mL IL-6 (Peprotech). Doubling time of approximate 24 hours. Cells were passaged every 2-3 days at a density of 100,000-200,000 cells/mL.

2.6.3. Bone marrow extraction.

Cleaned bones were crushed using a pestle and mortar. BM was collected in PBS 2% FBS and filtered through a 70 µm strainer (Miltenyi Biotec) to remove cell aggregates. This step was repeated 2 times until obtaining a 30 mL BM single cell suspension.

2.6.4. Red blood cell lysis.

PB red blood cell lysis was carried out using ammonium chloride (NH_4Cl) (STEMCELL technologies). When RBCs are in NH_4Cl under hypotonic conditions (0.8% NH_4Cl), H_2O enters the cells. Non-nucleated RBCs have weaker membrane tonicity, in comparison to leukocytes or nucleated RBCs therefore, they undergo osmotic lysis. 12 µL of blood were added into 600 µL of NH_4Cl, incubated for 6', mixed thoroughly by inverting the tube 3 to 4 times, and incubated for another 6'**.** Samples were then centrifuged for 10' at 370 xG at RT and supernatant containing lysed RBCs was removed to obtain a pellet of WBCs. For BM, cell pellet was resuspended in 1 mL of NH_4Cl and incubated 2' at RT. Red blood cell lysis was stopped by adding 9 mL of PBS 2% FBS.

2.6.5. Splenocyte and thymocyte extraction.

Spleen or thymus was placed into a 70 µm strainer in PBS 2% FBS inside a 6-cm petri dish. Spleen or thymus was then homogenised using a 5mL syringe plunger. Cell suspension was resuspended to eliminate cell aggregates. This step was repeated 2 times to obtain a 7-mL spleen or thymus single cell suspension.

2.6.6. AutoMACS magnetic cell separation – c-kit$^+$ enrichment.

BM WBC pellet (after red blood cell lysis) was resuspended in 300 µL of PBS 2% FBS containing 20 µL of mouse CD117 (c-kit) microbeads (Miltenyi Biotec), and incubated for 12' at 4°C while rotating. Cells were washed, filtered through a 30 µm strainer (CellTrics), and placed into the AutoMACS (Miltenyi Biotec). C-kit$^+$ cells were separated using the "posselds" program, which is ideal for rare cell populations with low or medium antigen expression. C-kit$^+$ cells were then used for downstream applications: cell sorting (to isolate HSCs/HPCs), HSPCs culture, or normal/leukaemic transduction experiments.

2.6.7. Colony forming cell (CFC) assay.

CFC assays were performed according to the manufacturer's protocol. Cells and media up to 250 µL were added into the methylcellulose (STEMCELL technologies), vortexed twice for 3'', incubated for 5 minutes, and plated using a 2 mL syringe and 19G blunt-end needle. MethoCult™ M3434 was used for wild-type murine haematopoiesis studies; MethoCult™ H4434 was used for human leukaemia experiments; and MethoCult™ M3231 supplemented with 20 mL of IMDM, 1 mL of Pen/Strep, 20 ng/mL of murine SCF, 10 ng/mL of murine IL-3, 10 ng/mL of murine IL-6, 10 ng/mL of murine GM-CSF (peprotech) was employed for murine leukaemogenic assays. Colonies were counted and scored using an inverted microscope (Leica) at day 12 for assays using M3434 and H4434, and at day 6 for M3231.

2.6.8. Culture of murine HSPCs for normal haematopoiesis experiments.

Murine HSPCs (after c-kit enrichment) were cultured in STEMSpan (STEMCELL technologies) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 ng/mL of murine SCF, 50 ng/mL of murine TPO (peprotech), 50 ng/mL of murine FLT3L (peprotech), and 5 ng/mL of IL-3.

2.6.9. Cryopreservation media.

Human cell lines were frozen down in 70% of media, 20% FBS and 10% DMSO (Sigma). Murine BM cells and leukaemia cell lines were frozen down in 90% FBS and 10% DMSO. All cells were frozen down by centrifuging at 4°C. Pellet was resuspended in ice-cold freezing media, transferred to a 1 mL cryovial (Camlab limited), and stored into an alcohol-free cell freezing container (Biocision) that allows for a -1ºC/minute freeze rate. Cryovials were stored at minus 80°C for short term storage (less than one month) or in liquid nitrogen (minus 175°C) for long term storage (longer than one month).

2.6.10. Generation of retroviruses and lentiviruses.

Calcium phosphate method was used to generate retroviruses (Kingston *et al.* 2003). 10 µg of retroviral vectors containing oncogenes (or empty vectors) were diluted in 437.5 µL of H_2O, and then mixed with 62.5 µL of calcium chloride (Sigma). This mix was added drop-wise to 500 µL of 2x hepes buffered saline (HBS; Sigma) and after 15' incubation, added to the media of Platinum E cells at 70% confluency in a 10-cm dish. 25 µM Chloroquine (Sigma) was added to Platinum E cells media before transfection. Supernatant-containing retroviruses was collected at 24 and 48 hours after transfection, passed through a 0.45 µm filter (Sigma), snap freeze in dry ice, and stored at minus 80°C.

To generate lentiviruses, 3 µg of VSV-G and 6.9 µg of psPAX2 plasmids were co-transfected with 10 µg of shGATA2 or Cre lentiviral plasmids using the calcium phosphate method in HEK293T cells.

For virus titre calculation, on day one, serial dilutions of viruses produced were added to 50,000 HEK293T cells while seeding into a 24-well plate. On day two, the media containing viral particles was replaced for fresh media. On day 4, cells were analysed by flow cytometry to measure efficiency of transduction. The titre was calculated from wells showing between 10-30% of positive cells since multiple integrations occurs at higher transduction rates (Fehse *et al.* 2004). Therefore, to obtain the titre (viral particles/mL), the number of plated cells (50,000) are multiplied by the proportion of transduced cells and divided by the volume of viruses added to the supernatant (in mL).

2.6.11. Lentiviral GATA2 knockdown in THP1.

Non-treated cell culture plates (Starlab) were coated with 100 µg/ml of retronectin (Clontech) overnight at 4°C. Retronectin was re-used (up to 10 times) and stored at minus 20°C. Lentiviruses encoding shGATA2 (31-34) and a GFP marker or scramble GFP control were bound to the retronectin-coated well by centrifugation for 2:30 hours at 2,100 xG at RT. Supernatant was discarded and 500,000 THP1 cells were bound

to the virus-retronectin-coated plate by centrifugation for 5' at 500 xG at RT. 8 hours later, THP1 cells were transferred to another plate and expanded for 5 days before sorting transduced THP1 live cells based on GFP fluorescence. To avoid contamination, sorted THP1 cells were maintained in media or plated in methylcellulose supplemented with amphotericin B (Sigma).

2.6.12. Lentiviral Cre deletion of Gata2 in murine HSPCs.

According to the method described in **2.6.11**, wild type murine c-kit$^+$ cells were transduced with lentiviruses carrying Cre-iVenus or empty vector. 72 hours after transduction, live cells were sorted based on GFP fluorescence and plated in their respective media or methylcellulose supplemented with amphotericin B (Sigma).

2.6.13. Retroviral mouse models of AML.

The day before transduction, 1 million c-Kit$^+$ cells were pre-stimulated with 40 ng/mL SCF, 20 ng/mL IL-3, and 20 ng/mL IL-6. Cell were transduced with retroviruses encoding Meis1a/Hoxa9, Mll-af9, or their respective control vectors according to the method described in **2.6.11** with minor changes. 6 hours after the first transduction, cells were transduced for a second time in a new retronectin-coated plate containing retroviruses. Next day, medium containing antibiotics geneticin 418 (G418) (1 mg/ml) (Gibco) and/or puromicyn (1.5 µg/ml) was added to the medium to select transduced Meis1a/Hoxa9 cells. 72 hours later live cells were plated in CFC1 in M3231 containing G418 and puromicyn to maintain selection. Mll-af9 transduced cells were left for 72 hours before sorting live cells based on GFP fluorescence into CFC1. Colonies were harvested and replated every 6 days up to 3 rounds of CFC. Cells at CFC3 were harvested and enriched for c-kit prior to transplant into lethally-irradiated primary recipients or generation of pre-LSCs cell lines. LSCs from moribund mice showing symptoms of AML were sorted and transplanted into lethally-irradiated secondary recipients.

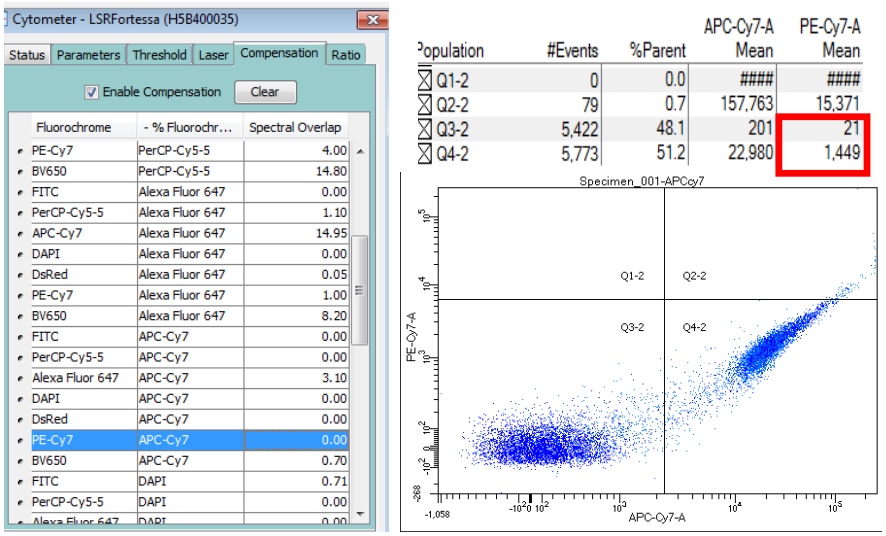
2.7. Flow cytometry.

All FACS analysis were performed in a 4 laser BD LSRFortessa™ (BD Biosciences), except the proliferation assay, which was analysed on the 2 laser BD Accuri. All FACS sorting experiments were performed in a 4 laser BD FACSAria™ Fusion (BD Biosciences). Full list of antibody clones, fluorochromes, and companies can be found in **Table 2.5**. All FACS data were analysed using FlowJo 10.0.8 (Tree Star, Inc) using the gating strategies for each assay that are described in **Figure 2.5** to **Figure 2.8**. Manual compensation in FACS Diva software was performed using single stains for each antibody-associated fluorochrome within each experiment. Compensation was adjusted by equalising the median fluorescence intensity (MFI) of the negative and positive population of each fluorochrome against all individuals fluorochrome within the experiment (**Figure 2.4**). For SLAM and progenitor staining, fluorescence minus one (FMO) controls were used to set the background fluorescence of each antibody as a guidance for gating of each population.

Table 2.5 Antibodies for FACS

Antigen	Clone	Reactive specie	Fluorochome	Concentration	Company
CD3	17A2	Mouse	Biotin, APC, FITC	1/1000	BioLegend
CD4	GK1.5	Mouse	Biotin, PE, PE-Cy7	1/1000	BioLegend
CD8a	53-6.7	Mouse	Biotin, PE, APC-Cy7	1/1000	BioLegend
CD9	MZ3	Mouse	PE	1/1000	BioLegend
CD11b	M1/70	Mouse/Human	Biotin, PE, APC	1/1000	BioLegend
CD14	M5E2	Human	Pacific blue	1/500	BioLegend
CD16/32	93	Mouse	PE-Cy7	1/25	BioLegend
CD19	6D5	Mouse	APC-Cy7	1/100	BioLegend
CD33	WM53	Human	APC	1/500	BD Biosciences
CD34	RAM34	Mouse	FITC, APC	1/25	eBiosciences
CD41	MWreg30	Mouse	FITC	1/1000	BioLegend
CD45.1	A20	Mouse	BV510, APC	1/500	BioLegend
CD45.2	104	Mouse	Pacific blue, BV510, PE	1/500	BioLegend
CD45R/B220	RA3-6B2	Mouse/Human	Biotin, FITC, APC	1/1000	BioLegend
CD48	HM48-1	Mouse	FITC	1/50	BioLegend
CD51	RMV-7	Mouse	PE	1/1000	BioLegend
CD71	RI7217	Mouse	PE	1/1000	BioLegend
CD117	2B8	Mouse	PE, APC	1/100	BioLegend
CD150	TC15-12F12.2	Mouse	PE-Cy7	1/100	BioLegend
Gr1	RB6-8C5	Mouse	Biotin, FITC, PE-Cy7	1/1000	BioLegend
Ter119	Ter119	Mouse	Biotin, APC-Cy7	1/1000	BioLegend
Sca-1	D7	Mouse	PE, APC-Cy7	1/25	BioLegend
Streptavidin		Mouse/Human	Pacific blue, PerCP	1/100	BioLegend, eBiosciences
γ-H2AX	2F3	Mouse/Human	APC	1/200	BioLegend
Mouse IgG1, κ Isotype Ctrl	MOPC-21		APC	1/200	BioLegend
Fc block	93	Mouse		1/100	BioLegend
GATA2	IC2046P	Human	PE	1/20	R&D systems

(A)



(B)

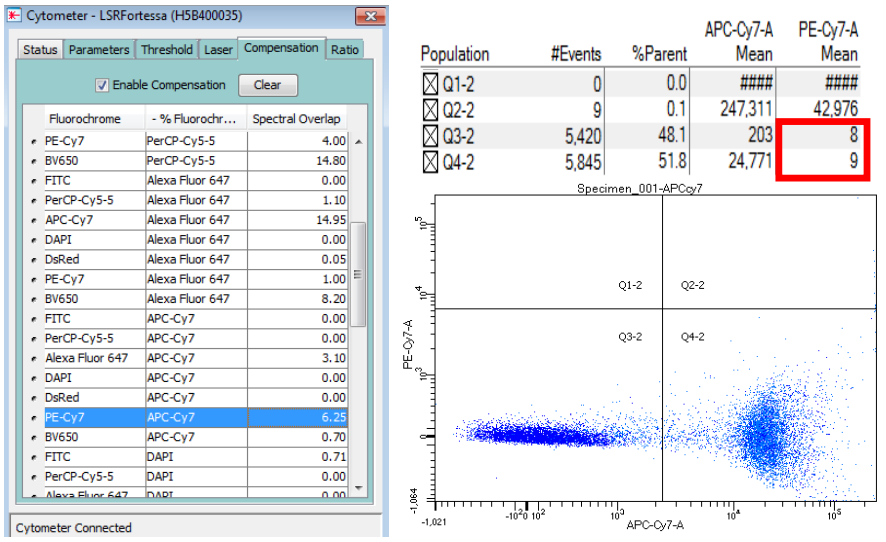


Figure 2.4. Compensation strategy in BD FACS DIVA. (a) Non-compensated FACS plot showing APC-Cy7 leaking into the PE-Cy7 channel. MFI APC-Cy7 negative (21); MFI APC-Cy7 positive (1,449); Spectral overlap 0. (b) FACS plot showing compensation of APC-Cy7 into the PE-Cy7 channel. MFI APC-Cy7 negative (8); MFI APC-Cy7 positive (9); Spectral overlap 6.25

2.7.1. Detection of apoptosis by annexin V.

Cells were incubated in annexin binding buffer (BD biosciences) containing annexin V (BioLegend) for 25' in the dark at RT. Annexin binding buffer was added to stop the reaction, and 2 µL of diamidino-2-phenylindole (DAPI, 20 µg/ml)(Molecular probes) was added to the samples before acquisition of 20,000 events in single cells gate.

In healthy cells, PS resides within the inner side of the plasma membrane, and it is translocated to the outer side of the membrane early during apoptosis. Loss of membrane integrity during late apoptosis allows DNA dyes such as DAPI to enter and stain the nuclei. Annexin V is a 35–36 kDa Ca^{2+}-dependent phospholipid-binding protein that recognises and binds phosphatidylserine (PS) residues. Annexin V binding buffer provides the Ca^{2+} required for its binding (**Figure 2.5**).

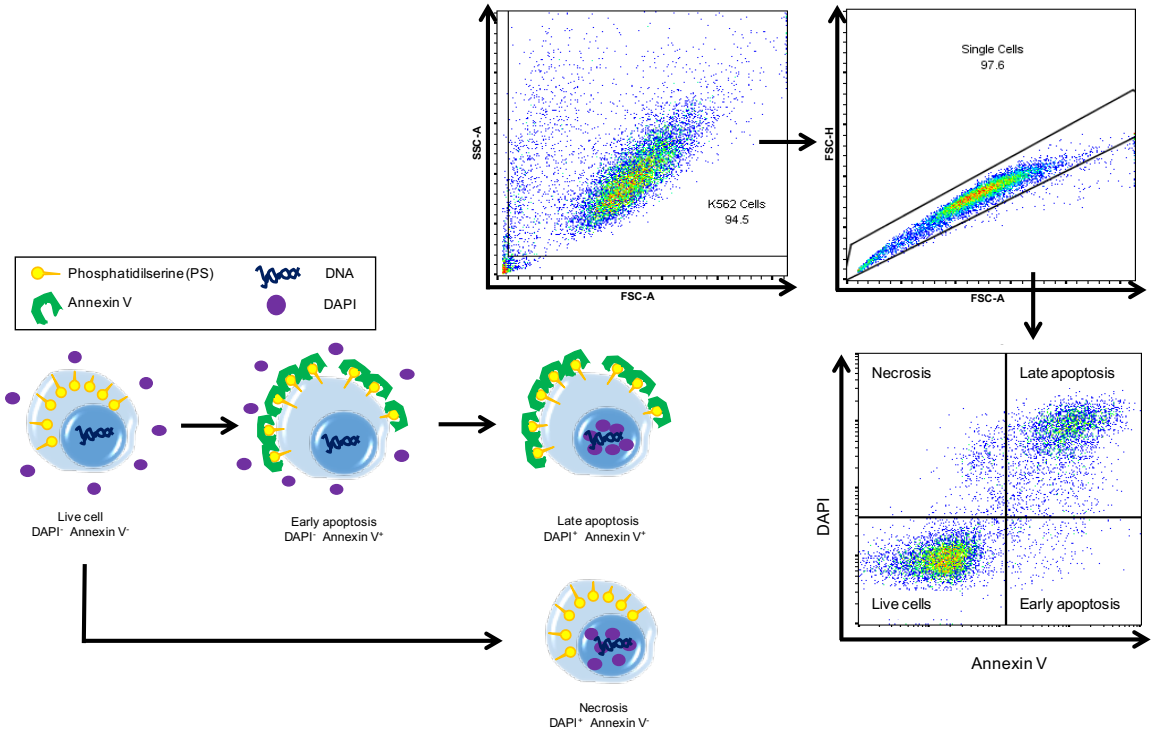


Figure 2.5 Detection of apoptosis using annexin V. (Left) Schematics of the apoptosis and necrotic death cell pathways in relation with annexin V and DAPI binding to PS and DNA. (Right) Representative FACS plots showing the Annexin V – DAPI staining: live cells ($DAPI^-$ Annexin V^-), early apoptosis ($DAPI^-$ Annexin V^+), late apoptosis ($DAPI^+$ Annexin V^+), necrosis ($DAPI^+$ Annexin V^-).

2.7.2. Proliferation assay.

Cells were stained with 2 µL of propidium iodide (PI, 20 µg/mL)(Sigma), vortexed, and 10 µL acquired with a BD Accuri™ C6 Plus (BD Biosciences). BD Accuri has the ability to quantify the exact number of cells within a specific volume of cells. PI negative cells within 10 µL were used to calculate the total number of cells in each well.

2.7.3. Assessment of cell cycle status.

Cells were stained with 100 µL of PBS containing DAPI (5 µg/mL) 0.1% NP40 (Sigma) and 50 µg/mL of RNAaseA (ThermoFisher). After 5' incubation in the dark, 20,000

events were acquired at low speed (< 400 events/sec). NP40 is a mild detergent that permeabilises the plasma and nuclear membrane allowing DAPI to bind the DNA. DAPI fluorescence in linear scale was used to measure the DNA content and distinguish the cell cycle phases (**Figure 2.6**).

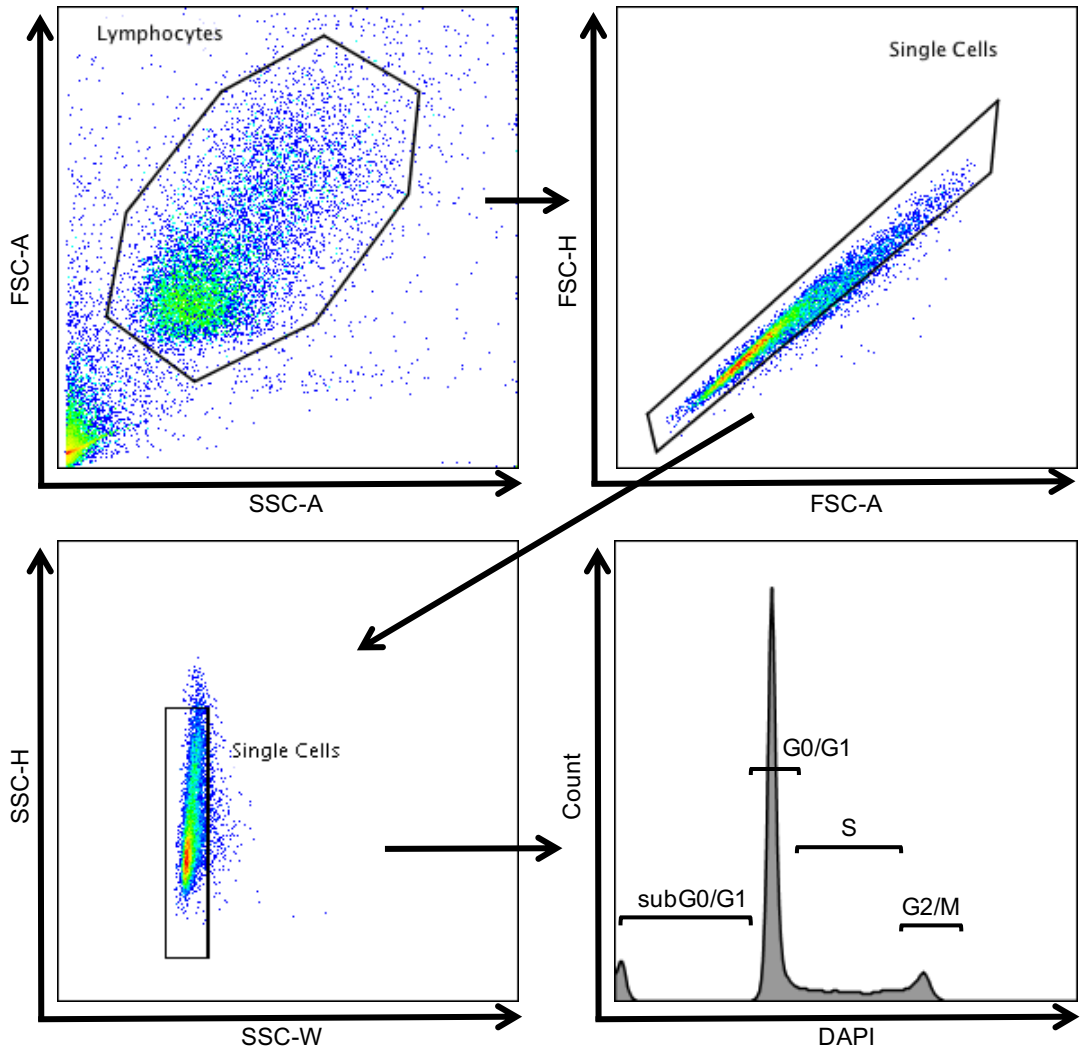


Figure 2.6 Cell cycle analysis using DAPI. DNA content shown as DAPI fluorescence in a FACS histogram. SubG0 corresponds to apoptotic cells (N < 2). G0 corresponds to quiescent cells. G1 corresponds to cells preparing for DNA replication (N = 2 copies of DNA), S corresponds to the DNA replication phase (N > 2), G2 corresponds to cells preparing for Mitosis (N = 4). M corresponds cells in mitosis (N = 4).

2.7.4. Intracellular staining.

Cells were washed twice with ice-cold PBS prior to fixation with 1% methanol-free PFA (ThermoFisher) for 15' on ice. Cells were then permeabilised in PBS 0.1% X-Triton (Sigma) 2% BSA (ThermoFisher) for 15' on ice, and stained for 30' on ice and in the dark with a fluorescence-labelled antibody diluted in the permeabilisation buffer. Cells

were washed twice and resuspended in PBS 2% BSA prior to acquisition. For cell cycle profile, DAPI was added at a final concentration of 5 µg/mL.

2.7.5. Extracellular staining.

Extracellular staining was carried out in PBS 2% FBS and with an incubation time of 25' in the dark at 4°C, except for streptavidin staining. Cells were washed and filtered through a 30 µm strainer. To exclude dead cells from the analysis, 2 µL of DAPI (20 µg/mL) was added prior to acquisition.

2.7.5.1. Lineage staining.

200,000 cells from BM, SP or thymus (or 12 µL of blood) were stained with antibodies to distinguish myeloid cells (Gr1, Mac1), T lymphocytes (CD3e, CD4, CD8a), B lymphocytes (CD19, B220), erythroid cells (Ter119, CD71), and megakaryocytes (CD41) (**Figure 2.7**). For transplantation experiments, CD45.1 and CD45.2 were included to separate donor cells from recipients and support cells. At least 20,000 events were recorded from live cells.

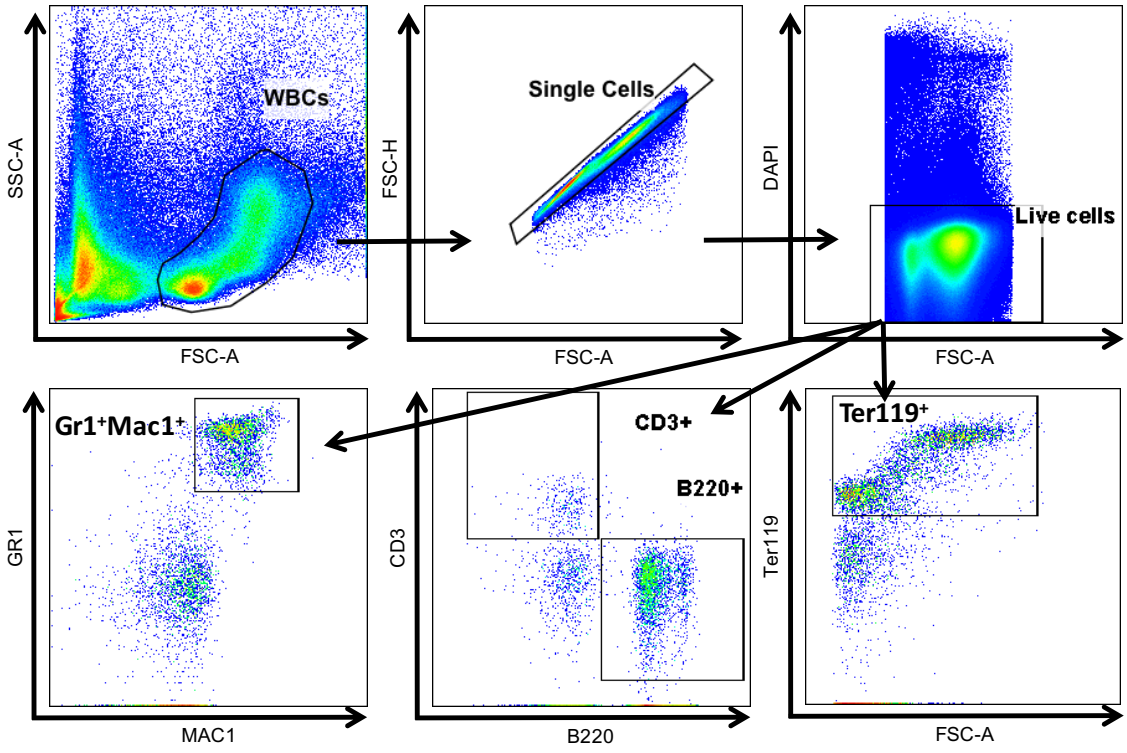


Figure 2.7. Gating strategy for myeloid and lymphoid lineage staining.

To assess the differentiation status of human THP1 cells, cells collected at the indicated time points were stained with anti-human CD14, CD11B and CD71.

2.7.5.2. HSC and progenitor staining.

10 million cells from BM or SP were stained with a cocktail of biotinylated antibodies against lineage-specific markers (Ter119, Mac1, Gr1, B220, CD4, CD8a, CD3e) plus antibodies to detect haematopoietic stem cells and multipotent progenitors (c-kit, Sca-1, CD48, CD150) or lineage-restricted progenitors (c-kit, Sca-1, CD34, CD16/32, CD127) (**Figure 2.8**). Cells were then washed and incubated with fluorescence-conjugated streptavidin for 15' in the dark at RT. For transplantation experiments, 20 million cells were stained with the above combinations together with CD45.1 and CD45.2 antibodies to separate donor cells from recipients and support cells. At least 10,000 LSK cells were recorded for SLAM staining, and 10,000 LK for progenitor staining.

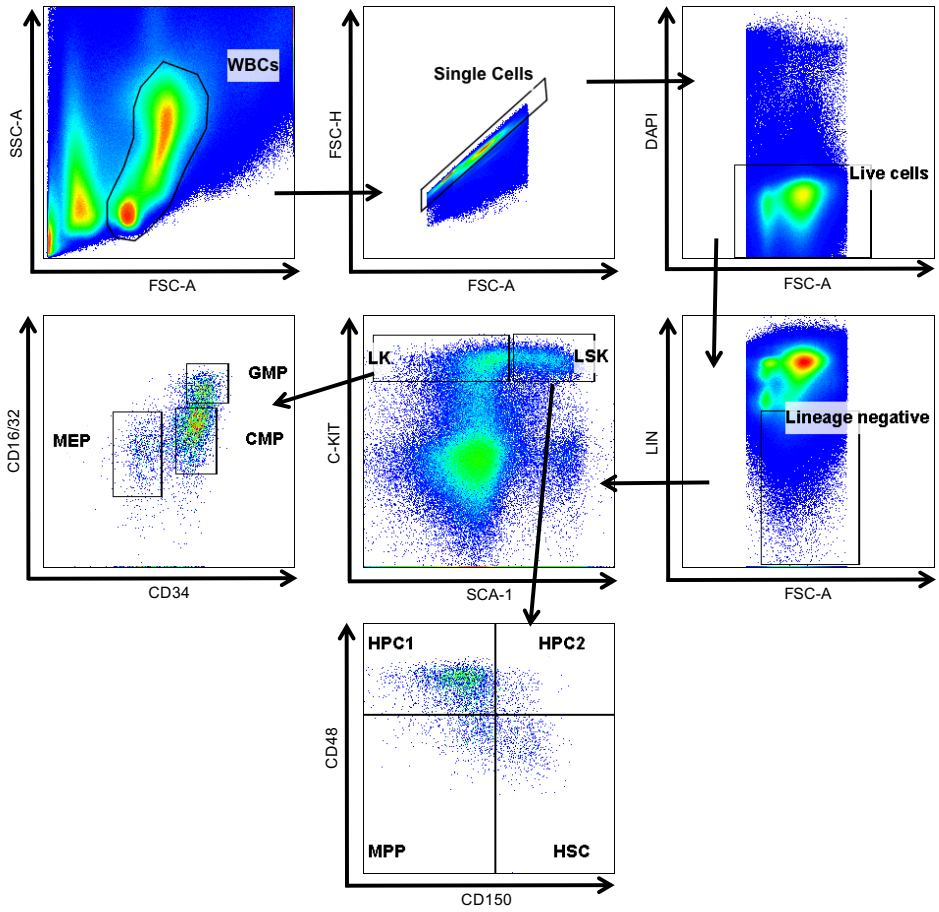


Figure 2.8. Gating strategy used for SLAM and progenitor staining.

2.7.5.3. LSC staining.

BM cells from moribund mice with AML were stained with antibodies to detect LSCs in the *Meis1a/Hoxa9* and *Mll-af9* models. According to previous publications, LSCs were immunophenotypically characterised as $DAPI^-$, $CD45.2^+$ (or GFP^+ for Mll-af9 model), c-kit^+, $CD16/32^+$, $CD34^{low}$.

2.7.6. Cell sorting.

Cells after c-kit enrichment were stained with antibodies as described in **2.7.5.** and sorted using a BD FACSAria™ Fusion. All sorting experiments were carried out with an 85-micron nozzle.

2.8. Transplantation assays.

C57BL/6 SJL (CD45.1) or *C57BL/6 (*CD45.2) adult recipient mice were irradiated 24 hours before transplantation. Mice received a split dose of 1,000 cGy (^{137}Cs source) four hours apart between doses. After second irradiation, mice were housed in individual ventilated cages (IVCs) and administered baytril water for four weeks. All transplants were carried out under Class 2 safety cabinet. Mice tail vein were dilated by heating the mice at 37°C for 15-30'. Cells were injected in PBS in a volume of up to 200 µL per mouse.

2.8.1. Leukaemia transplants.

For primary transplants of pre-leukaemic cells from Meis1a/Hoxa9 (or Mll-af9), 500,000 c-kit^+ $CD45.2^+$ (or GFP^+) cells were injected in the tail vein of lethally-irradiated $CD45.1^+$ mice, unless otherwise stated. For secondary transplants of LSCs, 10,000 c-kit^+ $CD45.2^+$ (or GFP^+) BM cells were injected in the tail vein of lethally-irradiated $CD45.1^+$ mice.

2.8.2. Normal haematopoiesis transplants.

For cell-autonomous transplant, 500,000 unfractionated BM cells from Gata2 mice were injected in the tail vein of lethally-irradiated CD45.1^{+} mice. For secondary transplant, 500,000 CD45.2^{+} BM cells were transplanted. For niche studies, 500,000 unfractionated BM cells from CD45.1^{+} mice were transplanted into the tail vein of lethally-irradiated Gata2 mice.

2.9. RNA extraction and gene expression analysis.

All samples were collected in RLT plus buffer (Qiagen) and stored at minus 80 until extraction. RNA extraction was carried out with the RNAeasy Plus Micro/Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was made using the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) for Gata2 expression experiments in murine haematopoiesis, and QuantiTec RT Kit (Qiagen) for the rest of experiments. Real time quantitative PCR (RT-qPCR) was performed in a QuantStudio® 7 Flex Real-Time PCR System (Applied biosystems) using Taqman (Applied Biosystems) or Sybr green method (PCR Biosystems). Full list of primers is listed in **Table 2.6**. Differences in input cDNA were normalised against the housekeeping gene (*Hprt* for mouse and *GAPDH* for human) and the mRNA expression levels were determined by the $2^{-\Delta\Delta CT}$ method of relative quantification (Schmittgen and Livak 2008).

Table 2.6 qRT-PCR primers

Primer name	ID name	Manufacturer
Gata2	Mm00492301_m1	Applied Biosystems
Hprt	Mm03024075_m1	Applied Biosystems
GATA2	Hs00231119_m1	Applied Biosystems
P21	Hs00355782_m1	Applied Biosystems
P27	Hs00153277_m1	Applied Biosystems
P53	Hs01034249_m1	Applied Biosystems
P57	Hs00175938_m1	Applied Biosystems
LIG4	Hs00172455_m1	Applied Biosystems
GAPDH	Hs02786624_g1	Applied Biosystems

2.10. Data and statistical analysis

FACS data were analysed using FlowJo 10.0.8 (Tree Star, Inc) software and all results graphed using GraphPad Prism 7 (GraphPad Software Inc, CA). Data are presented as mean ± standard error of mean (SEM). Significant differences were calculated using Mann–Whitney U test or TWO-WAY ANOVA (*$p<0.05$, **$p<0.01$, ***$p<0.001$, **** $p<0.0001$).

Chapter 3

Assessing the role of *Gata2* in adult murine haematopoiesis

3.1. Introduction

Haematopoietic stem cells (HSCs) are multipotent stem cells that give rise to progenitor cells, which differentiate into multiple blood lineages. External signals delivered from the BM niche are crucial for HSC homeostasis, alongside a cell intrinsic transcriptional program driven by transcription factors (TFs). To date, few TFs have been identified as essential for HSC survival in the mouse adult haematopoietic system; conditional deletion of *Mcl-1*, *Tel1/Etv6* and *Cited2* leads to a complete loss of immunphenotypically defined HSCs in adult mice (Kranc *et al.* 2009; Opferman 2005; Hock *et al.* 2004).

Gata2, a zinc finger TF expressed in the haematopoietic system (Tsai *et al.* 1994), is essential for the generation and survival of HSCs in development (de Pater *et al.* 2013). Constitutive deficiency of *Gata2* in *Gata2*$^{-/-}$ mice causes embryonic lethality (E10.5) due to severe anaemia, and profound effects on both primitive and definitive haematopoiesis (Tsai *et al.* 1994). Conditional deletion of *Gata2* before (*Vec-Cre*) and after (*Vav-iCre*) HSC generation identified that loss of *Gata2* leads to a detrimental impact on HSC function in the aorta-gonad-mesonephros (AGM) and foetal liver (FL) (de Pater *et al.* 2013).

The requirement for *Gata2* in adult HSC is less clear. Analysis of constitutive haploinsufficient *Gata2* (*Gata2*$^{+/-}$) mice showed quantitative and qualitative defects in transplantation assays of HSCs in adult BM (Rodrigues *et al.* 2005; Ling *et al.* 2004). Furthermore, *Gata2*$^{+/-}$ mice display defects in the number and functionality of granulocyte-macrophage progenitor (GMP) (Rodrigues *et al.* 2008). In humans, *GATA2* haploinsufficiency mutations have been linked with hereditary immunodeficient, MonoMac and Emberger syndromes that precede transformation to myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) (Hyde and Liu 2011), yet no features of *GATA2* clinical syndromes were observed in *Gata2*$^{+/-}$ mice. However, haematopoietic analysis of *Gata2*$^{+/-}$ mice have only been conducted in young adult mice, and it remains unclear whether aged *Gata2*$^{+/-}$ mice would provide a tractable model of *Gata2*-related syndromes. Therefore, a better understanding of the genetic interactions and downstream effectors of *Gata2* in normal adult haematopoiesis and malignant transformation is necessary.

3.2. Aims and Objectives

The specific aims for this chapter are:

1. To assess the expression of *Gata2* in the murine haematopoietic system.

2. To study the impact of acute, conditional *Gata2* deletion in HSCs using the Mx1-Cre system.

3. To establish a suitable model to investigate the molecular mechanism downstream *Gata2* in HSCs.

4. To characterise the long-term effects of *Gata2* haploinsufficiency employing the Mx1-Cre mouse model.

3.3. Results

3.3.1. *Gata2* is highly expressed in HSCs.

To date, expression of *Gata2* has been reported to be highest in HSCs and decreases throughout differentiation (Lim *et al.* 2012) yet this has not been formally demonstrated in highly purified HSCs and progenitor cell subsets (Oguro *et al.* 2013). We therefore purified HSCs and multipotent progenitors (MPP, HPC1, HPC2), lineage-restricted progenitors (CMP, GMP, MEP) and differentiated blood cells (T and B lymphocytes, myeloid cells and erythroid cells) by FACS (**Figure 3.1A**) and performed q-PCR to determine mRNA levels of *Gata2*. Our results indeed showed that *Gata2* is highly expressed in HSC and multipotent progenitors, and expression dramatically reduces during differentiation by 5-fold in CMP and 10-fold in GMP and MEP (**Figure 3.1B**). Finally, *Gata2* expression was reduced in T cells (80 fold), B cells (100 fold), myeloid cells (20 fold) and erythroid cells (25 fold) (**Figure 3.1B**).

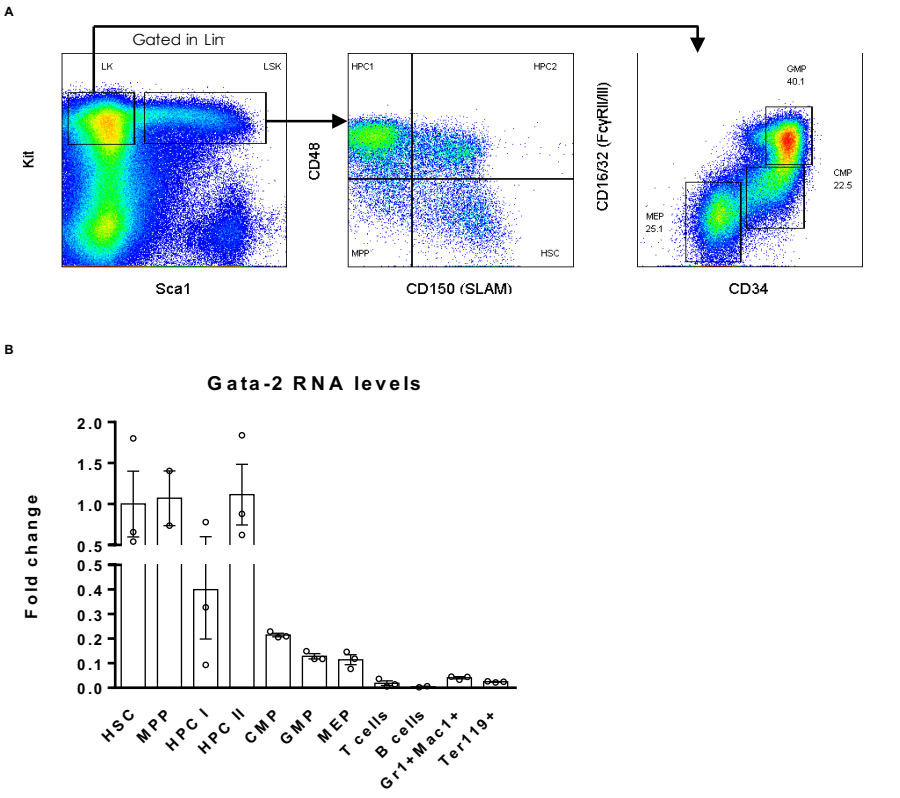


Figure 3.1. *Gata2* is highly expressed in HSCs. HSPCs populations (HSC, MPP, HPC-1, HPC-2, CMP,GMP, MEP) and mature blood cells (T, B, myeloid, erythroid) from adult BM mice were sorted to assess *Gata2* expression by Q-PCR. (A) Representative FACS plots showing sorting strategy for HSPCs. (B) Relative *Gata2* mRNA levels in haematopoietic cells in relation with expression in HSCs (HSC = 1) (n = 3-5). Two independent experiments were performed for the analysis. Data are mean ± SEM.

3.3.2. Acute deletion of *Gata2* leads to myeloid suppression

To directly assess the requirement for *Gata2* in adult haematopoiesis and to overcome the embryonic lethality in *Gata2*$^{-/-}$ mice (Tsai *et al.* 1994), we employed the *Mx1-Cre* system (Kühn *et al.* 1995) in which acute deletion of *Gata2* was achieved by the activation of the IFN pathway through pIpC injections. We bred *Gata2* floxed (fl/fl) mice with *Mx1-Cre* to obtain a cohort of *Gata2*$^{fl/fl}$*;Mx1-Cre*$^{+}$ or control mice (*Gata2*$^{fl/fl}$*;Mx1-Cre*$^{-}$ or *Gata2*$^{+/+}$*;Mx1-Cre*$^{+}$ respectively). Upon pIpC induction of 8-10-week-old mice, we harvested mice 24 days after the first dose of pIpC (**Figure 3.2A**) and confirmed complete *Gata2* deletion in *Gata2*$^{\Delta/\Delta}$*;Mx1-Cre*$^{+}$ BM cells (**Figure 3.2B**). We observed a reddish hue in the BM suspension of *Gata2*$^{\Delta/\Delta}$*;Mx1-Cre*$^{+}$ compared to control BM cells, indicating a reduction of white blood cells (**Figure 3.2E**). In addition, BM cellularity was significantly decreased in *Gata2*$^{\Delta/\Delta}$*;Mx1-Cre*$^{+}$ mice (**Figure 3.2C**). Immunophenotypic analysis of the BM revealed a complete reduction in the number of myeloid cells in the BM of *Gata2*$^{\Delta/\Delta}$*;Mx1-Cre*$^{+}$ mice (**Figure 3.2F**). Depletion of myeloid cells was also observed in the peripheral blood (PB) and spleen (SP) of *Gata2*$^{\Delta/\Delta}$*;Mx1-Cre*$^{+}$ mice (**Figure 3.2F**). The frequency and absolute number of erythroid cells were additionally decreased in the BM but not in the PB and SP of *Gata2*$^{\Delta/\Delta}$*;Mx1-Cre*$^{+}$ mice (**Figure 3.2G**). Red blood cell formation occurs in the BM and these cells lose their nuclei during their last differentiation step from reticulocyte to erythrocyte, and these erythrocytes are the only circulating red blood cells under homeostasis. Morphological and blood counts would have helped to confirm whether we actually detected nucleated red blood cells (Ter119^{+}) in the PB. In addition, the high percentage of CD41^{+} cells observed in the PB of control and KO mice could be due the lack of EDTA in the FACS buffer used for these experiments. Interestingly, the BM, but not the SP or PB, of *Gata2*$^{\Delta/\Delta}$*;Mx1-Cre*$^{+}$ mice showed a significant expansion in the frequency and absolute number (not statistically significant) of B cells (**Figure 3.2H** and **Figure 3.2J-M**). Therefore, these data indicate that *Gata2* is a critical regulator of myeloid lineages in haematopoiesis and of erythroid and B cell maintenance in the bone marrow.

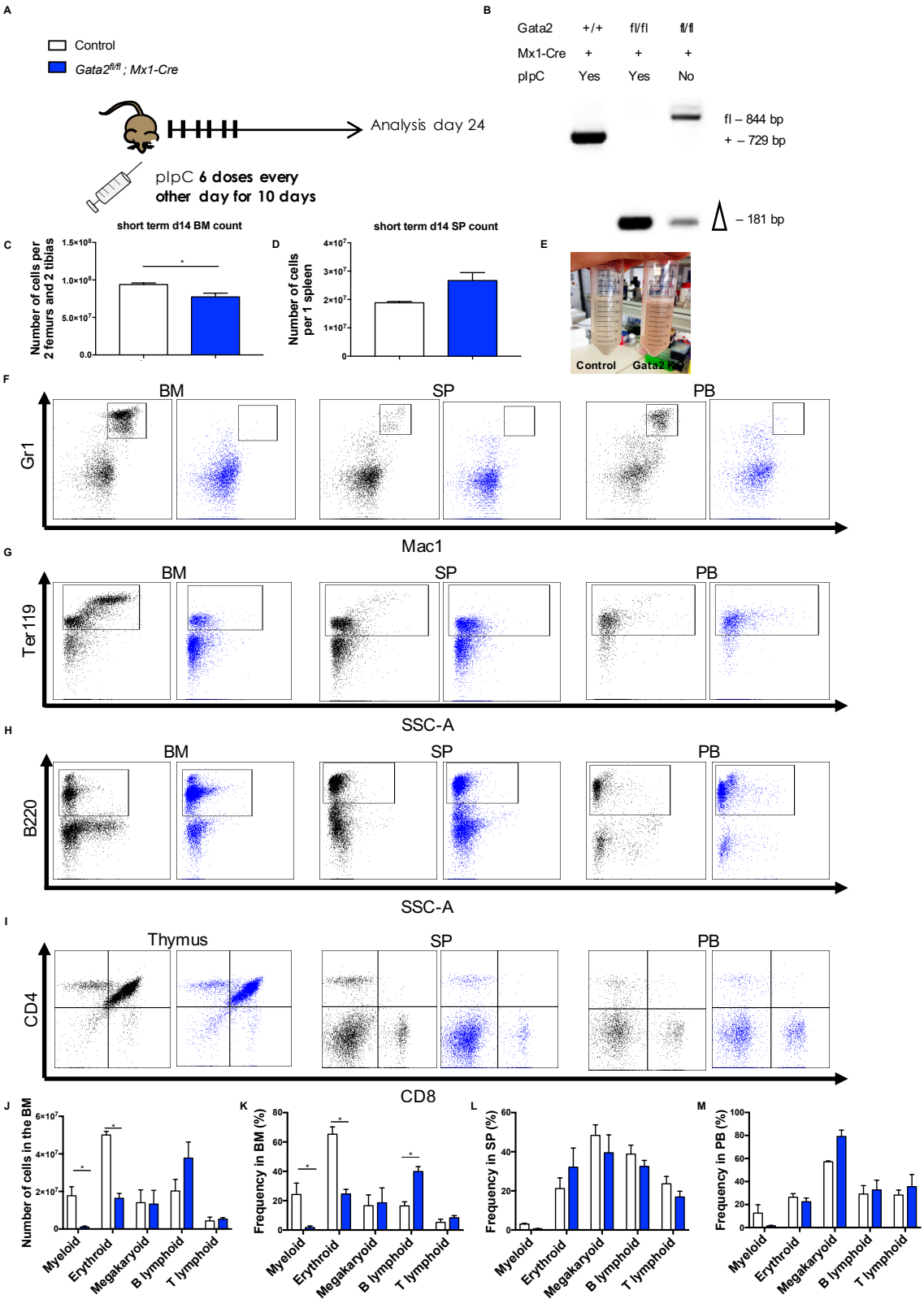


Figure 3.2. Acute deletion of *Gata2* leads to myeloid suppression and B cell expansion. (A) 8-to-12 week old *Gata2*$^{fl/fl}$;*Mx1-Cre*$^{+}$ control mice received six doses of pIpC every other day. 24 days after the initial dose, the mice were harvested for analysis. (B) Genomic PCR showing *Gata2* deletion (C) BM cellularity at day 24 (n = 4). (D) SP cellularity at day 24 (n = 4). (E) Picture showing representative BM cell suspension at day 24. (F-I) Representative FACS plots of BM, SP, PB and thymus at day 24 showing (F) myeloid, (G) erythroid, (H) B cells, and (I) T cells. (J) Number of cells of each lineage in the BM at day 24 (n = 4). (K-M) Frequency of cells of each lineage in the (K) BM, (L) SP, and (M) PB at day 24 (n = 4). Two independent experiments were performed for all analysis. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

3.3.3. *Gata2* is essential for the maintenance of adult HSCs.

Next, we addressed the impact of *Gata2* deletion in HSCs and haematopoietic progenitor cells. We analysed the BM of *Gata2*$^{\Delta/\Delta}$*;Mx1-Cre*$^{+}$ mice at day 24 as described in **Figure 3.2A**, and found a near complete loss of the LSK compartment, that comprises the HSC, MPP, HPC-1 and HPC-2 populations, and LK cells, which capture CMP, GMP and MEP progenitor populations (**Figure 3.3A** and **Figure 3.3B**). From these data, we infer that *Gata2* could regulate the survival of HSCs. However, since we have not performed a survival curve after *Gata2* deletion, we cannot confirm the survival phenotype, and it could be argued that *Gata2* regulates *C-kit* expression and therefore HSCs are still present but we were not able to detect them by the standard LSK definition.

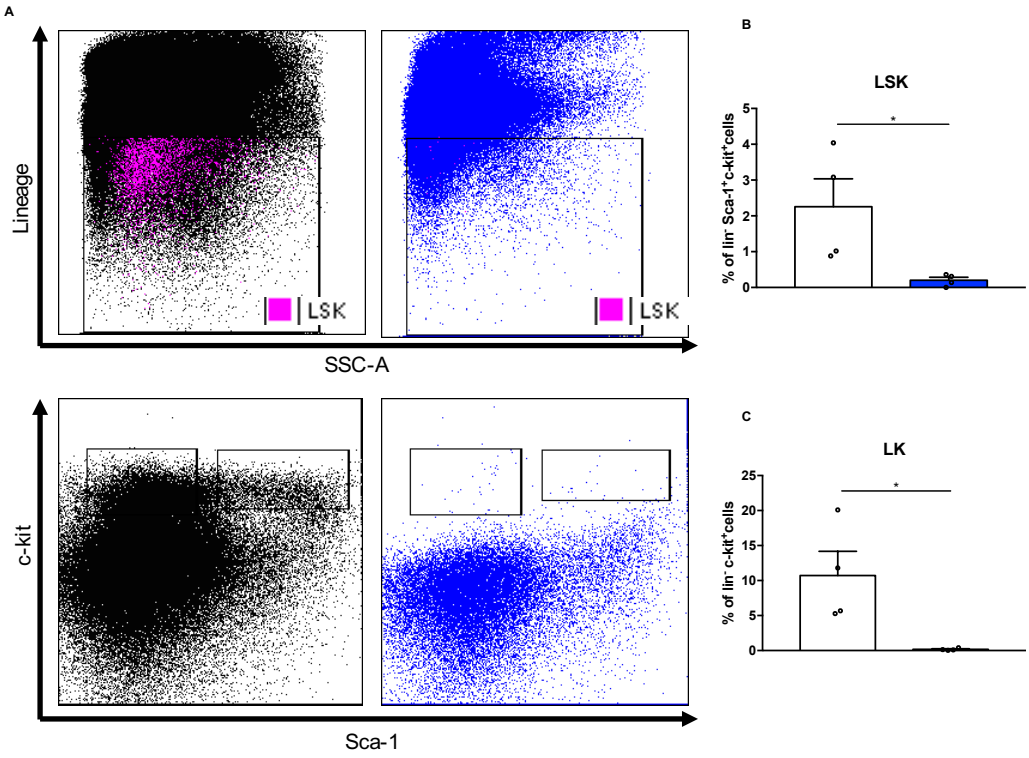


Figure 3.3. *Gata2* is essential for the maintenance of adult HSCs. Analysis of HSPCs at day 14 according to 3.2A schematics. (A) Representative FACS plots of showing loss of LSK and LK population in *Gata2* deleted mice (blue dots). (B) Frequency of LSK cells at day 14 (n = 4). (C) Frequency of LK cells at day 14 (n = 4). Two independent experiments were performed for all analysis. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

In order to study the molecular mechanism behind acute *Gata2* deletion in HSCs, we sought to analyse *Gata2*$^{\Delta/\Delta}$;*Mx1-Cre*$^{+}$ at day 15 after the first dose of pIpC (**Figure 3.4A**). After confirming complete *Gata2* deletion (**Figure 3.4B**) we were able to phenocopy our data at day 24 following the first dose of pIpC, including LSK and LK complete loss, myeloid depletion, and B cell expansion **(Figure 3.4C-G**). We also found a complete abolition in the CFC formation of *Gata2*$^{\Delta/\Delta}$;*Mx1-Cre*$^{+}$ BM cells in this experimental setting (**Figure 3.4E**). Therefore, acute deletion of *Gata2* leads to a rapid onset loss of HSCs, or at least, HSC immunophenotype.

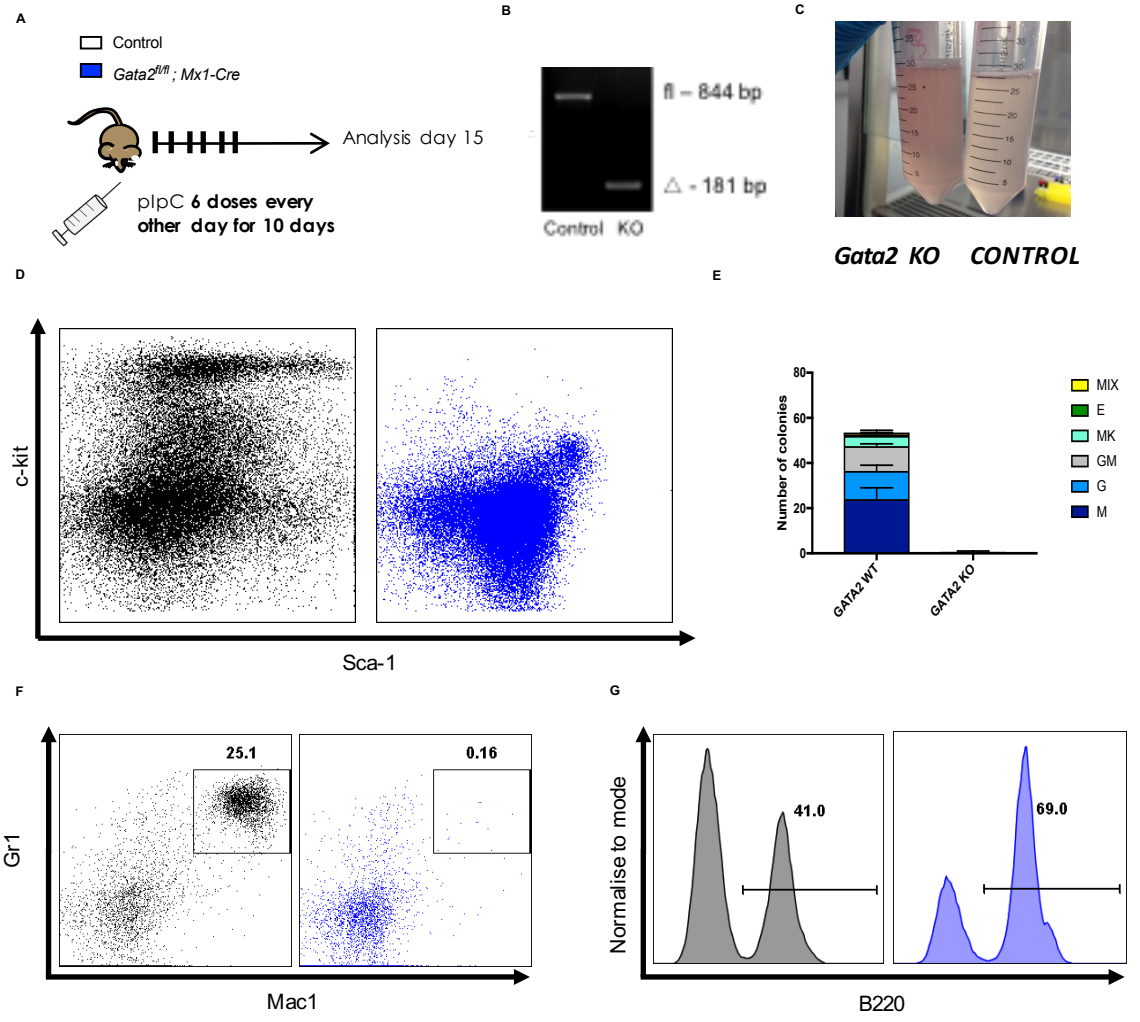


Figure 3.4. HSC loss at day 15 after *Gata2* deletion. (A) Following the same procedure than in figure 3.2A, we analysed the haematopoietic system at day 15 after pIpC induction. (B) Genomic PCR showing *Gata2* deletion. (C) BM cell suspension from *Gata2 KO* (left) and control (right) mice. (D-to-G) FACS plots from *Gata2 KO* and control mice BM showing a depletion of HSPCs in vivo (D) and in CFC assays in vitro (E), depletion of myeloid cells (F), and an increase in the frequency of B cells (G) at day 15 (n = 1). One experiment was performed for all analysis. Data are mean ± SEM.

3.3.4. *Gata2* acts in a cell-autonomous manner and independently of the niche to regulate HSCs.

The *Mx1-Cre* promoter is present both in haematopoietic cells and in the BM microenvironment (Kühn *et al.* 1995). To determine whether *Gata2* functions cell-autonomously in HSC maintenance, we transplanted untreated CD45.2$^+$ BM cells from *Gata2$^{fl/fl}$;Mx1-Cre$^+$* or control mice together with unfractionated CD45.1$^+$ BM cells in a competitive transplant (**Figure 3.5A**). Similar engraftment was observed in the PB of recipients transplanted with *Gata2$^{fl/fl}$;Mx1-Cre$^+$* and control cells (**Figure 3.5B**). Six-weeks after transplant *Gata2* deletion was induced and efficient *Gata2* deletion in CD45.2$^+$ BM cells of recipients transplanted with *Gata2$^{fl/fl}$;Mx1-Cre$^+$* BM cells (**Figure 3.5C**) was confirmed. We found a decrease in the frequency of CD45.2$^+$ cells in the BM, and to a lesser extent in the PB and SP of recipients transplanted with *Gata2$^{\Delta/\Delta}$;Mx1-Cre$^+$* BM cells (**Figure 3.5D**). Indeed, further immunophenotypic analysis revealed a complete loss of myeloid cells and significant reduction of erythroid cells in the BM, PB and SP (**Figure 3.5E-G**). Strikingly, CD45.2$^+$ *Gata2$^{\Delta/\Delta}$* cells within the LSK and LK compartments were undetectable (**Figure 3.5H** and **Figure 3.5I**). Finally, to test whether there was any residual haematopoietic activity in CD45.2$^+$ *Gata2$^{\Delta/\Delta}$* cells, we isolated CD45.2$^+$ BM cells from primary transplantation and transplanted them together with unfractionated CD45.1$^+$ competitor cells into secondary recipients. Four weeks after transplant we found no engraftment in the BM, PB, SP or thymus of recipients injected with CD45.2$^+$ *Gata2$^{\Delta/\Delta}$* cells **(Figure 3.5J**). Collectively these data demonstrate that *Gata2* regulates HSC maintenance in a cell-autonomous manner.

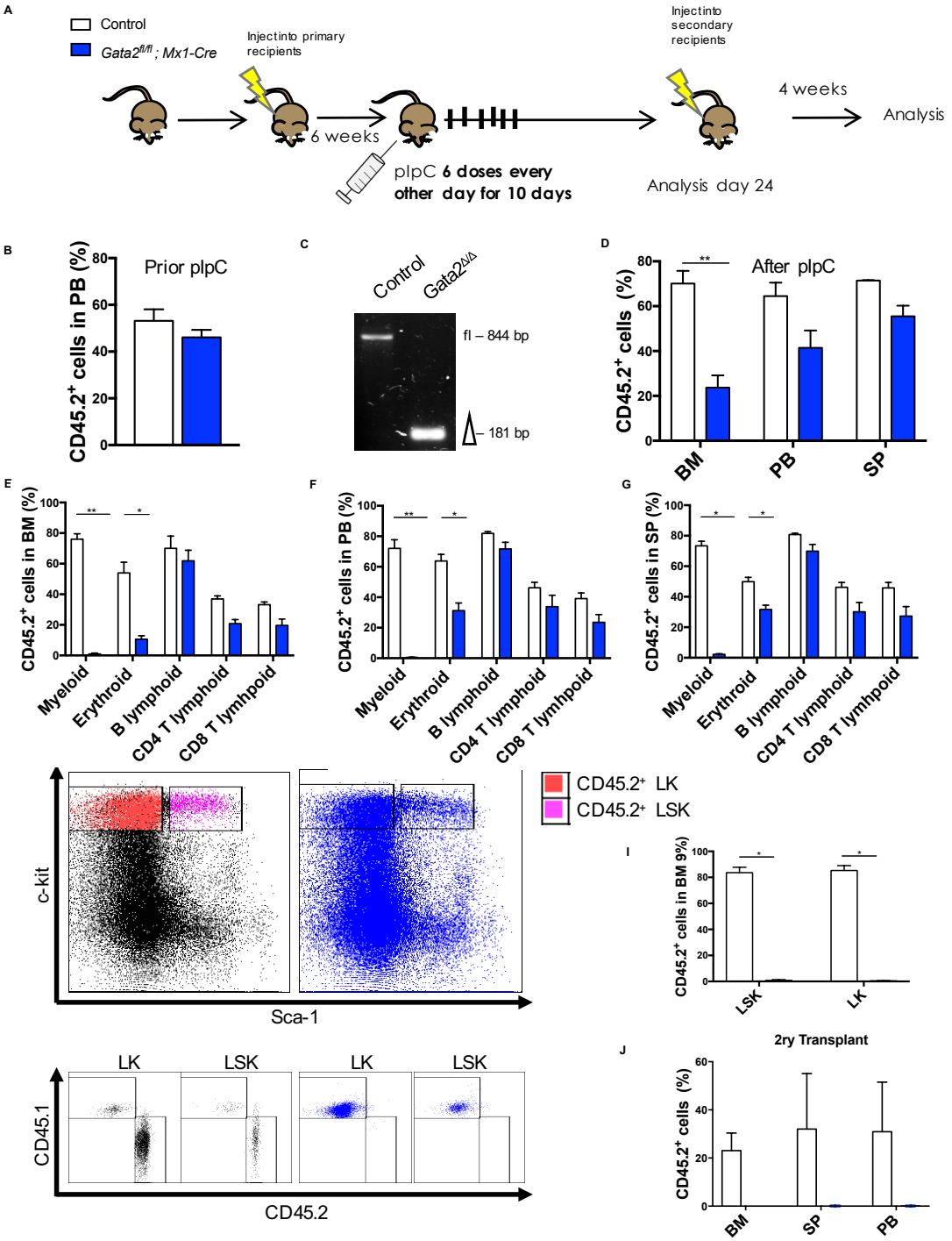


Figure 3.5. Gata2 acts in a cell-autonomous manner to regulate HSCs. CD45.2+ BM cells from *Gata2*$^{fl/fl}$;*Mx1-Cre*+ or control mice were transplanted into lethally-irradiated mice together with unfractionated CD45.1+ BM cells. Six weeks later *Gata2* deletion was induced as described in 3.2A, and 24 days later BM, SP, and PB were analysed. CD45.2+ BM cells from primary recipients were transplanted into lethally-irradiated mice, and four weeks later the contribution of CD45.2+ cells in BM, SP, and PB was measured. (B) Engraftment before pIpC induction (week six) (n = 7-8). (C) PCR gel showing *Gata2* deletion in CD45.2+ BM cells. (D) Engraftment at day 24 after pIpC in BM, SP and PB (n = 7-8). (E-G) Engraftment of specific blood cells at day 24 in (E) BM, (F) PB, (G) SP (n = 7-8). (H) Representative FACS at day 24 plot showing the contribution of *Gata2*$^{-/-}$ (blue) or control (black) CD45.2+ cells in the LSK and LK compartments of primary recipients (I) Frequency of CD45.2+ cells in LSK and LK populations (n = 7-8). (J) Frequency of CD45.2+ cells in the BM, PB and SP of secondary recipients four weeks after transplant. At least two independent experiments were performed for all analysis. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

To independently confirm the cell-autonomous role of *Gata2* in the regulation of HSPCs, we transduced c-kit^{+} cells from *Gata2*$^{fl/f}$ or control mice with lentivirus containing a bicistronic Cre and GFP reporter (**Figure 3.6A**). After confirming complete *Gata2* deletion (**Figure 3.6B**), we plated GFP^{+} cells into a CFC assay. We found that GFP^{+} *Gata2*$^{\Delta/\Delta}$ cells were unable to generate colonies in methylcellulose (**Figure 3.6C**). These data indicate a dependency for *Gata2* in HSPCs survival *ex vivo*, however we would need to validate this result by using *Gata2*$^{+/+}$ infected with Cre compared to empty vector control to gauge the potential Cre toxicity in colony formation of HSPCs.

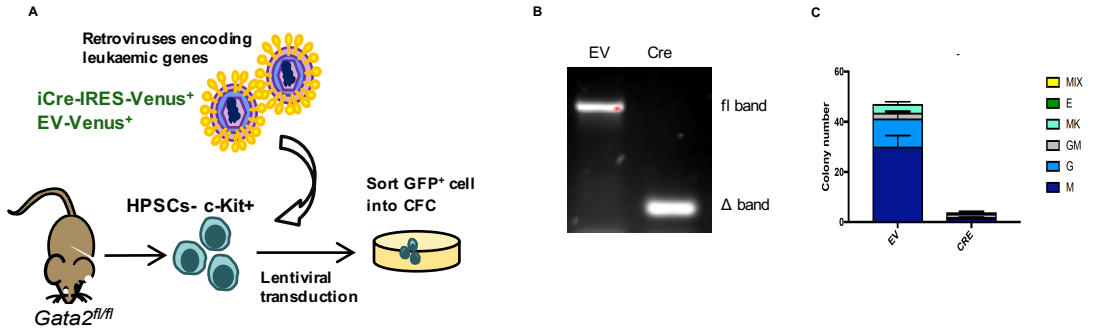


Figure 3.6. *Ex-vivo* deletion of *Gata2* in HSPCs using lentiviral Cre. (A) C-kit+ cells from *Gata2*$^{fl/fl}$ mice were transduced with iCre-IRES-Venus or control vector, and 72-hours later 2,000 Venus+ cells sorted into CFC. (B) Genomic PCR showing *Gata2* deletion (C) Number of CFU-C at day 14 (n = 2). One experiment was performed for all analysis. Data are mean ± SEM. Statistical analysis: Mann-Whitney test. All statistical analysis were non-significant (NS).

To gauge the contribution of any *Gata2*-dependent impact of the niche on HSC maintenance, *wild-type* CD45.1^{+} BM cells were transplanted into *Gata2*$^{fl/fl}$*;Mx1-Cre*$^{+}$ and control mice. After confirming stable reconstitution, we induced *Gata2* deletion in the BM microenvironment containing *wild-type* HSCs (**Figure 3.7A**). No engraftment or lineage distribution defects were observed in the PB of the *Gata2* deleted niche recipients at weeks 3, 6 and 9 after pIpC-mediated *Gata2*-niche deletion (**Figure 3.7B-F**). We found a slight increase in the chimerism of BM cells transplanted into *Gata2*-deficient niche mice, which can be explained by the depletion of residual haematopoietic *Gata2*$^{\Delta/\Delta}$ cells in *Gata2*-deficient niche mice after pIpC induction, while the remaining haematopoiesis from control-niche mice remained, at low levels, after pIpC deletion (**Figure 3.7D**). Thus, the *Gata2* deficient niche does not impact HSC engraftment ability and multi-lineage distribution. Despite that Mx1-Cre is known to delete niche in the BM microenvironment from previous publications (Kuhn *et al.*

1996), we did not formally test the protein levels of *Gata2* in niche cells in comparison to normal levels from WT BM cells in this transplantation setting.

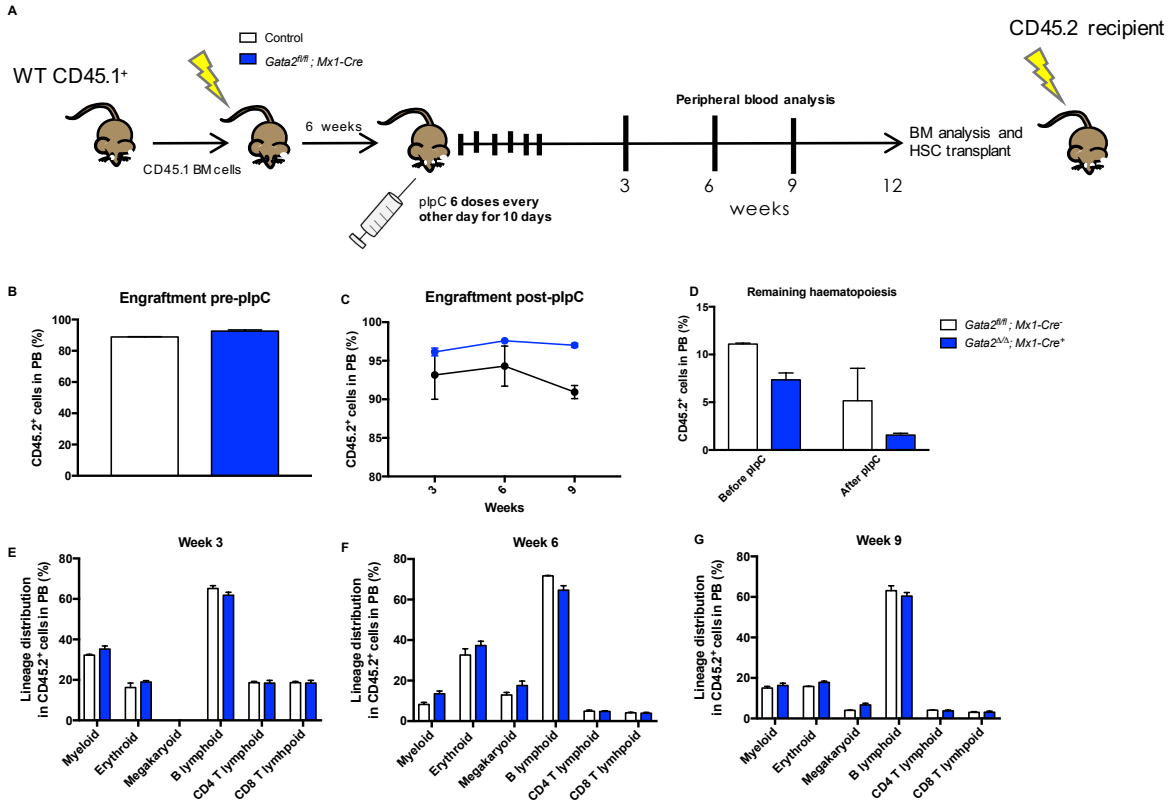


Figure 3.7. PB monitoring of *Gata2* mice transplanted with WT BM cells. (A) *wild-type* CD45.1+ BM cells were transplanted into lethally-irradiated *Gata2*$^{fl/fl}$*;Mx1-Cre*+ or control mice. Six weeks later *Gata2* niche deletion was induced with plpC as indicated in Figure 3.2A. Engraftment was measured every three weeks in the PB. At week 12 (ongoing experiment) CD45.1+ HSCs that have been surrounded by *Gata2* deficient or control BM microenvironment will be transplanted into lethally-irradiated CD45.2+ mice. (B) Engraftment at week 6 prior to plpC (n = 2-5). (C) Engraftment in the PB at the indicated time points after plpC induction (n = 2-5). (D) Remaining haematopoiesis before and after plpC induction (n = 2-5). (E-G) Engraftment in the PB of the indicated populations at (E) 3, (F) 6, and (G) 9 weeks after plpC induction (n = 2-5). One experiments was performed for all analysis. Data are mean ± SEM. Statistical analysis: Mann-Whitney test. All statistical analysis were non-significant (NS).

3.3.5. *Gata2* heterozygous mice display reduced HSCs in young and aged haematopoietic systems.

As we were unable to detect any HSCs after acute *Gata2* deletion, we sought to determine whether *Gata2* haploinsufficient mice would be a tractable model system to study the HSC survival mechanism seen in *Gata2*$^{\Delta/\Delta}$*;Mx1-Cre*$^{+}$ mice. To validate previous studies showing a reduced number of LSK cells in constitutive *Gata2* haploinsufficient (Gata2$^{+/-}$) mice (Rodrigues *et al.* 2005; Ling *et al.* 2004), we induced *Gata2* deletion in *Gata2*$^{fl/+}$*;Mx1-Cre*$^{+}$ or control mice and confirmed deletion of one *Gata2* allele in BM and LSK cells 24 days after the first dose of plpC (**Figure 3.8A** and

Figure 3.8B). We found a decrease in the frequency of LSK cells in *Gata2*$^{fl/\Delta}$*;Mx1-Cre*$^{+}$ (**Figure 3.8C** and **Figure 3.8D**). Therefore, *Gata2* haploinsufficient mice will allow further investigation of the molecular mechanisms downstream of *Gata2* in HSCs.

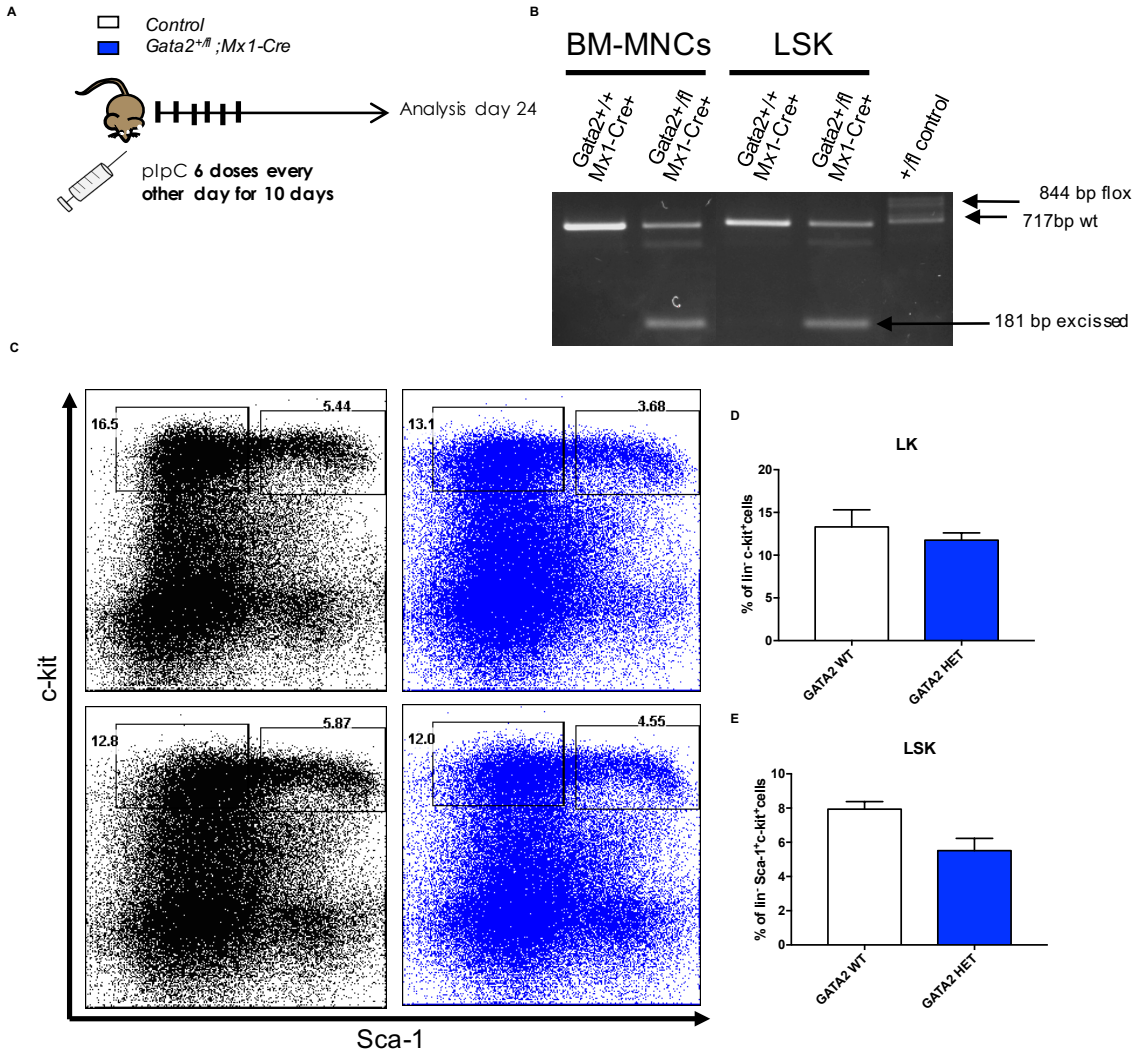


Figure 3.8. LSK reduction at 24 days after deletion in *Gata2* heterozygous mice. (A) *Gata2*$^{+/fl}$*;Mx1-Cre*$^{+}$ or control mice were administered pIpC as outlined in Figure 3.2A, and 24 days later the mice were analysed. (B) PCR gel showing complete deletion of *Gata2* in BM and LSK cells. (C) FACS plots showing LSK and LK compartment in *Gata2* HET (blue) and control (black) mice at day 24 after pIpC. (D and E) Frequency of (D) LK and (E) LSK populations (n = 2). One experiment was performed for all analysis. Data are mean ± SEM. Statistical analysis: Mann-Whitney test. All statistical analysis were non-significant (NS).

Next, we asked whether ageing *Gata2* haploinsufficient mice would serve to model *GATA2* hereditary mutations found in humans that develop immunodeficiency, MonoMAC or Emberger syndrome and which progresses to MDS and AML (Hyde and Liu 2011). We induced *Gata2*$^{fl/+}$*;Mx1-Cre*$^{+}$ or control mice and monitored them for 6 months (**Figure 3.9A**). Efficient deletion of one *Gata2* allele in the BM of *Gata2*$^{fl/\Delta}$*;Mx1-*

Cre^{+} mice was confirmed (**Figure 3.9B**). A non-significant decrease was observed in BM, but not SP, cellularity from *Gata2*$^{fl/\Delta}$*;Mx1-Cre*$^{+}$ mice (**Figure 3.9C-E**). We detected a decrease in the numbers of myeloid cells in BM and erythroid cells in the SP of *Gata2*$^{fl/\Delta}$*;Mx1-Cre*$^{+}$ mice **(Figure 3.9F-I**). In addition, we found a non-significant decrease in the abundance of MPP and HSCs from the BM *Gata2*$^{fl/\Delta}$*;Mx1-Cre*$^{+}$ mice **(Figure 3.9J**). Interestingly, we detected an enhancement in the frequency and numbers of HPC-2 cells in the BM of *Gata2*$^{fl/\Delta}$*;Mx1-Cre*$^{+}$ mice **(Figure 3.9J**). Finally, we plated BM cells into a CFC assay and unexpectedly found that *Gata2*$^{fl/\Delta}$ BM cells gave rise to more colonies than control BM cells **(Figure 3.9K** and **Figure 3.9L**). Collectively, these data indicate that aged *Gata2* haploinsufficient mice have perturbed haematopoiesis. Further investigation will be required to determine if these mice develop MDS/AML akin to clinical *GATA2* haploinsufficiency syndromes.

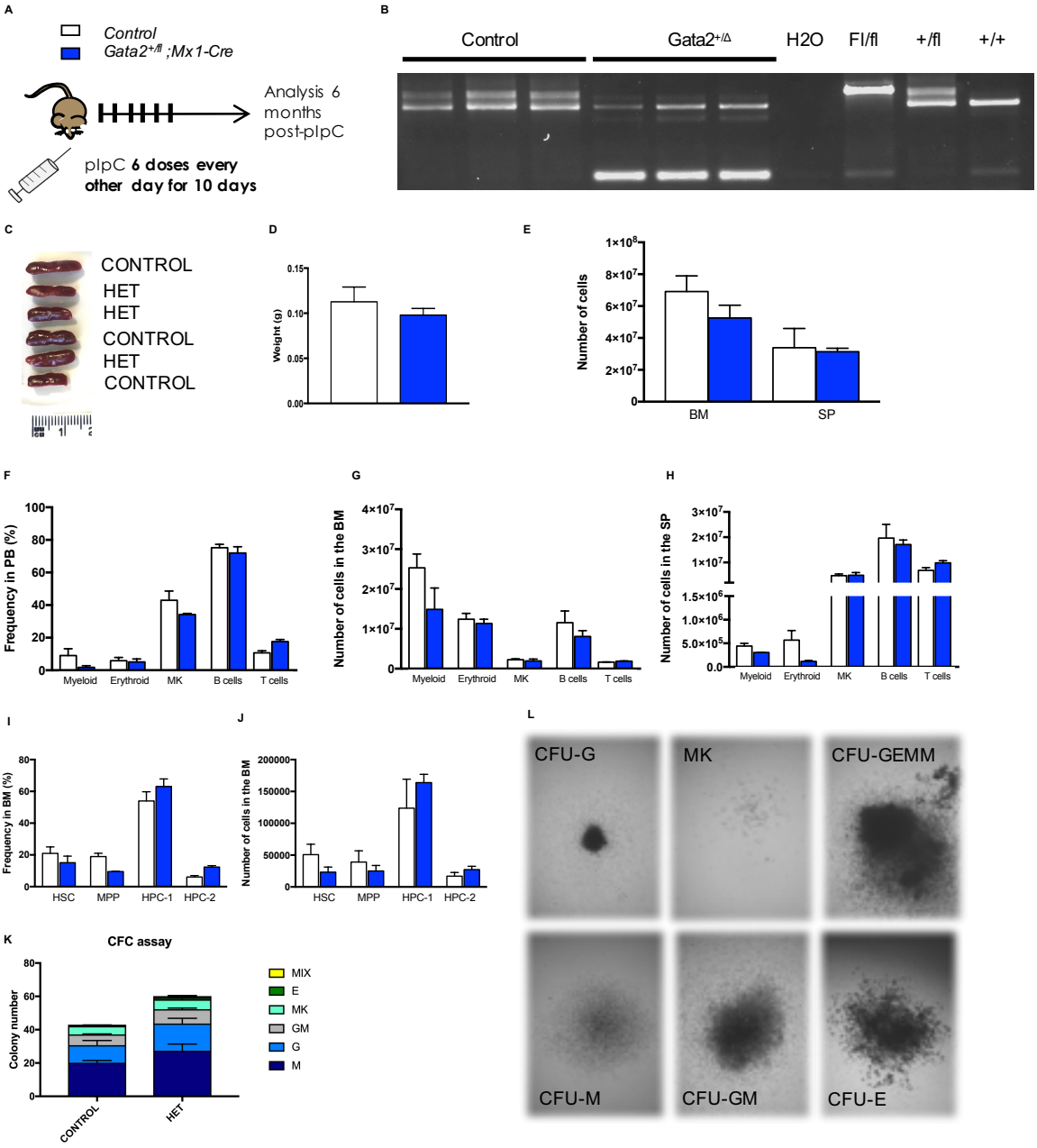


Figure 3.9. Long term analysis of *Gata2* heterozygous mice. (A) Following the same approach is in Figure 3.8A, we monitor the mice for long term (6 months) prior to analysis. (B) PCR gel showing complete deletion of *Gata2* (C) Spleen pictures. (D) BM cellularity (n = 3). (E) SP cellularity (n = 3). (F) Frequency of each population in the PB (n = 3). (G-H) Total number of each the indicated populations in the (G) BM and (H) SP (n = 3). (I) Frequency and (J) total numbers of the indicated populations within the LSK compartment (n = 3). (K) Number of CFU-C at day 14 (n = 3). (L) Representative CFU-C pictures at day 14. One experiment was performed for all analysis. Data are mean ± SEM. Statistical analysis: Mann-Whitney test. All statistical analysis were non-significant (NS).

3.4. Discussion

In this study, we explored the function of *Gata2* in the maintenance of adult HSCs and haematopoiesis. In addition, we have investigated the long-term effects of *Gata2* loss of function of one allele in relation to the development of previously reported *GATA2*-haploinsufficiency related syndromes.

Our data identifies the crucial role of *Gata2* in HSCs. *Gata2* was one of the first TFs to be studied in the haematopoietic system. In addition to the anaemic phenotype observed in *Gata2*$^{-/-}$ mice, they also displayed a lack of multipotential reconstitution in chimera experiments, suggesting a HSPC defect (Tsai *et al.* 1994). By means of the conditional Cre-lox system it was later found that *Gata2* KO mice were unable to generate or maintain HSCs in the embryo (de Pater *et al.* 2013). Indeed, this rapid loss of *Gata2* deficient HSCs in this setting was due to the induction of apoptosis (de Pater *et al.* 2013). Adult *Gata2 (Gata2*$^{+/-}$*)* haploinsufficient mice have reduced HSCs coupled with a reduction of the anti-apoptotic protein *Bcl-xL*, highlighting *Gata2* as major regulator of HSC survival (Rodrigues *et al.* 2005).

In another study, loss of c-kit in haematopoietic progenitors and BM failure was observed upon conditional deletion of the *Gata2* C-terminal zinc finger domain in adult mice, using a tamoxifen-inducible Cre system to delete the *Gata2* C-terminal zinc finger domain in all tissue-types (H. S. Li *et al.* 2016). To circumvent the nonspecific tissue effects of the tamoxifen-Cre system, the authors performed a cell-autonomous experiment to delete *Gata2* in haematopoietic cells after tamoxifen induction and detected a reduction of c-kit expression in the BM of recipients with *Gata2* KO cells (H. S. Li *et al.* 2016). These findings are consistent with our results using the Mx1-Cre system, although some differences can be seen between these two Cre-lox models. The authors described a multi-lineage defect at 10-days after *Gata2* deletion in both non-cell autonomous and cell-autonomous experiments, including B cell and T cell lineages, while deletion in the *Mx1-Cre* system used here showed myeloid and erythroid abolition but not lymphoid 24 days after deletion. Long-term cell-autonomous follow-up of *Gata2* KO cells will be used to further assess multi-lineage failure (which is an ongoing experiment). However, lethality of *Gata2* KO mice treated with tamoxifen can also be explained by non-haematopoietic *Gata2* effects, since *Gata2* has been

reported to regulate prostate, lung, neurons, thyroid gland, and pituitary gland (He *et al.* 2014; Kumar *et al.* 2012; El Wakil *et al.* 2006; Charles *et al.* 2006).
The differences observed in the frequency of mature haematopoietic lineages upon *Gata2* deletion with the *Mx1-Cre* promoter can also be explained by the survival kinetics of each haematopoietic lineage, rather than a specific dependency of *Gata2* for myeloid and erythroid lineage maturation. In support of this, specific *Gata2* deletion using *LysM-Cre* in myeloid cells did not impact haematopoiesis (H. S. Li *et al.* 2016). Also, lymphoid cells are long-lived while myeloid and erythroid are short-lived (Ho *et al.* 2015), potentially explaining the existence of residual lymphoid cells in the BM of *Gata2* KO mice. Importantly, we found an increase in the frequency and number of B cells in the BM of Mx1-Cre *Gata2* KO mice at day 24, and this increase was even stronger at day 15 after deletion. Since this increase was not seen in the cell-autonomous setting, it is postulated that *Gata2* deletion mediates niche-dependent expansion of B cells. For instance, reduction of *Gata3* levels upon *Gata2* acute deletion could induce a B cell response, since *Gata3* is a known negative regulator of B cells (Rothenberg 2013). In support of this, *Gata3* is able to regulate haematopoiesis through sympathetic innervation present in the niche (Fitch *et al.* 2012), and at the same time *Gata2* has been shown to be responsible to *Gata3* activation in the nervous system (Tsarovina 2004).

Gata2 joins a select list of TFs that are required for HSC maintenance. Conditional acute deletion of *Cited2* and *Tel/Etv6* using the Mx1-Cre system led to HSC loss and multilineage failure, although they were indispensable for mature haematopoietic cells (Kranc *et al.* 2009; Hock *et al.* 2004). In contrast, Mcl-1 affects lymphoid lineages in addition to its role in HSC survival (Opferman *et al.* 2003). Further experiments with lineage-specific Cre should determine whether *Gata2* is required for erythroid and lymphoid lineages since it has been already reported that is dispensable for myeloid mature cells (H. S. Li *et al.* 2016). Exploring the *Gata2*-mediated HSC survival molecular mechanisms and genetic interactions will be of interest to understand the pathways governing adult HSCs, which will provide a better insight of the deregulation of HSCs and malignant transformation into pre-LSC/LSCs.

Finally, we attempted to model *GATA2*-related syndromes by studying the long-term effects of *Gata2* heterozygous deletion using the Mx1-Cre system. Our preliminary

data indicates a decrease in the total number of myeloid and B cells, characteristics found in *GATA2*-related syndromes (Collin *et al.* 2015). However, we did not analyse the levels of DCs or NK cells, which are other key immune cell types deficient in *GATA2*-deficient syndromes (Collin *et al.* 2015). Surprisingly, *Gata2* heterozygous mice had increased CFC capacity *in vitro*. In addition, we found a decrease in the number of HSCs and MPPs together with an increase in HPC-2, and possibly HPC-1 cells, perhaps presaging the development of MPN/MDS; this result mirrors what was observed in *Atg7* KO mouse that develop myeloproliferative neoplasms (MPN)/MDS (Mortensen *et al.* 2011). Taken together, these findings indicate a potential for *Gata2* haploinsufficient mice to model *GATA2*-deficiency clinical syndromes. Longer monitoring and detailed immunophenotypic and histological examination will be required to confirm these results. Since *GATA2* mutations in these hereditary syndromes occur at an early developmental stage, use of alternative mouse models to induce the deletion during HSC development would perhaps provide a more faithful model to investigate *GATA2*-related syndromes and provide a drug platform that would help to treat diagnosed *GATA2*-deficient family members at early stages. Moreover, since *GATA2* deregulation has been reported in non-hereditary MDS/AML (Vicente *et al.* 2012; Maaike Luesink *et al.* 2012) cases, and in other cancer types (Kumar *et al.* 2012; Rodriguez-Bravo *et al.* 2016; Wang *et al.* 2015; Xu *et al.* 2016), elucidating the role of *Gata2* in the context of *Gata2*-heterozygous mice will shed light in the molecular mechanisms involved in malignant transformation.

Chapter 4

Exploring the function of *Gata2* in murine models of *Meis1a/Hoxa9* and *Mll-af9* driven AML

4.1. Introduction

AML is driven by a subset of leukaemia cells termed leukaemia stem cells (LSCs) (Bonnet and Dick 1997). It is generally acknowledged that some subsets of LSCs are resistant to eradication, they ultimately cause relapse and, importantly, need to be therapeutically targeted to effect cure (Dick 2005; Tabe and Konopleva 2015; Jung *et al.* 2015). Inducing cell death and/or differentiation of LSCs are attractive targets for therapy, as they would eliminate the source of leukaemia cell growth/accumulation.

Gata2 has been established as a critical, dose dependent regulator of haematopoiesis (see **chapter 3)** (Rodrigues *et al.* 2005; Ling *et al.* 2004), yet its role in the initiation, maintenance and progression of acute myeloid leukaemia (AML) remains highly controversial. Both down-regulation and overexpression of *Gata2* are considered to be involved in AML pathogenesis. For instance, sporadic and hereditary *GATA2* mutations give rise to myelodysplastic syndrome (MDS) and AML (Hyde and Liu 2011). Conversely, low-level overexpression of *Gata2* is able to immortalise murine BM cells *in vitro,* with myeloid skewing without progressing to full-blown leukaemia (Nandakumar *et al.* 2015). In addition, overexpression of *GATA2* has been linked with poor prognosis in AML patients (Vicente *et al.* 2012; Luesink *et al.* 2012). Recently, *GATA2* has also been linked with poor prognosis and aggressiveness in other cancer types, including prostate, lung and breast cancer (Kumar *et al.* 2012; Rodriguez-Bravo *et al.* 2016; Wang *et al.* 2012). Though several studies have shown that overexpression of *Gata2* reduces the clonogenic potential of *Mll-af9* pre-LSCs *in vitro* (Danis *et al.* 2015; Bonadies *et al.* 2011), how such deregulation of *GATA2* expression impacts the functionality of LSCs is not understood.

Evi1 is an essential TF for HSCs and has been shown to bind and activate *Gata2* directly in HSCs and leukaemia cells (Yuasa *et al.* 2005; Goyama *et al.* 2008; Sato *et al.* 2008). *Evi1* overexpression in mice generates a MDS/AML phenotype (Buonamici *et al.* 2004), and its overexpression is associated with poor outcome in both diseases (Barjesteh van Waalwijk van Doorn-Khosrovani *et al.* 2003; Lugthart *et al.* 2008). In agreement with this, *Evi1* is a transcriptional target of mixed lineage leukaemia (*Mll*) translocations (Arai *et al.* 2010). *Mll* oncoproteins comprises a subset of leukaemia (10% overall leukaemia-subtypes (Krivtsov and Armstrong 2007)) with aggressive

clinical features. The *Mll* gene encodes a methyltransferase that when aberrantly fused to another protein triggers epigenetic reprogramming through activation of *Meis1a/Hoxa9* and *Evi1* in HSC or progenitor cells giving rise to LSCs, which generate lymphoid, myeloid, or mixed leukaemia (Krivtsov and Armstrong 2007; Bindels *et al.* 2012; Faber *et al.* 2009). Pre-LSCs and LSCs derived from *Mll* oncogene expression have been well characterised in retroviral and transgenic mouse models (Krivtsov *et al.* 2006; Somervaille and Cleary 2006). Therefore, these models provide an amenable system to study the dysregulated expression of *Gata2* in Pre-LSC and LSC biology. This knowledge can then be translated in order to appropriately target pre-LSCs and LSCs in MDS and AML, and perhaps cancer stem cells (CSCs) in other cancer types where *Gata2* function is also affected.

4.2. Aims and Objectives

The specific aims and objectives for this chapter are:

1. To assess the impact of genetic deletion of *Gata2* in LSC development and maintenance in the *Mll-af9* and *Meis1a/Hoxa9* retroviral mouse models *in vivo*.

2. To assess the *in vitro* efficacy of a small molecule inhibitor of *Gata2*, K-7174, to target pre-LSCs and LSCs from *Mll-af9* and *Meis1a/Hoxa9* retroviral mouse models.

3. To investigate the co-operation between K-7174 and standard chemotherapeutic drugs in targeting *Mll-af9* and *Meis1a/Hoxa9* derived pre-LSCs and LSCs in vitro.

4.3. Results

4.3.1. *Gata2* is downregulated in pre-LSCs and LSCs from *Meis1a/Hoxa9* and *Mll-af9* retroviral AML mouse models.

Preliminary data obtained in collaboration with investigators at Edinburgh University showed that *Gata2* was increased in pre-leukaemia LSK cells from a 10-week-old transgenic *Mll-af9* knock-in (*Mll-af9*^{KI}) mouse (**Figure 4.1A-C**). In this model, the *Mll-af9* translocation is under the control of the endogenous *Mll* promoter thus generating a translational model to study the pathogenesis of *Mll-af9* AML (W. Chen *et al.* 2008; Vukovic *et al.* 2015). This data demonstrates overexpression of Gata2 in pre-LSCs and supports the idea of a role for *Gata2* in leukaemia initiation in *Mll-af9*-mediated AML.

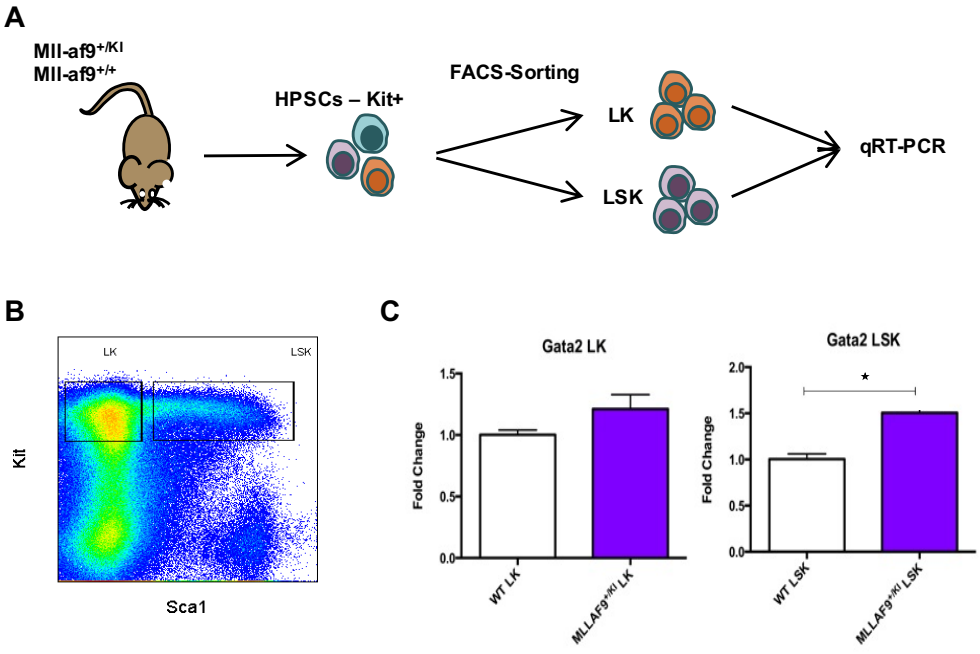


Figure 4.1 *Gata2* is overexpressed in pre-leukaemic cells from a *Mll-af9*$^{+/KI}$ transgenic mouse. (A) LK and LSK cells from 10 week-old Mll-af9$^{+/KI}$ or littermate control (*Mll-af9*$^{+/+}$) mice were sorted and *Gata2* levels measured by qRT-PCR. (B) Representative FACS plot showing LK and LSK populations. (C) *Gata2* gene expression in LK and LSK cells. Data are mean ± SEM (n = 4-5). Statistical analysis: Mann-Whitney test. **Experiment performed by Dr. Milica Vukovic (SCRM, Edinburgh).**

Next, we sought to establish well-characterised retroviral mouse models of *Mll-af9* driven AML (Krivtsov *et al.* 2006; Somervaille and Cleary 2006). Here, HSPCs (c-kit$^+$ cells) were transduced with retroviruses encoding the *Mll-af9* translocation or co-transduced with its direct downstream targets *Meis1a9* and *Hoxa9*, that are overexpressed in 40% of AML patients (Lawrence *et al.* 1993). Next, transduced cells were serially-replated in methylcellulose CFC assays, and pre-LSCs obtained after three rounds of CFC were injected into lethally-irradiated primary recipients. Within the BM environment pre-LSCs acquire secondary mutations and become LSCs that initiate the onset of AML in mice. LSCs from moribund mice with AML were then able to propagate the disease upon transplantation into lethally-irradiated secondary recipients (**Figure 4.2**).

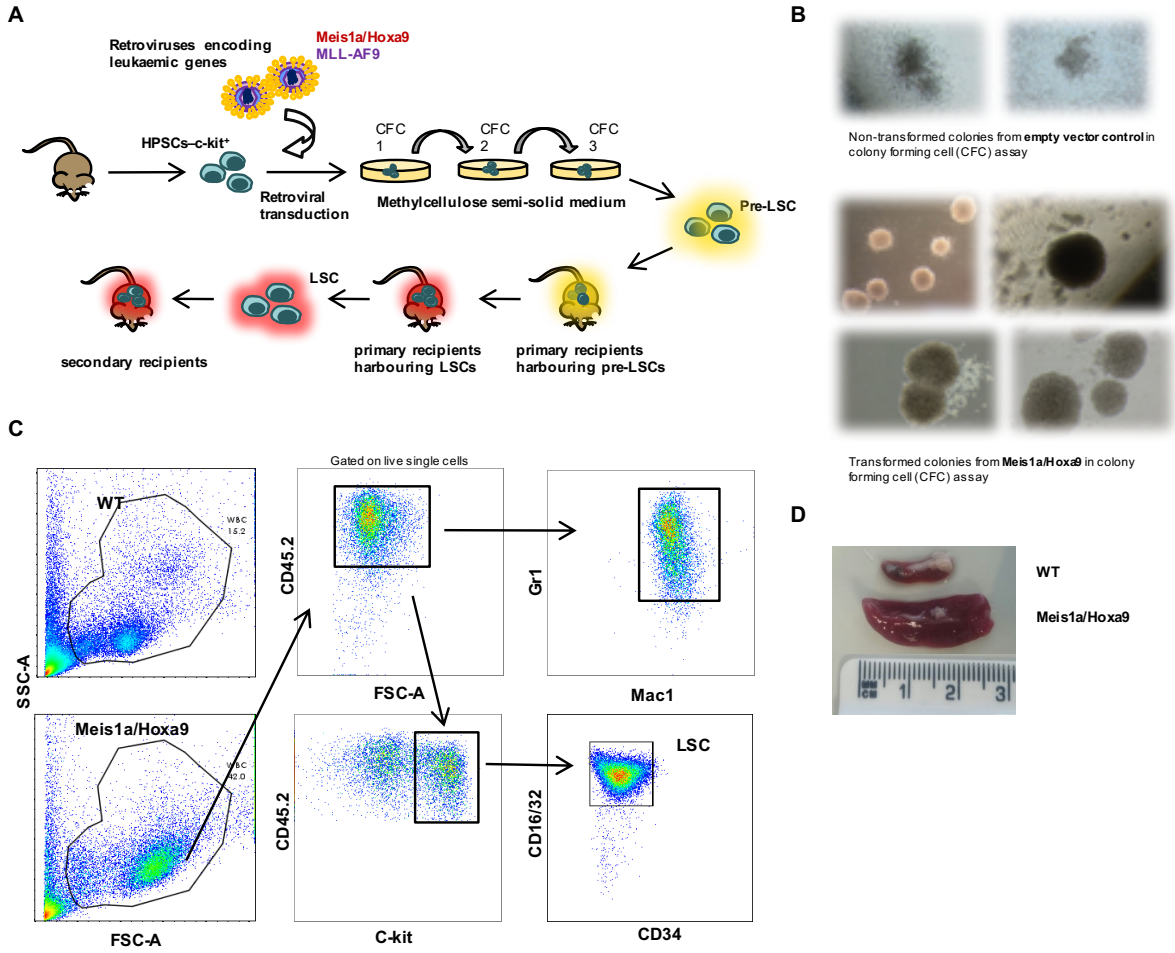


Figure 4.2. Establishment of the *Meis1a/Hoxa9 and Mll-af9* AML mouse models. (A) C-kit$^+$ cells were retrovirally transduced with *Mll-af9* or *Meis1a/Hoxa9* oncogenes and plated into methylcellulose under colony forming cell (CFC) assay. After CFC3, pre-LSCs (c-kit$^+$) were transplanted into lethally-irradiated recipients. LSCs from primary recipients were transplanted into secondary recipients. (B) Colonies at CFC1 from non-transformed empty vector (top) and transformed with Meis1a/Hoxa9 oncogenes (bottom).(C) FACS plots showing immunophenotypic leukaemic cells (CD45.2$^+$ (GFP$^+$) Gr1^{low} Mac1$^+$) and LSCs (CD45.2$^+$ (GFP$^+$), c-kit$^+$, CD16/32$^+$, CD34^{low}). (D) Spleenomegaly observed in leukaemic mice compared to WT control mice.

We analysed the expression of *Gata2* in *Mll-af9* or *Meis1a/Hoxa9* pre-LSCs (c-kit$^+$) compared to empty vector transduced c-kit$^+$ cells at CFC3 and to freshly-isolated c-kit$^+$ cells. Surprisingly, we found that *Gata2* expression from pre-LSCs was downregulated compared to the empty vector control (**Figure 4.3A** and **4.3C**). When compared to freshly-isolated c-kit$^+$ cells, *Mll-af9* pre-LSCs maintained similar *Gata2* levels (**Figure 4.3C**) while *Gata2* levels were greatly reduced in *Meis1a/Hoxa9* pre-LSCs (**Figure 4.3A**). Thus, downregulation of *Gata2* is an early event for *Meis1a/Hoxa9* mediated leukaemia transformation, and possibly in the *Mll-af9* model. To investigate whether downregulation of *Gata2* was also seen after LSC development and maintenance, we analysed freshly-isolated LSC from moribund mice with AML according to **Figure 4.2C**. In agreement with the results found in pre-LSCs, we observed a profound decrease in *Gata2* expression in *Mll-af9* and *Meis1a/*Hoxa9 LSCs compared to freshly isolated c-kit$^+$ cells (**Figure 4.3B** and **4.3D**). Therefore, downregulation of *Gata2* is maintained during the *in vivo* development of LSCs.

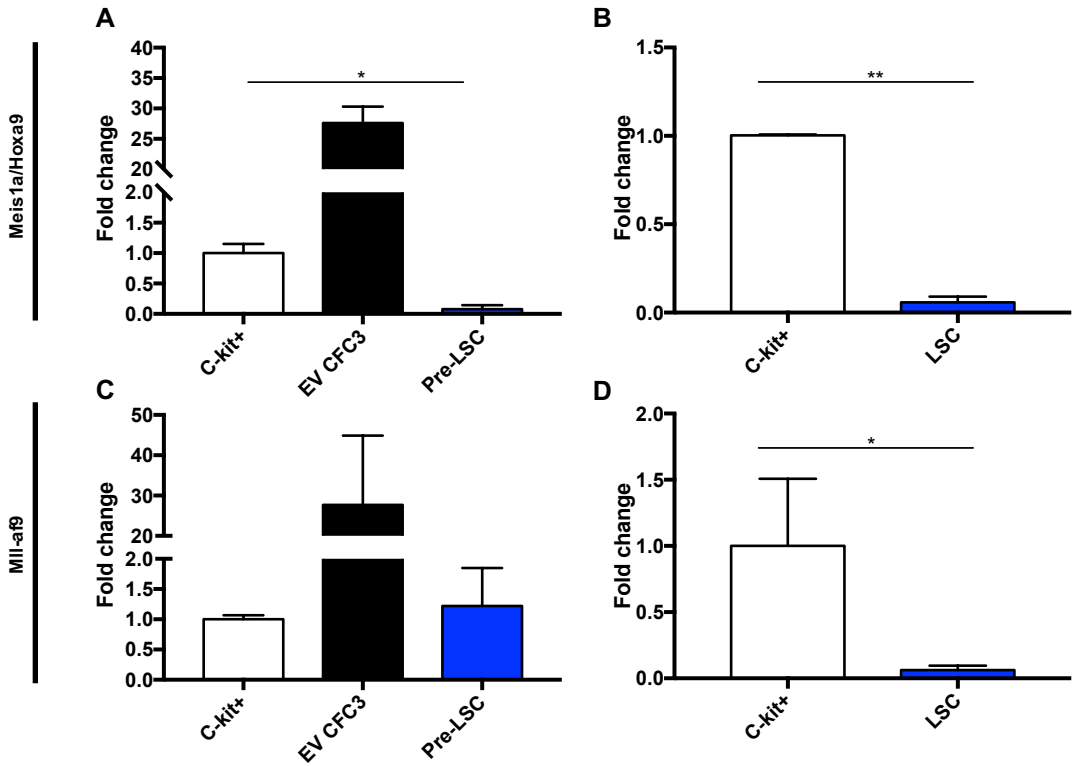
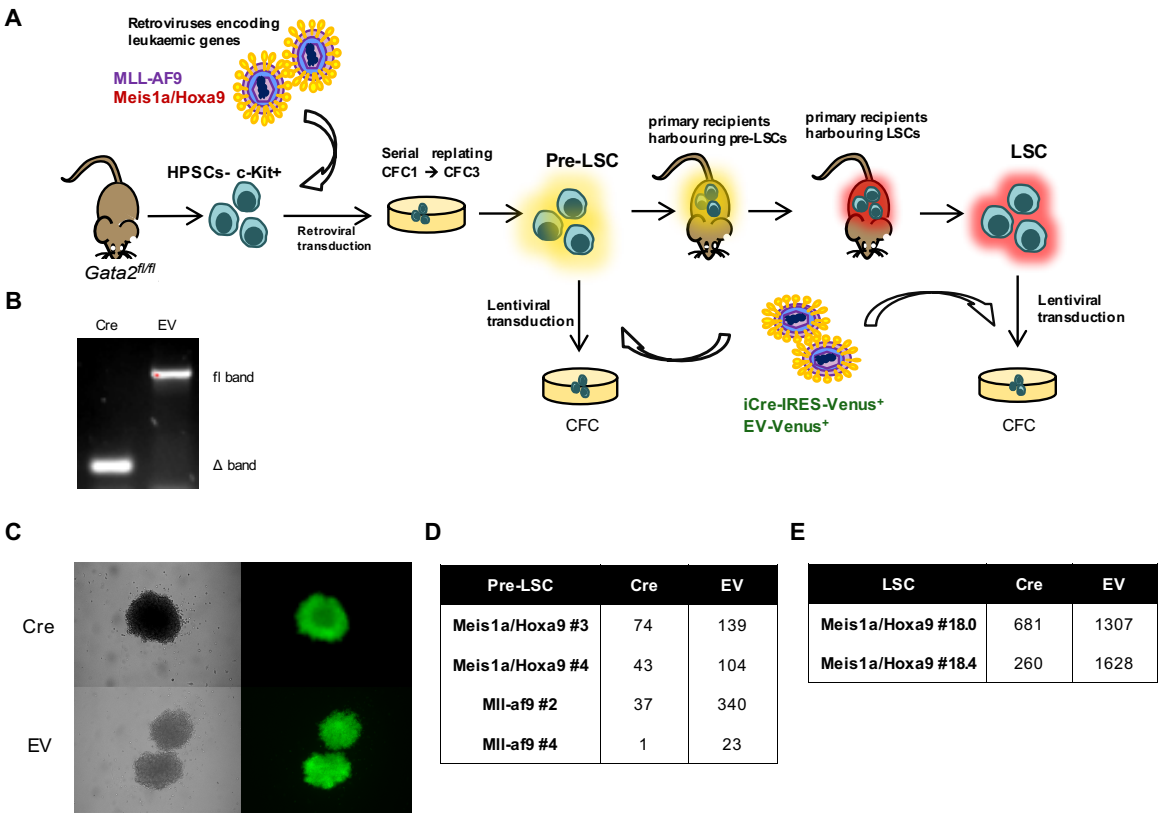


Figure 4.3. *Gata2* is downregulated in pre-LSCs and LSCs from *Meis1a/Hoxa9* and *Mll-af9* retroviral AML mouse models. RNA was extracted and Gata2 expression assessed by qRT-PCR at the Pre-LSC and LSC stage of the leukaemogenic model. (A) Meis1a/Hoxa9 Pre-LSCs (n = 3-5). (B) Meis1a/Hoxa9 LSCs (n = 3 for control and n = 11 for LSCs). (C) Mll-af9 Pre-LSCs (n = 4-5). (D) Mll-af9 LSCs (n=3-5). Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

4.3.2. *In vitro* deletion of *Gata2* reduces the clonogenic capacity of Pre-LSCs in the *Meis1a/Hoxa9* and *Mll-af9* models.

While observing a downregulation of *Gata2* expression in pre-LSC and LSC from *Mll-af9* and *Meis1a/Hoxa9* models (**Figure 4.2A and 4.2C**), previous research has shown that despite a low threshold of *Gata2* expression (see **section 2.3.1**) and activity in GMP (which is 10-fold reduced compared to HSCs), *Gata2* is still essential for GMP function when expression if reduced by half (Rodrigues *et al.* 2008). Thus, we elected to test the requirement for *Gata2* in the clonogenic growth of pre-LSCs *in vitro*. We transduced *Gata2*$^{fl/fl}$ HPSCs with *Meis1a/Hoxa9* or *Mll-af9* retroviruses, serially replated for three rounds of CFC, and transduced pre-LSC with lentiviruses expressing Cre (**Figure 4.4A**). Full deletion of the *Gata2* allele was confirmed (**Figure 4.4B**) and *Meis1a/Hoxa9* pre-LSCs expressing Cre formed less colonies (2-to-3 fold) compared to empty vector transduced *Meis1a/Hoxa9* pre-LSCs (**Figure 4.4D**). In addition, it was found that *Mll-af9* pre-LSCs expressing Cre formed less colonies (**Figure 4.4D**). Therefore, *Gata2* appears to be required for the clonogenic capacity of pre-LSCs in both AML models, however we would need to validate this result by using *Gata2*$^{+/+}$ *Meis1a/Hoxa9* transformed cells infected with Cre compared to empty vector control to gauge the potential Cre toxicity in colony formation of pre-LSCs.



Pre-LSC	Cre	EV
Meis1a/Hoxa9 #3	74	139
Meis1a/Hoxa9 #4	43	104
Mll-af9 #2	37	340
Mll-af9 #4	1	23

LSC	Cre	EV
Meis1a/Hoxa9 #18.0	681	1307
Meis1a/Hoxa9 #18.4	260	1628

Figure 4.4. In vitro deletion of *Gata2* reduce the clonogenic capacity of Pre-LSCs and LSCs in the *Meis1a/Hoxa9* and *Mll-af9* models. (A) C-kit+ cells from *Gata2*$^{fl/fl}$ mice were retrovirally transduced with *Mll-af9* or *Meis1a/Hoxa9* oncogenes and plated into methylcellulose under colony forming cell (CFC) assay. After CFC3, pre-LSCs (c-kit+) were infected with iCre-IRES-Venus and 10,000 Venus+ cells sorted into CFC. *Meis1a/Hoxa9* pre-LSCs were transplanted into lethally-irradiated recipients. LSCs from primary recipients were infected with iCre-IRES-Venus and 10,000 Venus+ cells sorted into CFC. (B) Genomic PCR showing *Gata2* deletion (C) Representative colonies from Cre (top) and EV (bottom) with (right) or without (left) GFP. (D) Table showing number of colonies of Pre-LSC after 6 days in CFC (n=2). (E) Table showing number of colonies of LSC after 6 days in CFC (n=2).

4.3.3. Acute deletion of *Gata2 in vivo* does not impact LSC development and maintenance in the *Mll-af9* retroviral AML mouse model.

Given the requirement for *Gata2* in pre-LSC growth *in vitro*, we sought to assess the requirement for *Gata2* in LSC development and maintenance in *Mll-af9* driven AML *in vivo*. We demonstrated that deletion of *Gata2* using the *Mx1-Cre* promoter leads to the rapid loss of HSPCs (see **Chapter 3**); this therefore precludes use of the system to study the role of *Gata2* in leukaemia initiation and establishment of pre-LSCs. As a method to circumvent this issue, we transduced HSPCs from *Gata2*$^{fl/fl}$;*Mx1-Cre* or control mice with *Mll-af9* retroviruses. After three rounds of CFC we harvested *Mll-af9* pre-LSC and transplanted them into primary recipients (**Figure 4.5A**). We monitored leukaemia development by periodical bleedings and induced *Gata2* deletion with pIpC before the percentage of leukaemic cells in the PB reached 20-25%, a threshold for the diagnosis of AML (**Figure 4.5B**). After pIpC induction we found that the levels of

leukaemic cells in the PB remained similar between *Gata2* excised (*Gata2*$^{\Delta/\Delta}$) or control pre-LSC transplanted mice (**Figure 4.5C**). In agreement with this, recipients of *Gata2*$^{\Delta/\Delta}$ pre-LSC succumbed to AML with similar latency to transplant recipients receiving control pre-LSCs (**Figure 4.5F**). Next, to study the requirement of *Gata2* in LSC propagation in the *Mll-af9* model, we isolated LSCs from *Gata2*$^{\Delta/\Delta}$ or control mice and transplanted them into secondary recipients (**Figure 4.5A**). We observed no differences in the survival of recipients transplanted with *Gata2*$^{\Delta/\Delta}$ LSC compared to recipients transplanted with control LSC (**Figure 4.5G**). Analysis of BM from moribund mice with AML revealed that not all recipients transplanted with *Gata2*$^{fl/fl}$;*Mx1-Cre* pre-LSC/LSCs and induced with pIpC achieved full deletion of the *Gata2* allele (40% for primary transplant, and 33% for secondary transplant) (**Figure 4.5D** and **4.5E**). Despite the caveats arising from incomplete deletion of the *Gata2* allele, it appears that *Gata2* is dispensable for LSC generation and maintenance in the *Mll-af9*-driven AML mouse model.

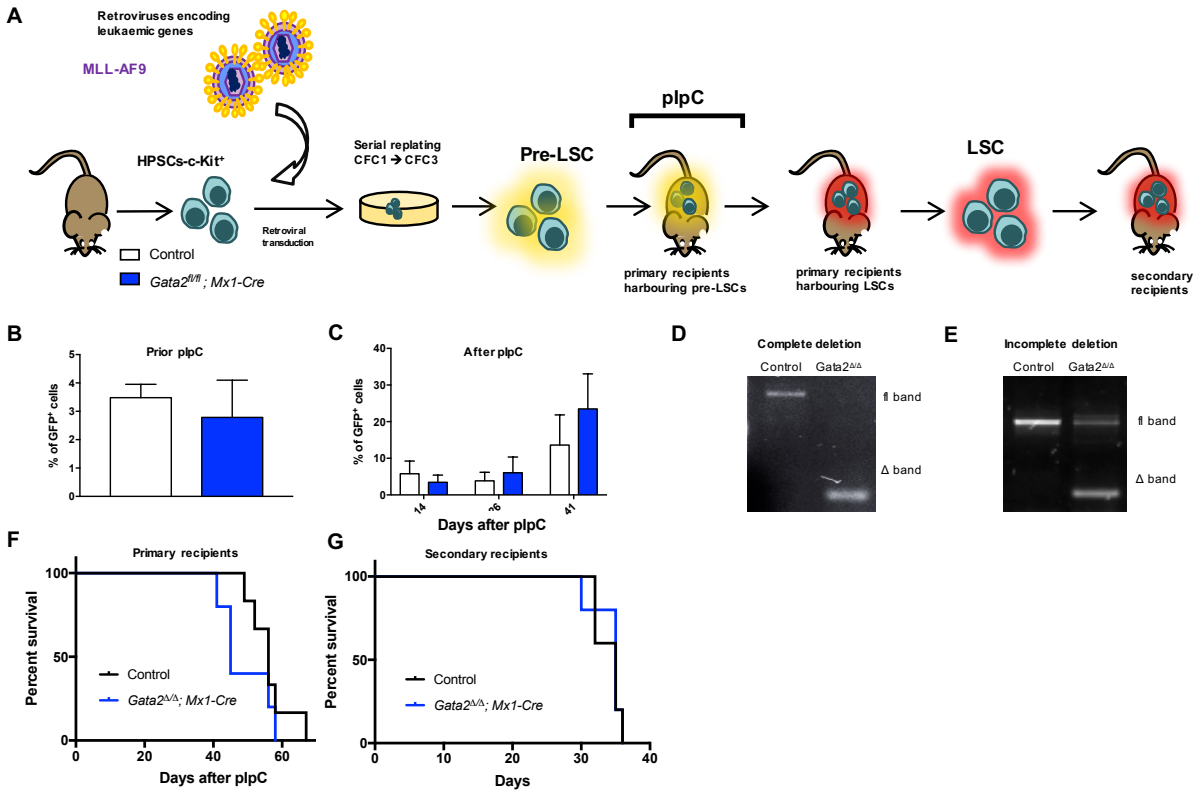


Figure 4.5. Acute deletion of *Gata2* does not impact LSC development and maintenance in the *Mll-af9* retroviral AML mouse model. (A) C-kit+ cells from *Gata2*$^{fl/fl}$*;Mx1-Cre* or control mice were retrovirally transduced with *Mll-af9* oncogenes and plated into methylcellulose under CFC assays. After CFC3, pre-LSCs (GFP+ c-kit+) were transplanted into lethally-irradiated recipients. At day 20 after transplant, mice were administered 6 doses of pIpC to induce gene deletion. LSCs from primary recipients were transplanted into secondary recipients. (B) % of leukaemic cells (GFP+) in the PB at day 20 after transplantation, prior to pIpC (n = 6 recipients per genotype from n = 4 biological replicates). C) % of leukaemic cells (GFP+) in the PB after pIpC (n = 6 recipients per genotype from n = 4 biological replicates). (D) and (E) Genomic PCR showing deletion of *Gata2*. (F) Kaplan-Meier survival curve of primary recipients transplanted with pre-LSCs (n = 6 recipients per genotype from n = 4 biological replicates). (G) Kaplan-Meier survival curve of secondary recipients transplanted with LSCs (n = 6 recipients per genotype from n = 2 biological replicates). Analysis performed from one independent experiment. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

4.3.4. Acute deletion of *Gata2* delays LSC development and maintenance in the *Meis1a/Hoxa9* retroviral AML mouse model.

Next, we sought to investigate the *in vivo* role of *Gata2* in a retroviral AML model driven by co-expression of *Meis1a* and *Hoxa9* (Kroon *et al.* 1998). Following the same methodology as the *Mll-af9* model (**Figure 4.5A**) we initiated and transplanted into primary recipients pre-LSCs from *Gata2*$^{fl/fl}$;*Mx1-Cre* or control mice (**Figure 4.6A**). Surprisingly, we found that upon *Gata2* deletion leukaemia cells in the PB of recipients of *Gata2*$^{\Delta/\Delta}$ pre-LSCs were dramatically reduced at week 4 and 6 after pIpC treatment (**Figure 4.6C** and **Figure 4.7**). Consistent with this, recipients of *Gata2*$^{\Delta/\Delta}$ pre-LSCs succumbed to AML with a significant delay (**Figure 4.6F**). Immunophenotypic analysis of primary recipients transplanted with *Gata2*$^{\Delta/\Delta}$ pre-LSCs that succumbed to AML showed no differences in the frequency of bulk leukaemia cells or the frequency of LSCs in the BM when compared to primary recipients transplanted with control pre-LSC (**Figure 4.6G** and **Figure 4.6H**).

To test whether *Gata2* deletion compromises LSC propagation in the *Meis1a/Hoxa9* model, we transplanted LSCs from *Gata2*$^{\Delta/\Delta}$ or control mice and transplanted them into secondary recipients (**Figure 4.6A**). Interestingly, recipients of *Gata2*$^{\Delta/\Delta}$ LSCs developed AML slower than recipients of control LSCs (**Figure 4.6I**). Immunophenotypic analysis of secondary recipients with AML revealed no differences in either the frequency of the bulk of leukaemia cells or LSCs (**Figure 4.6J** and **Figure 4.6K**). As observed in the *Mll-af9* model, full *Gata2* deletion was not achieved in all primary and secondary recipients transplanted with *Gata2*$^{fl/fl}$;*Mx1-Cre* pre-LSC (22% primary recipients; 40% secondary recipients) (**Figure 4.6D** and **Figure 4.6E**). To corroborate the requirement for *Gata2* in *Meis1a/Hoxa9* LSCs by an independent method, we transplanted *Gata2*$^{fl/fl}$ *Meis1a/Hoxa9* pre-LSCs into primary recipients. *Gata2*$^{fl/fl}$ LSCs from primary recipients were transduced with lentiviruses expressing Cre and plated into CFC (**Figure 4.4A**). Full deletion of the *Gata2* allele was confirmed (**Figure 4.4B**) and a reduction in the colony forming capacity of *Meis1a/Hoxa9* LSCs expressing Cre was observed (**Figure 4.4E**). Collectively, these data demonstrate the requirement for *Gata2* in LSC development and maintenance in the *Meis1a/Hoxa9*-driven AML mouse model.

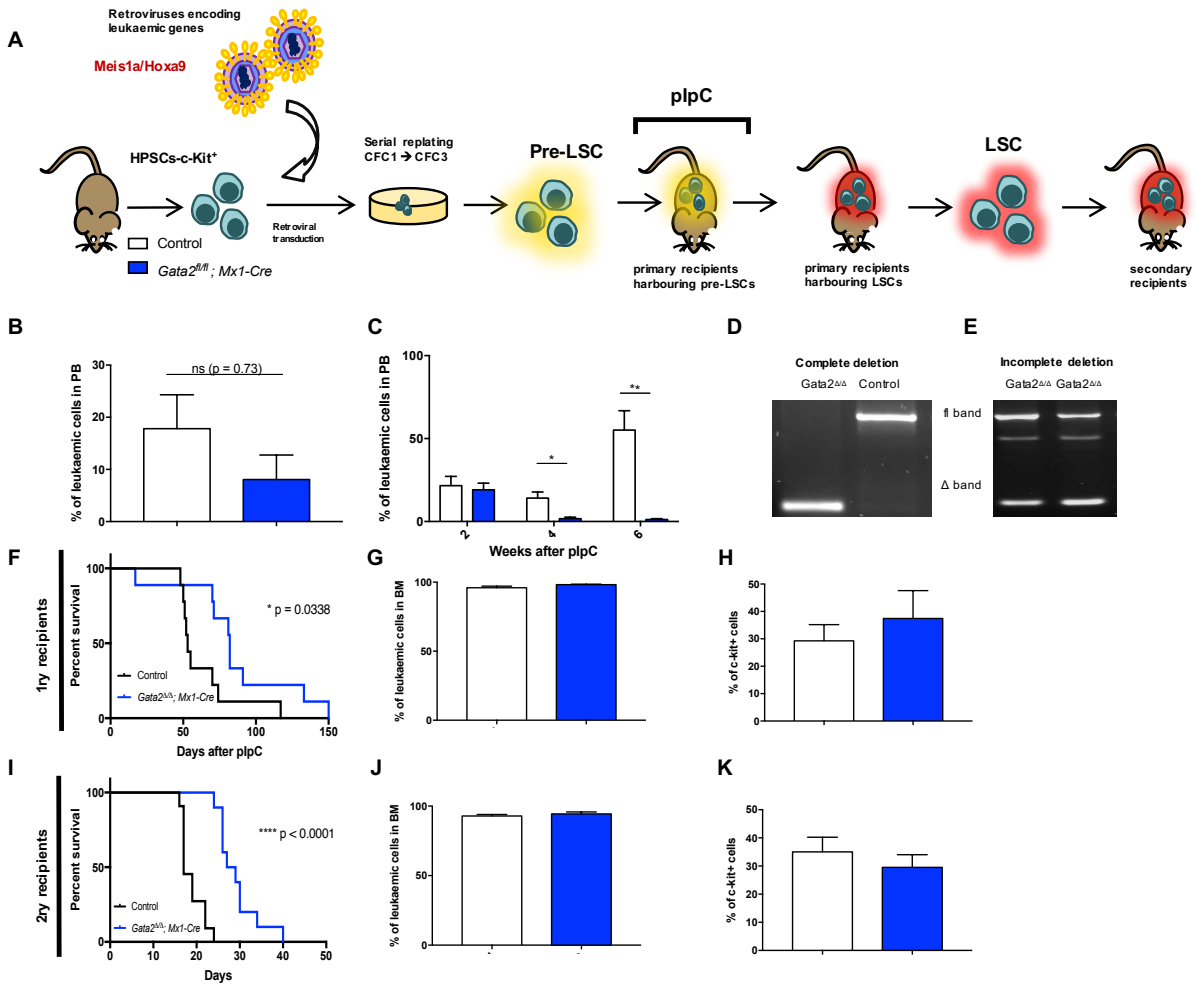


Figure 4.6. Acute deletion of *Gata2* delays LSC development and maintenance in the *Meis1a/Hoxa9* retroviral AML mouse model. (A) C-kit+ cells from *Gata2*$^{fl/fl}$*;Mx1-Cre* or control mice were retrovirally transduced with *Meis1a/Hoxa9* oncogenes and plated into methylcellulose under CFC assays. After CFC3, pre-LSCs (CD45.2+ c-kit+) were transplanted into lethally-irradiated recipients. At day 20 after transplant, mice were administered 6 doses of pIpC to induce gene deletion. LSCs from primary recipients were transplanted into secondary recipients. (B) % of leukaemic cells (CD45.2+) in the PB prior to pIpC (n = 9 recipients per genotype from n = 6-7 biological replicates). C) % of leukaemic cells (CD45.2+) in the PB after pIpC (n = 9 recipients per genotype from n = 6-7 biological replicates). (D) and (E) Genomic PCR showing deletion of *Gata2*. (F) Kaplan-Meier survival curve of primary recipients transplanted with pre-LSCs (n = 9 recipients per genotype from n = 6-7 biological replicates). (G) % of leukaemic cells (CD45.2+) in the BM of primary recipients with AML (n = 5 recipients per genotype from n = 3 biological replicates). (H) % of c-kit+ cells in the BM of primary recipients with AML (n = 5 recipients per genotype from n = 3 biological replicates). (I) Kaplan-Meier survival curve of secondary recipients transplanted with LSCs (n = 10-11 recipients per genotype from n = 2 biological replicates). (J) % of leukaemic cells (CD45.2+) in the BM of secondary recipients with AML (n = 10-11 recipients per genotype from n = 2 biological replicates). (K) % of c-kit+ cells in the BM of secondary recipients with AML (n = 10-11 recipients per genotype from n = 2 biological replicates). Analysis performed on at least two independent experiments. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

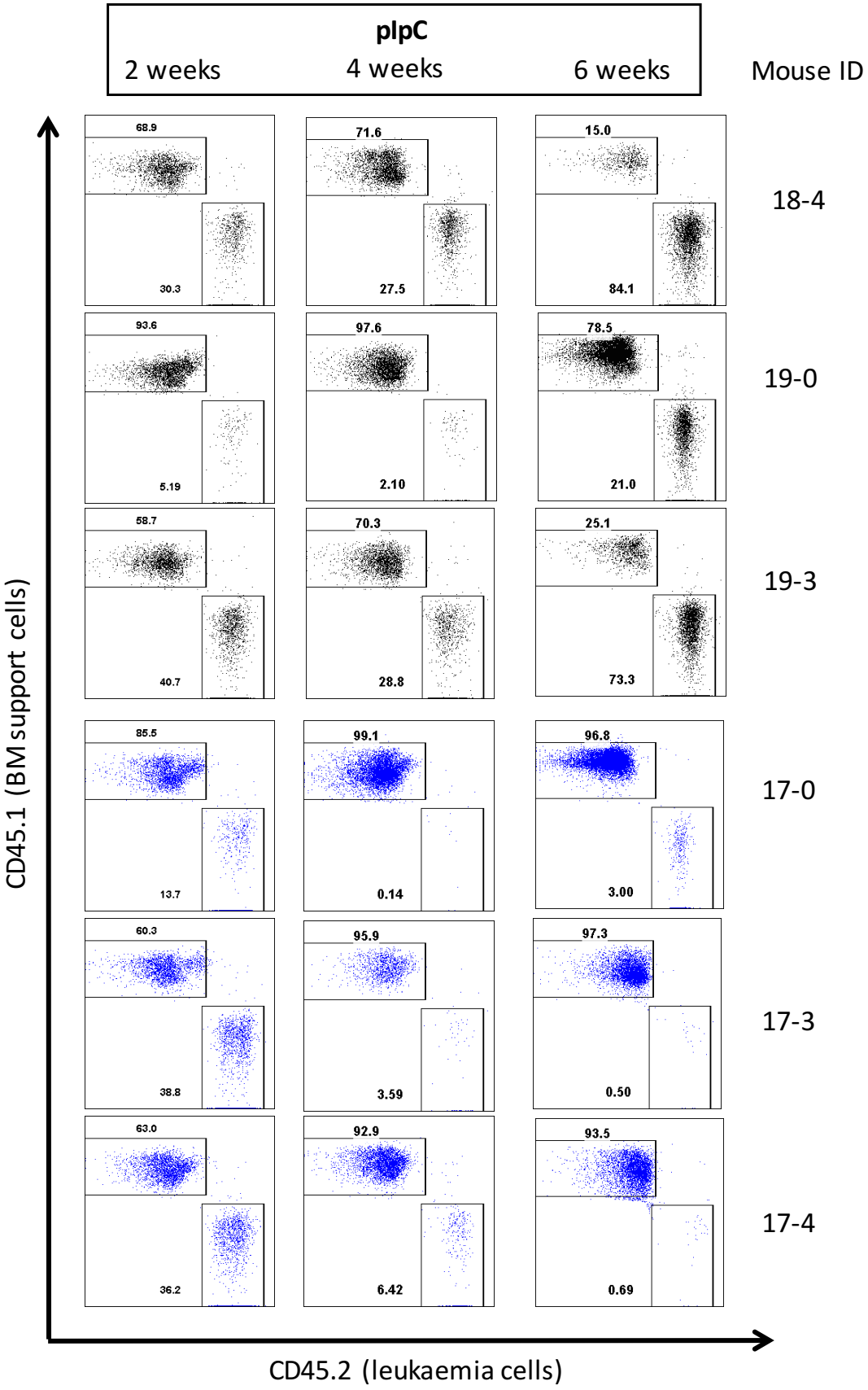


Figure 4.7. Leukaemia monitoring of *Meis1a/Hoxa9* primary recipients. FACS plots showing frequency of leukaemia cells in the PB of *Meis1a/Hoxa9* primary recipients injected with control (black) or *Gata2* deleted pre-LSCs (n = 3).

4.3.5. Pharmacological inhibition of *Gata2 in vitro* impacts the survival of the c-kit$^+$ population in *Meis1a/Hoxa9* and *Mll-af9* Pre-LSCs.

Our results suggest that pharmacological targeting of Gata2 may be effective in curtailing pre-LSC growth. Thus, we tested the efficacy of a validated small molecule inhibitor of *Gata2*, K-7174, to target *Meis1a/Hoxa9* pre-LSCs (Majik *et al.* 2012). K-7174 was first screened in a compound library by Kowa Co. Ltd for the search of adhesion inhibitory compounds (Umetani *et al. 2000*). It was found that K-7174 reduced specifically *VCAM1* mRNA and protein expression in HUVEC and U937 cells (Umetani *et al. 2000*). An electrophoretic mobility shift assay (EMSA) for all the known and potential binding sites to the *VCAM1* promoter discarded any effects of K-7174 to the binding of *Octamer binding site*, *AP1*, *SP1*, *ets1*, *IRF1*, or *NFkB* sites in the *VCAM1* promoter. However, the *GATA* binding to the *VCAM1* decrease in a dose-dependent manner after addition of K-7174. To corroborate the specificity of K-7174 to block *GATA* DNA binding, it was also found that K-7174 reduced *GATA* binding in the promoter of the *Preproendothelin1*, a well-known target of *GATA2* in endothelial cells (Umetani *et al. 2000*). In addition, western blot analysis of human prostate cancer cell lines treated with K-7174 showed decreased protein levels, suggesting that K-7174 binding to *GATA2* can induce protein degradation (He *et al.* 2014), however the exact mechanism by which K-7174 binds *GATA2* has not been described. Nevertheless, K-7174 has been also reported to bind to subunits of the proteasome in a similar way than Bortezomib (Kikuchi *et al.* 2013), and to induce cell death in multiple myeloma cell lines (Kikuchi *et al.* 2013). Bearing in mind that proteasome inhibitors are also able to target AML cells, it cannot be discarded that the impact of K-7174 on our AML cell lines is exclusively and/or solely mediated by *GATA2* inhibition.

First, we plated Meis1a/Hoxa9 pre-LSCs in methylcellulose containing different concentrations of K-7174 or DMSO control (**Figure 4.8A**). We observed a reduction in the number of compact colonies (**Figure 4.8B**), which are considered to contain the more immature blast-like cells while the number of diffuse colonies, which contain the more differentiated cells (So and Cleary 2002), remained unchanged (**Figure 4.8B**). Consequently, we observed a decrease in the frequency of the c-kit$^+$ population from CFCs treated with K-7174 (**Figure 4.8C**).

To confirm the effect of K-7174 in the c-kit$^+$ population independently, we generated *Meis1a/Hoxa9* pre-LSCs cell lines in liquid culture as previously reported (Vukovic *et al.* 2015). We then treated 100,000 pre-LSCs with K-7174 or DMSO control for 48 hours (**Figure 4.8A**) and found a dose-dependent decrease in the number of live cells at 24 and 48-hour time points (**Figure 4.8D**). The proliferation defect observed in K-7174 treated *Meis1a/Hoxa9* was associated with a slight decrease in the frequency of cells in S and G2/M phases of the cell cycle, although not statistically significant (**Figure 4.8E**). However, we noticed an increased frequency of treated *Meis1a/Hoxa9* pre-LSC in the subG0 phase, which corresponded to apoptotic or dead cells (**Figure 4.8E**) (Pucci *et al.* 2000). In agreement with this, we detected a dose-dependent increase in the number of late apoptotic cells from K-7174 treated *Meis1a/Hoxa9* pre-LSCs (**Figure 4.8F** and **Figure 4.8G**). When the frequency of apoptotic cells within the c-kit$^+$ and c-kit$^-$ population was examined, it was found that K-7174 specifically targeted the c-kit$^+$ rather than the c-kit$^-$ population in *Meis1a/Hoxa9* pre-LSCs (**Figure 4.8H** and **Figure 4.8I**). In fact, we observed that K-7174 treated *Meis1a/Hoxa9* pre-LSCs had an increased MFI and frequency of c-kit$^+$ within the late apoptotic phase (**Figure 4.8J** and **Figure 4.8K**). To discard any off-target effect due to antibody bound to dead cells, we also observed that the MFI and frequency of c-kit$^+$ was decreased in the live cell population of K-7174 treated *Meis1a/Hoxa9* pre-LSC (**Figure 4.8L** and **Figure 4.8M**). Taken together, we infer that pharmacological inhibition of *Gata2* specifically targets c-kit$^+$ cells that hold leukaemia initiating potential in *Meis1a/Hoxa9* pre-LSCs.

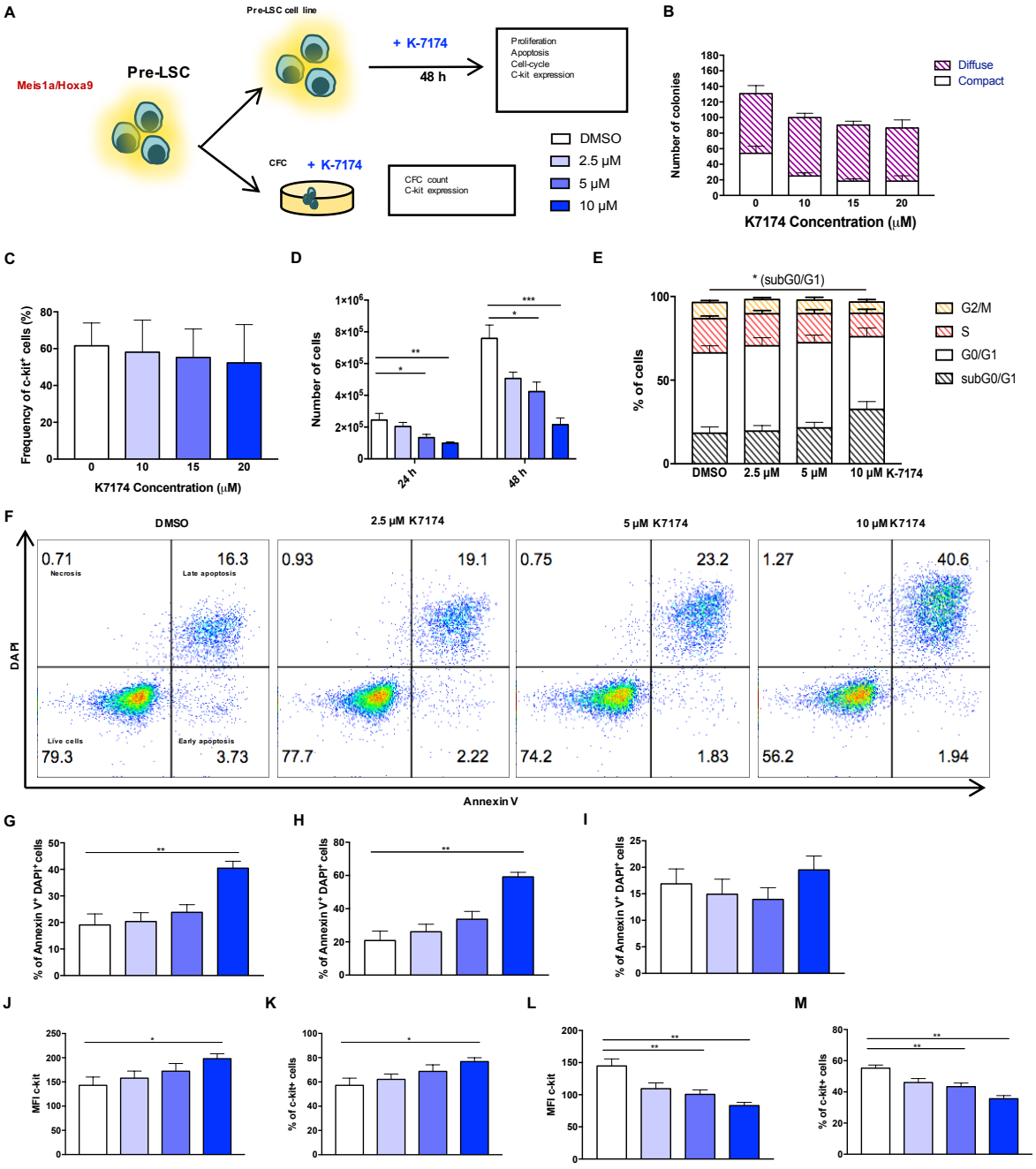


Figure 4.8. Pharmacological inhibition of *Gata2* impacts the survival of the *C-kit*⁺ population in *Meis1a/Hoxa9* Pre-LSCs. (A) 1,250 *Meis1a/Hoxa9* pre-LSCs were plated in CFC assays with K-7174. Alternatively, pre-LSCs cell lines in liquid culture were treated with K-7174 in a 48 hour assay. (B) Number of colonies at day 6 of CFC (n = 3 biological replicates). (C) MFI of c-kit at day 6 of CFC (n = 3 biological replicates). (D) Number of live cells at 24 and 48 hours time points (n = 5 biological replicates). (E) Cell cycle distribution after 48 hour treatment (n = 5 biological replicates). (F) Representative FACS plots showing annexin-V-DAPI staining of *Meis1a/Hoxa9* pre-LSCs at 48 hours. (G) (H) and (I) show the % of late apoptotic cells at 48 hours time point from single cells (G), c-kit⁺ cells (H), and c-kit⁻ cells (I) (n = 5 biological replicates). (J) MFI of c-kit in late apoptotic cells (n = 5 biological replicates). (K) % of c-kit⁺ cells in late apoptotic cells (n = 5 biological replicates). (L) MFI of c-kit in live cells (n = 5 biological replicates). (M) % of c-kit⁺ cells in live cells (n = 5 biological replicates). At least two independent experiments were performed for all analysis. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

Similarly, *Mll-af9* pre-LSCs cell lines were generated to test the efficacy of K-7174 (**Figure 4.9A**). 48-hour time point assays were performed as described for *Meis1a/Hoxa9* pre-LSCs and very similar results were observed. K-7174 treatment decreased the S and G2/M phases coupled with an increase of the sub G0 cell cycle phase (**Figure 4.9B**), reduced proliferation (**Figure 4.9C**), and increased apoptosis, especially within the c-kit$^+$ population (**Figure 4.9D-F**). In addition, K-7174 increased the MFI and frequency of c-kit$^+$ cells in apoptosis phase, coupled with a decrease within live cells confirming that the specific targeting for c-kit$^+$ cells is not due non-specific binding of antibody to dead cells (**Figure 4.9G-J**). Together, these findings suggest that *Gata2* is required for protecting the leukaemia initiating population in *Mll-af9* pre-LSCs.

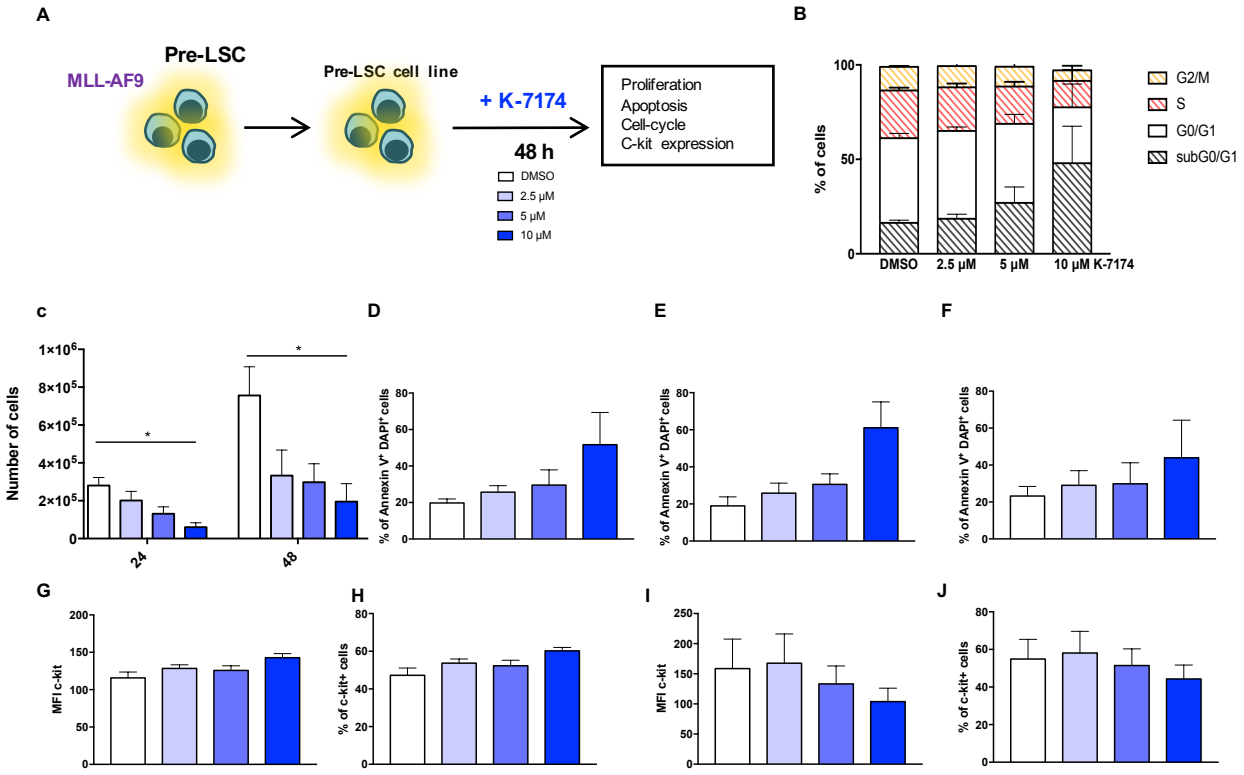


Figure 4.9. Pharmacological inhibition of *Gata2* impacts the survival of the *C-kit*$^+$ population in *Mll-af9* Pre-LSCs. *Mll-af9* pre-LSCs cell lines in liquid culture were treated with K-7174 in a 48 hour assay. (B) Cell cycle distribution after 48 hour treatment (n = 3 biological replicates) (C) Number of live cells at 24 and 48 hours time points (n = 3 biological replicates). (D) (E) and (F) show the % of late apoptotic cells at 48 hours time point from single cells (D), c-kit$^+$ cells (E), and c-kit$^-$ cells (F) (n = 3 biological replicates). (G) MFI of c-kit in late apoptotic cells (n = 3 biological replicates). (H) % of c-kit$^+$ cells in late apoptotic cells (n = 3 biological replicates). (I) MFI of c-kit in live cells (n = 3 biological replicates). (J) % of c-kit$^+$ cells in live cells (n = 3 biological replicates). At least two independent experiments were performed for all analysis. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

4.3.6. VP16 and Ara-C synergise with K-7174 to eradicate *Meis1a/Hoxa9* and *Mll-af9* pre-LSCs *in vitro*.

To test the cooperation between K-7174 and standard chemotherapeutic drugs, such as VP16 and Ara-C (Kudo *et al.* 2007; Reese and Schiller 2013), we incubated *Meis1a/Hoxa9* pre-LSCs with K-7174 alone or in combination with VP16 or Ara-C in a 48-hour assay (**Figure 4.10A**). As expected, the proliferation capacity of *Meis1a/Hoxa9* pre-LSCs (**Figure 4.10B**) with treatment of K-7174, VP16 or Ara-C alone was reduced. Interestingly, when K-7174 was combined with VP16 or Ara-C, proliferation of *Meis1a/Hoxa9* pre-LSCs was further reduced (**Figure 4.10B**). When we examined the apoptotic status of these pre-LSCs, we observed that while K-7174 specifically targets the c-kit$^+$ population, VP16 and, to a lesser extent Ara-C, induced apoptosis in both c-kit$^+$ and c-kit$^-$ populations (**Figure 4.10C-E**). Combination of K-7174 with either VP16 or Ara-C considerably increased the frequency of apoptotic cells, with almost a two-fold increase compared to single treatments with K-7174, VP16 or Ara-C (**Figure 4.10C**). Of note, while K-7174 had no impact in the c-kit$^-$ population, addition of K-7174 to VP16 or Ara-C boosted their killing potency independently of c-kit expression in *Meis1a/Hoxa9* pre-LSCs, suggesting a synergistic mode of action between chemotherapeutics and K-7174 (**Figure 4.10C-E**). Concurrently, the MFI and frequency of c-kit$^+$ cells were increased within the late apoptotic phase, and consequently, diminished within the live cells after K-7174 treatment alone or in combination with VP16 or Ara-C in comparison to either the DMSO control, VP16 or Ara-C single treatments (**Figure 4.10F-I**). Importantly, treatment with VP16 or Ara-C alone did not affect the MFI or frequency of c-kit$^+$ within live cells, highlighting the non-specific killing effects of standard chemotherapeutics (**Figure 4.10H** and **Figure 4.10I**).

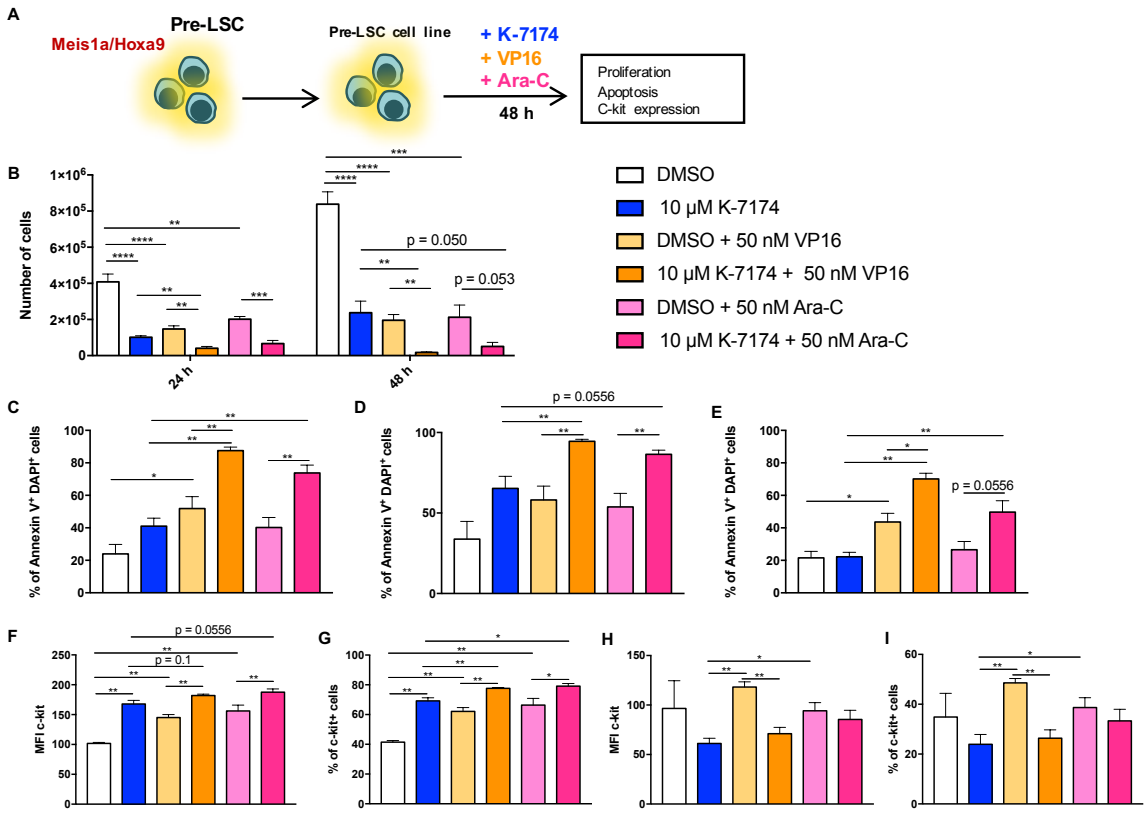


Figure 4.10. VP16 and Ara-C synergise with K-7174 to eradicate *Meis1a/Hoxa9* pre-LSCs. (A) *Meis1a/Hoxa9* pre-LSCs cell lines in liquid culture were treated with K-7174 and VP16 or Ara-C in a 48 hour assay. (B) Number of live cells at 24 and 48 hour time points (n = 5 biological replicates). (C), (D) and (E) show the % of late apoptotic cells at 48 hours time point from single cells (C), c-kit$^+$ cells (D), and c-kit$^-$ cells (E) (n = 5 biological replicates). (F) MFI of c-kit in late apoptotic cells (n = 5 biological replicates). (G) % of c-kit$^+$ cells in late apoptotic cells (n = 5 biological replicates). (H) MFI of c-kit in live cells (n = 5 biological replicates). (I) % of c-kit$^+$ cells in live cells (n = 5 biological replicates). At least two independent experiments were performed for all analysis. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

We also applied this combination of treatments to *Mll-af9* pre-LSCs (**Figure 4.11A**). Overall, we observed the same trend as with *Meis1a/Hoxa9* pre-LSCs, consisting of cooperative effects between K-7174 and chemotherapeutics VP16 or Ara-C in the reduction of proliferation (**Figure 4.11B**), induction of apoptosis in the c-kit$^+$ (**Figure 4.11C** and **Figure 4.11D**), and decrease of the MFI and frequency of c-kit$^+$ within live cells (**Figure 4.11H** and **Figure 4.11I**). We also observed a moderate trend in the increase of MFI and c-kit$^+$ expression in late apoptotic cells (**Figure 4.11F** and **Figure 4.11G**), and most importantly, K-7174 appeared not to potentiate the killing activity of VP16 and Ara-C within the c-kit$^-$ population of *Mll-af9* pre-LSCs (**Figure 4.11E**). Collectively, these data indicate that pharmacological inhibition of *Gata2* potentiates the activity of standard chemotherapeutics against *Meis1a/Hoxa9* and *Mll-af9* pre-LSCs.

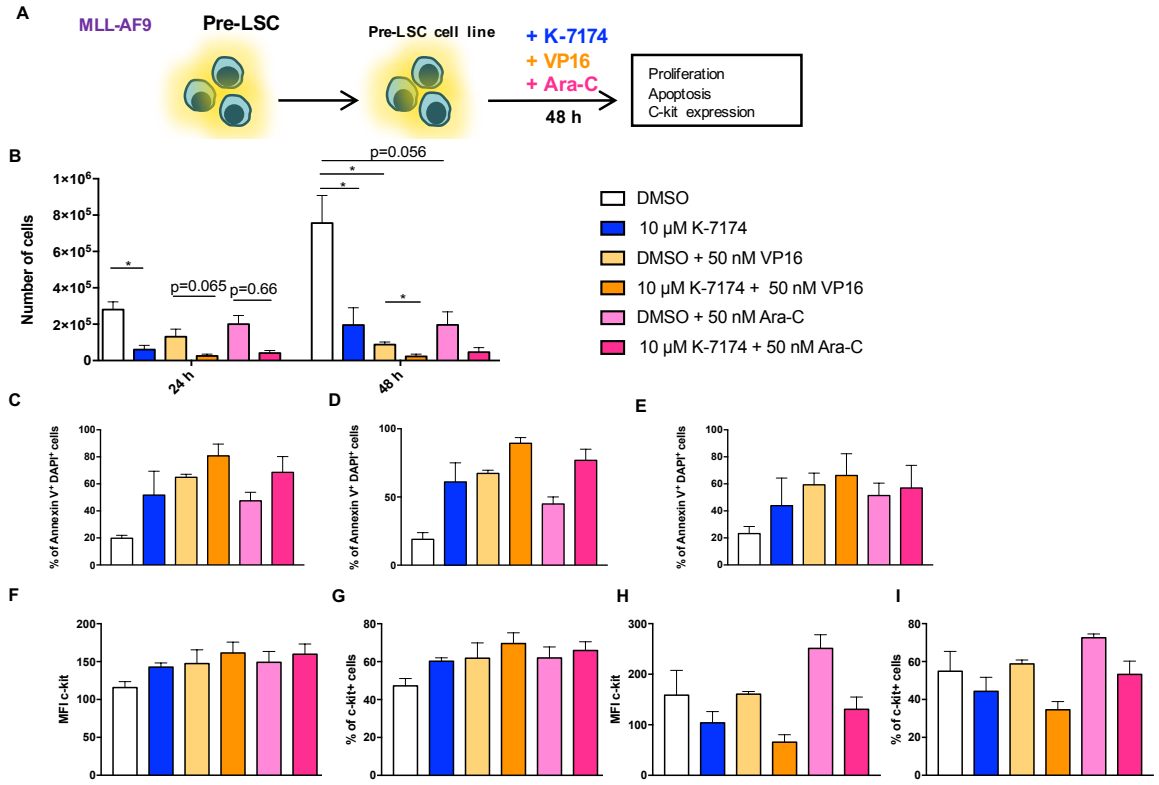


Figure 4.11. VP16 and Ara-C synergise with K-7174 to eradicate *Mll-af9* pre-LSCs. (A) *Mll-af9* pre-LSCs cell lines in liquid culture were treated with K-7174 and VP16 or Ara-C in a 48 hour assay. (B) Number of live cells at 24 and 48 hours time points (n = 3 biological replicates). (C), (D) and (E) show the % of late apoptotic cells at 48 hours time point from single cells (C), c-kit$^+$ cells (D), and c-kit$^-$ cells (E) (n = 3 biological replicates). (F) MFI of c-kit in late apoptotic cells (n = 3 biological replicates). (G) % of c-kit$^+$ cells in late apoptotic cells (n = 3 biological replicates). (H) MFI of c-kit in live cells (n = 3 biological replicates). (I) % of c-kit$^+$ cells in live cells (n = 3 biological replicates). At least two independent experiments were performed for all analysis. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

4.3.7. Pharmacological inhibition of *Gata2* impacts the survival of the c-kit$^+$ population in *Meis1a/Hoxa9* LSCs *in vitro*.

In order to investigate whether the K-7174 is able to directly target LSCs, we sorted *Meis1a/Hoxa9* LSCs (CD45.2$^+$ C-kit$^+$ CD34^{low} CD16/32$^+$) from primary recipients into methylcellulose treated with K-7174 (**Figure 4.12A**). At day 6 of the CFC assay, we found a significant reduction in the number of compact colonies, while diffuse colonies remain unchanged (**Figure 4.12B**). In addition, the frequency of c-kit$^+$ LSCs from colonies treated with K-7174 was reduced compared to DMSO control treated colonies (**Figure 4.12C**). Finally, we treated *Meis1a/Hoxa9* LSCs with K-7174 alone, or in combination with VP16 or Ara-C (**Figure 4.12A**). As seen with *Meis1a/Hoxa9* pre-LSCs, treated LSC displayed a dose dependent reduction in proliferation (**Figure 4.12D**), and a further reduction when K-7174 was combined with VP16 or Ara-C compared to single treatments of K-7174, VP16, or Ara-C (**Figure 4.12E** and **Figure 4.12F**). From this single experiment, it appears that K-7174 treatment on its own or in

combination with standard chemotherapeutics could be a feasible therapeutic strategy to target *Meis1a/Hoxa9* LSCs.

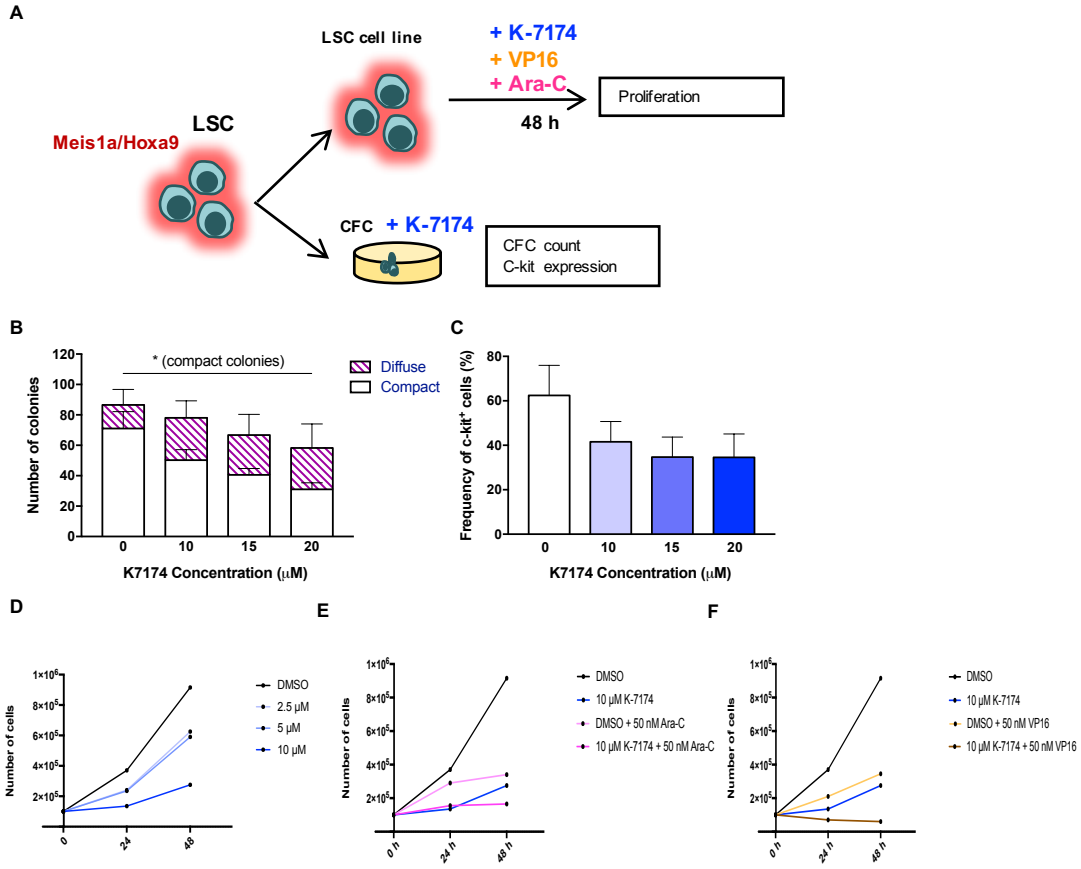


Figure 4.12. Pharmacological inhibition of *Gata2* reduces the clonogenic potential of *Meis1a/Hoxa9* LSCs. (A) 1,250 *Meis1a/Hoxa9* LSCs were plated in CFC assays with K-7174. Alternatively, LSCs cell lines in liquid culture were treated with K-7174 in a 48 hour assay. (B) Number of colonies at day 6 of CFC (n = 4 biological replicates). (C) MFI of c-kit at day 6 of CFC (n = 4 biological replicates). (D), (E) and (F) show the number of live cells at 24 and 48 hour time point treatment with K-7174 alone (D) or in combination with Ara-C (E) or VP16 (F). Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

4.4. Discussion

In this chapter, we explored the requirement for *Gata2* in pre-LSCs and LSCs from two different genetic mouse models of AML. To achieve this, we used different approaches to delete or knockdown *Gata2*, including *in vivo* and *in vitro* genetic deletion via the Cre-lox system, and pharmacological inhibition employing a small molecule inhibitor, K7174. Overall, our findings highlight that despite being expressed at relatively low threshold levels, *Gata2* plays a major role in pre-LSCs and LSCs driven by *Meis1a/Hoxa9*.

An increase of *Gata2* expression in a pre-leukaemia population from a transgenic *Mll-af9*^{KI} mouse was observed, in disagreement with our retroviral *Mll-af9* mouse model. These differences across models with the same genetic background could be explained by several factors, including cell culture conditions versus BM microenvironment, and oncogenic dose. In the *Mll-af9*^{KI} mouse model, the pre-leukaemia LSK cells are surrounded by a pre-malignant BM niche, which can secrete cytokines, growth factors or cell-to-cell interact with pre-LSCs and thus modulate its properties. In contrast, in the retroviral model, *Mll-af9* transduced cells are maintained in semisolid medium containing the required but not exhaustive combination of Scf, IL-3, IL-6, and GM-CSF cytokines. Discrepancies between the impact of the oncogenic dose of *Mll-af9* between the knock-in and the retroviral mouse models in relation to the cell of origin and differential expression of downstream effectors of *Mll-af9* pathogenesis, such *Evi1*, have been already interrogated (W. Chen *et al.* 2008). In the retroviral model, the copies of *Mll-af9* integrated into the genome of the cells can be higher than one and are regulated by a strong MSCV promoter, while the knock-in mouse closely recapitulates the human scenario by expressing the *Mll-af9* translocation under control of the endogenous *Mll* promoter.

There has been substantial controversy regarding the role of *Gata2* at the different stages of AML. For instance, sporadic or hereditary MDS/AML patients with *GATA2* haploinsufficiency indicates that downregulation of *Gata2* is required for leukaemia initiation (Hahn *et al.* 2011). In addition, Bonadies *et al*. reported downregulation of *Gata2* in retroviral mouse models driven by the expression of *Mll-enl* or *Moz-tif2* translocations (Bonadies *et al.* 2011). This is in line with our result showing

downregulation of *Gata2* in pre-LSC and LSCs, although we have not formerly quantified *Gata2* protein levels or activity in this study. Bonadies *et al*. also described that upon overexpression of *Gata2* in *Mll-enl* pre-LSCs, there is a lack of CFC capacity and a proliferation defect in culture. However, this overexpression system led to a supraphysiological 3,000-fold induction of *Gata2* (Bonadies *et al.* 2011). As exemplified in overexpression studies of *Gata2* in murine and human HSPCs (Persons *et al.* 1999; Tipping *et al.* 2009), tight regulation of *Gata2* levels is crucial for normal haematopoiesis and supraphysiological activity of *Gata2* could therefore be shutting down the homeostatic machinery in leukaemia cells. In agreement with this, more recently it has been published that low-level overexpression of *Gata2,* employing an inducible doxycycline-based system, is able to immortalize BM cells in culture with a myeloid lineage bias (Nandakumar *et al.* 2015). A chimeric mouse from ESCs microinjected with the same *Gata2* construct was also generated in this study and myeloid lineage skewing was observed *in vivo*, though never achieving full-blown leukaemia (Nandakumar *et al.* 2015). Therefore, disruption of *Gata2* steady-state levels leads to perturbed haematopoiesis in both murine and human cells. As shown with other TFs (Johnson 2000; Yang *et al.* 2007; Turner and Watson 2008), the idea of *Gata2* being able to act as both tumour suppressor and oncogene in HSCs is not mutually exclusive.

Studies addressing the importance of *Gata2* downregulation in leukaemia transformation, at least in murine retroviral mouse models of AML, merit further investigation. Bearing in mind that *Gata2* deletion using the *Mx1-Cre* system leads to rapid HSPC loss (see **chapter 3.3**), the use of *in vitro* methods to delete *Gata2* in HSPCs, prior or during leukaemia initiation would be required. For instance, a few studies using the *Mx1-Cre* model have reported that addition of *IFN*α into liquid or semi-solid culture achieved full deletion of the floxed allele (Kerenyi *et al.* 2013; Kranc *et al.* 2009). Doxycycline-induced Cre, lentiviral cre, or CRISPR-Cas9-mediated deletion of *Gata2* before/during/after leukaemia transformation with oncogenes (i.e. *Mll-af9* or *Meis1a/Hoxa9*) could also be used as experimental approaches to address this question. Conversely, inducible systems to overexpress *Gata2* before/during/after leukaemia transformation would help to clarify the impact and target genes of *Gata2* dose dependent leukaemia initiation. Curiously, *Hoxa9* was one of the top genes

upregulated following low-level overexpression of *Gata2* in BM cells (Nandakumar *et al.* 2015). Together with our Gata2 gene expression results in *Mll-af9* and *Meis1a/Hoxa9* models, it appears that there is a reciprocal regulation and co-operation of *Gata2* and *Hoxa9* genes in leukaemia initiation.

Surprisingly, we observed a differential requirement for Gata2 in two interrelated AML models driven by Mll-af9 and Meis1a/Hoxa9. So how does *Gata2* deletion have an impact in leukaemia initiation when driven by Meis1a/Hoxa9 overexpression, but not *Mll-af9* oncogenic translocation, which is an upstream activator of Meis1a/Hoxa9? Part of the explanation may be the heterogeneous starting HSPC (c-kit$^+$) population used for leukaemia transformation in these retroviral transduction models. In fact, several publications have demonstrated that the cell of origin (LSK, CMP, GMP) in the *Mll-af9* model has an impact in the leukaemia initiating capacity, gene dependence, drug-resistance, and gene expression profiles (Krivtsov *et al.* 2013). In this study, we have no control over the cell of origin driving leukaemogenesis in the *Mll-af9* model since c-kit+ cells can initiate the leukaemia from either LSK, CMP or GMP, while, in contrast, *Meis1a/Hoxa9* are only capable of transforming LSK cells but not CMP or GMP (Arai *et al.* 2010; Krivtsov *et al.* 2013). Thus, we hypothesise that the differences arising between the *Meis1a/Hoxa9* and the *Mll-af9* models may be driven by the cell of origin. *Meis1a/Hoxa9* leukaemia arise from LSK cells, which are highly dependent on *Gata2* levels and therefore could explain why even low levels of *Gata2* observed in pre-LSCs and LSCs are important for their functions. Bearing in mind that the step-wise leukaemogeneic assay used in this study favours the establishment of a dominant clone during the replating assays, it is interesting to speculate that *Mll-af9* pre-LSCs, and consequently *Mll-af9* LSCs, arise from a single clonal population. While *Mll-af9* LSK cells might possess a clonal advantage over CMP or GMP cells, the numbers of CMP and GMP transduced cells would be much higher than transduced LSK cells within the *c-kit*$^+$ starting population, which in turn could exclude the LSK competition in the CFC assays. The disparity between the impact of *Gata2* deletion in the *Mll-af9* and *Meis1a/Hoxa9* models could also be explained by the additional downstream pathways that govern leukaemia development in the *Mll-af9* model. It has been reported that *Evi1 is* a critical effector in *Mll-af9* leukaemia but not *Meis1a/Hoxa9* (Bindels *et al.* 2012). In addition, another study found that while *Mll-af9* binds and activates the *Evi1* promoter*, Meis1a/Hoxa9* has a low *Evi1* expression (Arai *et al.*

2010). *Evi1* is a zinc-finger TF that has been closely related to *Gata2*. In fact, *Evi1* knockout mice have a similar phenotype than *Gata2 KO* mice (Sato *et al.* 2008) and *Evi1* is also able to activate the *Gata2* promoter in HSCs (Goyama *et al.* 2008). Thus, *Evi1* could replace and rescue the function of *Gata2* in *Gata2* deleted *Mll-af9* pre-LSCs/LSCs. Investigating the requirement for *Gata2* in prospectively isolated subsets of HSPCs should shed light on the stage-specific requirements and molecular mechanisms operating in Gata2 driven leukaemogenesis.

Coexistence of molecularly distinct clones of pre-LSCs and LSCs have been found before and after treatment in AML patients (Goardon *et al.* 2011; Dick 2005). Generally, the LSCs in the retroviral model employed are defined by c-kit^{+} expression (Somervaille and Cleary 2006; Vukovic *et al.* 2015), or the putative L-GMP including CD16/32^{+} and CD34^{low} markers (Krivtsov *et al.* 2006). As seen in this and other studies, all or most of the leukaemia population within the c-kit^{+} LSC population express the CD16/32^{+} and CD34^{low} markers, although variation in gating strategies of c-kit^{+} and CD34^{low} is present in the field. Single-cell omics advances in the past years has shed light towards the identification of (epi)genetic heterogeneity within the established haematopoietic hierarchy, and challenged the conventional markers used to isolate HSPCs by flow cytometry (Ema *et al.* 2014; Guo *et al.* 2013; Corces *et al.* 2016). In addition, this approach has been also applied to leukaemia studies (Jung *et al.* 2015; Thomas and Majeti 2017). Specifically, a study employing single-cell analysis has identified heterogeneity within the L-GMP LSC population in the *Mll-af9* retroviral mouse model (Guo *et al.* 2013). For instance, the authors described that CD24^{-} L-GMP LSCs induced AML *in vivo* faster than CD24^{+} L-GMP LSCs. It could be hypothesised that in the *Meis1a/Hoxa9* model, *Gata2*-dependent and *Gata2*-independent L-GMP LSCs exist. Therefore, deletion of *Gata2* reduces the proportion of leukaemia cells in the PB by killing the *Gata2*-dependent LSCs, but eventually relapse of AML is driven by *Gata2*-independent LSCs. Our gene expression analysis within the “bulk” pre-LSC/LSC could be hiding the true expression of *Gata2* in specific subsets of *Meis1a/Hoxa9* pre-LSCs/LSCs with greater leukaemia initiating activity. It would be worth investigating whether *Gata2* defines a “true” pre-LSC/LSC subset within the *Meis1a/Hoxa9* mouse model. For instance, a *Gata2-Venus* reporter mouse model was recently engineered to study the activity of *Gata2* in HSC development (Kaimakis *et al.* 2016). Therefore, transformation of the *Gata2-Venus* reporter mice

with *Meis1a/Hoxa9* oncogenes would facilitate the tracking and molecular characterisation of *Gata2*-*Venus*$^{+}$ leukaemia cells, if any, within the established *Meis1a/Hoxa9* pre-LSC/LSC populations.

In both AML models, we obtained complete and incomplete deletion of *Gata2* employing the *Mx1-Cre* system in recipients transplanted with pre-LSCs. However, we found that recipients of *Meis1a/Hoxa9* pre-LSCs succumbed to AML slower than control pre-LSCs, regardless of the efficiency of *Gata2* deletion. In the Mx1-Cre system, incomplete deletion due to strong selective pressure has already been reported in *Tel1* in normal HSCs or *Jmjd1c* in *Mll-af9* cells (Zhu *et al.* 2015). In the leukaemia setting, the authors found complete *Jmjd1c* deletion 14 days after pIpC in the PB of leukaemic mice, while moribund mice presented incomplete *Jmjd1c* deletion in the BM. The authors found a delay in AML development, as per our case, however they did not mention complete deletion in any of the mice that succumbed of AML, nor did they report any mice that survived had complete *Jmjd1c* deletion (Zhu *et al.* 2015). In our experiments, we found complete *Gata2* deletion in mice that succumb to AML, indicating that the selective pressure phenomenon cannot be applied in our data. Instead, PB analysis showed a massive drop in the frequency of leukaemia cells, initially in leukaemic mice upon acute deletion of *Gata2* in *Meis1a/Hoxa9* pre-LSCs, suggesting that *Gata2* protects *Meis1a/Hoxa9* pre-LSCs from cell death. As alluded to above, the fact that even recipients with complete *Gata2* deletion succumb to AML indicates that a sub-population of *Meis1a/Hoxa9* pre-LSCs are *Gata2* independent. Alternatively, since we have not assessed the impact of *in vivo* acute deletion of *Gata2* in established LSCs (i.e. in secondary recipients) it could be hypothesised that LSCs are already present by the time of pIpC-mediated deletion, and that consequently *Meis1a/Hoxa9* LSCs, but not pre-LSCs, are resistant to acute *Gata2* deletion. In support of this, we detected a dramatic reduction in the clonogenic capacity of *Meis1a/Hoxa9* pre-LSCs after *Gata2*-mediated deletion with lentiviruses expressing Cre. This would further support the idea of the presence of *Gata2*-resistant LSCs at the time of deletion. Further experiments are needed to validate the *in vitro* results with lentiviral Cre to assess the leukaemia initiating capacity of *ex-vivo* deleted pre-LSCs and LSCs in both AML models.

Finally, we tested the impact of pharmacological inhibition of *Gata2* with a small molecule inhibitor, K-7174, against *Meis1a/Hoxa9* and *Mll-af9* pre-LSC. K-7174 inhibition of *Gata2* has been shown in several publications and in Chapter 5 (Imagawa *et al.* 2003; Majik *et al.* 2012). Nevertheless, we should formally test the inhibition of *Gata2* in our mouse models. K-7174 reduced the proliferation and induced apoptosis in *Meis1a/Hoxa9* and *Mll-af9* pre-LSC cell lines, specifically in the c-kit$^+$ population. Induction of apoptosis by K-7174 treatment could be related to apoptotic genes affecting both *Gata2* and these mouse models of AML. For instance, downregulation of the anti-apoptotic gene *Bcl-xL* was seen in constitutive *Gata2* haploinsuficient mice (Rodrigues *et al.* 2005). In addition, data in Chapter 5 showed that knockdown of *GATA2* increases apoptosis and *P21* expression in THP1 cells harbouring a *MLL-AF9* translocation (see **Figure 5.**). There is evidence in the literature linking downregulation of *Bcl-xL* and induction of *p21* with apoptosis in retroviral mouse models, driven by *Mll-af9* and *Meis1a/Hoxa9* expression (Wu and O'Reilly 2011; Mizukawa *et al.* 2011). Decrease of *c-kit* expression upon K-7174 treatment can be explained by the fact that *Gata2* is a well-known transcriptional activator of the *c-kit* promoter in haematopoietic cells (Maeda *et al.* 2010). Specific targeting of the leukaemia initiating population (c-kit$^+$) allowed for the synergism between K-7174 and standard chemotherapeutics such as Ara-C and VP16, that targeted both the c-kit$^+$ and c-kit$^-$ pre-LSCs populations. Synergism between these two and other chemotherapeutics used in AML should be validated with different concentrations of each agent. Furthermore, *in vivo* studies should be conducted to explore the ability of K-7174, alone or in combination chemotherapeutics, to eradicate *Meis1a/Hoxa9* and *Mll-af9* pre-LSCs/LSCs. In addition, direct targeting of *GATA2* with regards to HSPC toxicity should be evaluated. In section 3.3. we have shown that K-7174 induces apoptosis of murine HSPCs *in vitro*. However, to date, the effects of *GATA2* KD or KO in human HSCs have not been reported. Future studies should address the toxicity of K-7174 (or *GATA2* KD) in human HSCs, and in comparison, to the targeting efficiency of AML cells.

To conclude, we have shown that pharmacological inhibition of *Gata2* is effective against the leukaemia initiating population within *Meis1a/Hoxa9* and *Mll-af9* pre-LSCs *in vitro*. The caveats resulting from off-target effects of the small molecule inhibitor K-7174 on other *Gata family* genes and the proteasome will be thoroughly discussed in the human AML chapter (see **section 5.4**). These caveats aside, our data indicate

that K-7174 (or other means of GATA-2 targeting) hold potential as a therapeutic to treat *Mll-af9* or *Meis1a/Hoxa9* driven AML, either alone or as an adjunct to current standard AML chemotherapy.

Chapter 5

Investigating the function of *GATA2* overexpression in human AML

5.1. Introduction

GATA2 is a key transcriptional regulator of haematopoietic stem and progenitor cells (HSPCs). Strict regulation of *GATA2* level is crucial for HSC homeostasis. *Gata2*$^{+/-}$ mice display lower numbers and qualitative defects of FL HSCs in transplantation experiments (Ling *et al.* 2004). Similarly, BM HSC from *Gata2*$^{+/-}$ mice are also diminished, perform poorly in transplantation assays (Rodrigues *et al.* 2005) and after 5-FU myeloablative treatment (Ling *et al.* 2004) indicating a proliferation defect of *Gata2*$^{+/-}$ BM HSCs. In fact, dysregulation of *GATA2* levels in HSCs leads to the development of BM failure. A case in point is the decrease in *GATA2* levels observed in BM CD34^{+} cells (Fujimaki *et al.* 2001) and BM MSCs (Xu *et al.* 2009b) from aplastic anaemia patients, a condition that presents with BM aplasia and fat replacement in the BM (Brodsky and Jones 2005). Furthermore, *GATA2* loss of function mutations have been linked to immunodeficiency of DC, B, NK cells, MonoMac and Emberger syndrome, that can develop into MDS and AML (Hyde and Liu 2011). Over 100 different types of mutations have been described in the ZF domains (ZF1 and ZF2) and untranslated regions of the *GATA2* gene. Conversely, overexpression of *Gata2* in murine or human HSCs inhibit differentiation and induce quiescence both *in vitro* and *in vivo* (Persons *et al.* 1999; Tipping *et al.* 2009). More recently, a study reported that low-level overexpression of *Gata2* in murine BM cells leads to BM immortalisation *in vitro,* characterised with an increase of myeloid markers and block in lymphoid differentiation. *In vivo* experiments confirm myeloid lineage skewing after low-level overexpression of *Gata2*; however, these mice remain asymptomatic without progression to AML (Nandakumar *et al.* 2015).

Over the last 5 years, several publications have pinpointed an oncogenic role for *GATA2* in cancer. *GATA2* overexpression has been associated with poor prognosis and aggressiveness in several cancer types including: prostate, lung, breast, and colorectal cancer. *GATA2* overexpression was found in human breast carcinoma, and *PTEN* was inhibited directly by *GATA2*-promoter binding or indirectly by blocking androgen receptor (AR)-dependent PTEN activation (Wang *et al.* 2012). In non-small cell lung cancer (NSCLC), *GATA2* expression was found in association with the poor prognosis *KRAS* mutation (Anon 2012), and *GATA2* inhibition was able to reduce tumours size in *Kras*-driven NSCLC murine model (Kumar *et al.* 2012). *GATA2*

overexpression has also been reported with chemotherapy resistance and aggressiveness in prostate cancer, whilst RNAi of *GATA2* reduced prostate tumours *in vivo*. The *IGFR-1-AKT/ERK/JNK* signalling pathways activate *GATA2,* which in turn activates the transcription of *IGF2* that binds to *IGFR-1,* thereby configuring a positive feedback loop in prostate cancer cells (Vidal *et al.* 2015).

Of relevance to haematological cancer, overexpression of *GATA2* has been found in 40% of adult AML patients (Vicente *et al.* 2012) correlating with cytogenetic grouping and overexpression of *EVI* and *WT1,* both of which confer poor prognosis (Vicente *et al.* 2012). Shorter overall survival (OS) and event-free survival (EFS) have been shown to correlate with higher *GATA2* expression (Vicente *et al.* 2012). In addition, overexpression of *GATA2* has also been documented in 60-to-85% of paediatric AML patients (M Luesink *et al.* 2012). Interestingly, *GATA2* expression was normalised in patients in complete remission, while remaining elevated in those with resistant disease (M Luesink *et al.* 2012). Therefore, *GATA2* expression could help stratify AML patients and operate as an independent poor prognosis marker for AML however, the biological impact of *GATA2* overexpression on AML cell fate is currently poorly defined.

5.2. Aims and objectives

The specific aims and objectives for this chapter were:

1. To confirm that *GATA2* is overexpressed in human AML by combining and analysing large gene expression datasets published previously.

2. To investigate the role of *GATA2* in human AML (M5) THP-1 cells harbouring MLL-AF9 translocation employing shRNA-mediated knockdown.

3. To assess the impact of K-7174, a validated small molecule inhibitor of *GATA2*, in human AML cells.

4. To study the synergism between K-7174 and standard chemotherapeutic drugs used in AML.

5.3. Results

5.3.1. *GATA2* is overexpressed in human AML

In order to validate previous studies showing the overexpression of *GATA2* in AML patients, we sought to analyse a combination of 13 published AML databases. Briefly, AML (n = 2611) and control (n = 77) patient datasets were downloaded from the GEO to create a case/control cohort hybridised to the same array (Affymetrix Human Genome U133 Plus 2.0 GeneChip) and run through R using bioconductor packages. Data was normalised using RMA. We found that *GATA2* levels were higher in AML patients compared to healthy controls (BM MNCs) (**Figure 5.1A**). Specifically, *GATA2* was overexpressed in 511 out of 2061 (25%) AML patients based on Log2FC cut-off (**Figure 5.1B**). Higher expression of *GATA2* was observed across different mutations, FAB-subtypes, and Karyotypes (**Figure 5.1C-E**). Higher overexpression was observed in FAB-M2, M3 and M6 subtypes while M5 subtype had a similar *GATA2* expression than healthy controls (**Figure 5.1D**). Thus, these results indicate *GATA2* overexpression across multiple subtypes of AML.

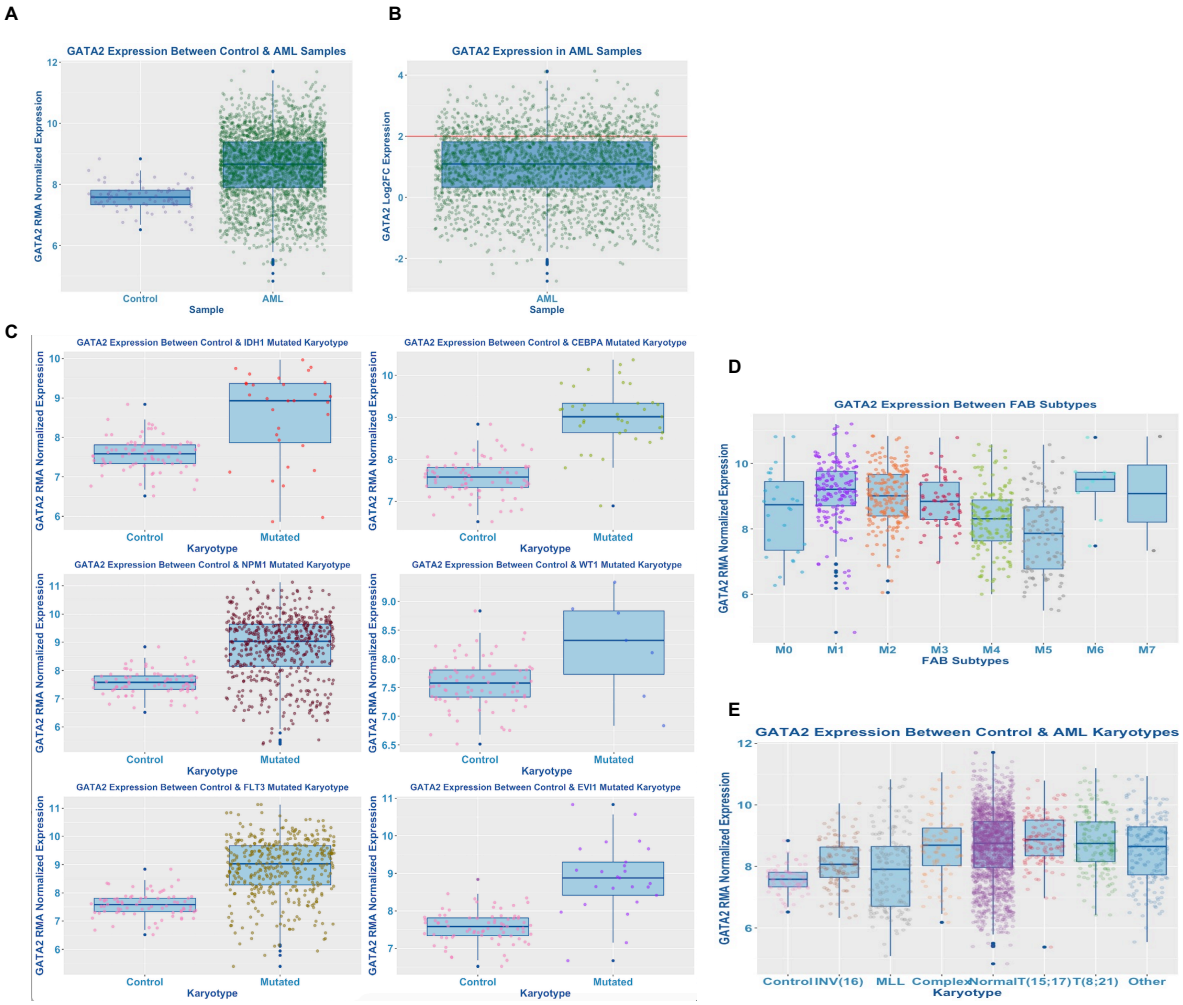


Figure 5.1. GATA2 expression in AML. a) GATA2 expression between control and AML samples. b) GATA2 expression in AML samples. Orange line marks GATA2 overexpressing samples (25%) based on Log2FC. c) GATA2 expression among the most common mutations in AML d) GATA2 expression in FAB subtypes of AML. e) GATA2 expression among cytogenetics groups in AML. **Bioinformatics analysis performed by Mr. Alex Gibbs (Cardiff University)**.

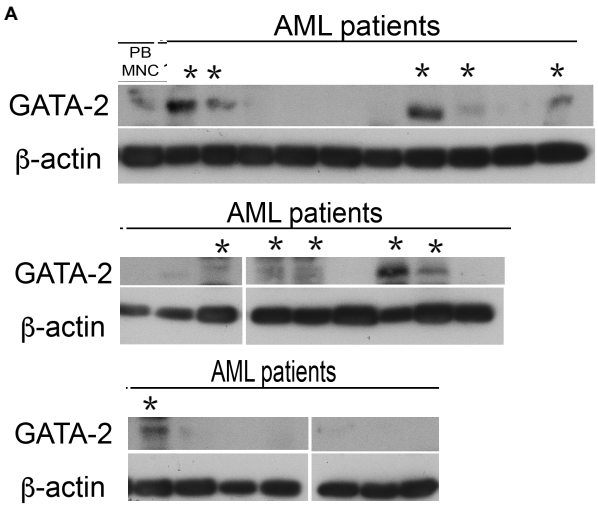


Figure 5.2. GATA2 is (over)expressed in AML patient. a) Western blot image of total BM MNCs detecting GATA2 protein levels in AML patients. (* indicates AML samples (over)expressing GATA2). **Western blot performed by Dr. Rhys Morgan (Bristol University).**

To corroborate this data, *GATA2* levels in several human leukaemia cell lines were assessed. Compared to human BM MNCs, *GATA2* RNA was increased by 30-fold in the K562 cells and 20-fold in KG1 cells (**Figure 5.3A**). In addition, we also assessed two monocytic cell lines harbouring a *MLL-AF9* translocation; THP1 cells showed a 4-fold increase in *GATA2*, while NOMO1 cells had similar *GATA2* RNA levels compared to control BM MNCs (**Figure 5.3A**). Intracellular FACS was performed to measure the *GATA2* protein levels in these cell lines which correlated with the RNA expression profile seen in NOMO1, THP1 and KG1 cells (**Figure 5.3B** and **Figure 5.3C**). We also detected *GATA2* (over)expression in 11 out of 26 samples (42%) in primary AML patient samples (**Figure 5.2**).

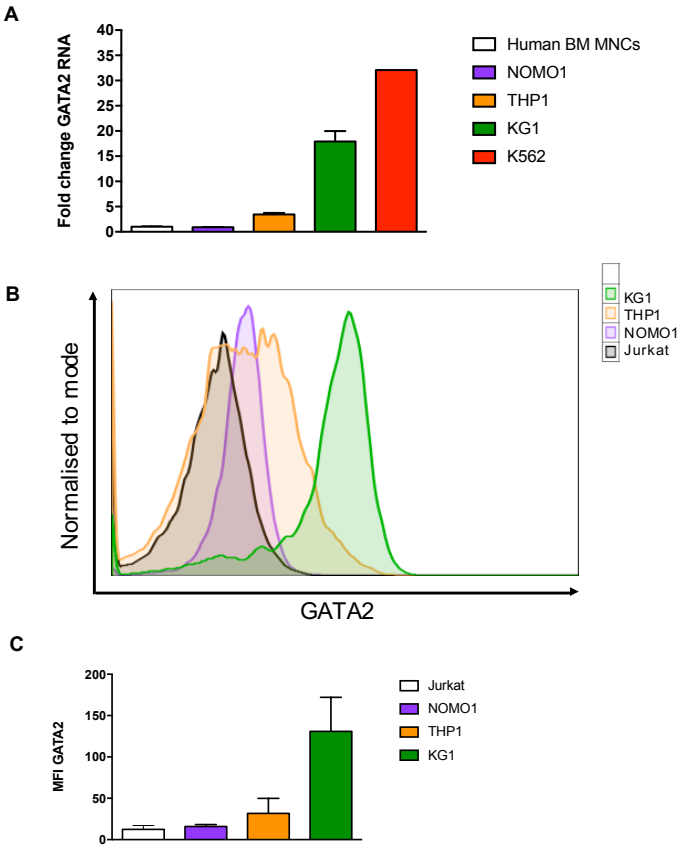


Figure 5.3. GATA2 expression in human leukaemia cell lines. (A) RNA from NOMO1, THP1, KG1a and K562 was extracted and GATA2 levels assessed by qPCR. Human BM MNCs were used as a control. GAPDH was used as a housekeeping gene. N = 2-5 from 2 independent experiments. (B) Intracellular FACS histogram showing GATA2 protein expression in KG1, THP1, and NOMO1 compared to Jurkat cells (negative control) (C) MFI of GATA2 from B (n = 2). Data are mean ± SEM.

5.3.2. *GATA2* knockdown impairs the proliferation and clonogenic capacity of THP1 AML cells.

To investigate the biological impact of *GATA2* in human AML cell fate, we used a bicistronic lentiviral vector system carrying shRNA against human *GATA2* and GFP reporter (See methods, **Figure 2.1** and **Table 2.2**) to attenuate GATA2 expression. We validated the efficiency of GATA-2 knockdown of four constructs targeting *GATA2* (named 31, 32, 33 and 34). Knockdown efficiency of shRNA 31, 32 and 34 was between 50-60% compared to scramble control at the RNA level in K562 and THP1 cells (**Figure 5.4A** and **Figure 5.4B**). Western blot of THP1 cells revealed no *GATA2* protein levels in shRNA 31 and 32, although decreased *B-ACTIN* loading was observed, especially in shRNA 32, compared to scramble control (**Figure 5.4C**). shRNA 34 showed a very faint *GATA2* protein band, however *B-ACTIN* loading was comparable to the control (**Figure 5.4C**). For further experimentation, we used shRNA number 31 and 34. To corroborate the knockdown efficiency at protein level, we also validated the decrease of *GATA2* protein levels in THP1 cells transduced with shRNA 31 and 34 (**Figure 5.4D**).

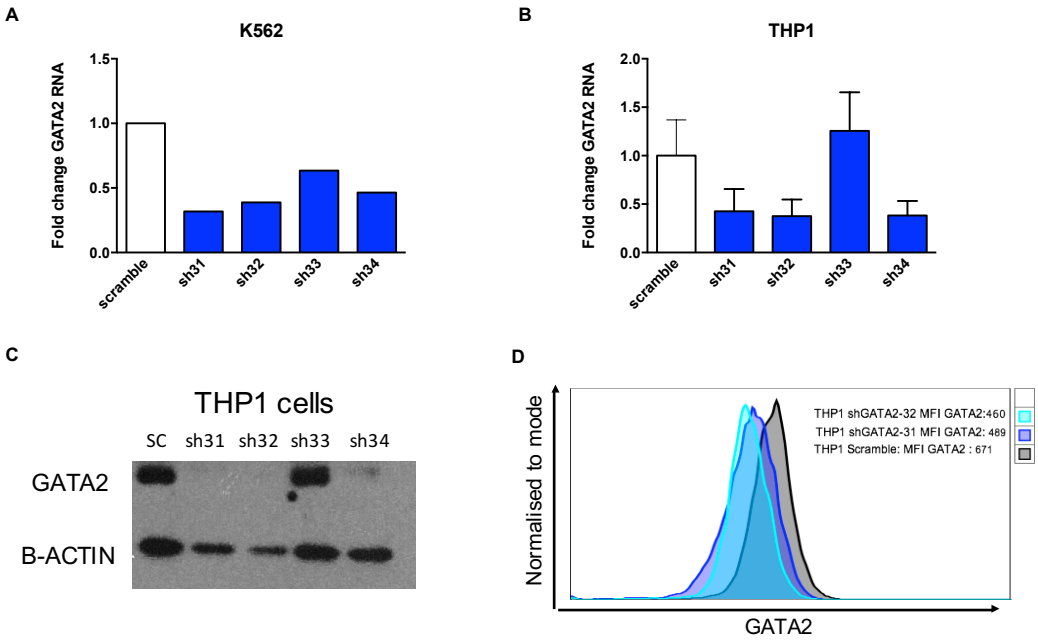


Figure 5.4. Knockdown validation of human short hairpin against GATA2. Human cell lines were transduced with lentiviruses encoding a short hairpin against human GATA2 (or scramble control) and a GFP reporter. GFP$^+$ cells were FAC-sorted and GATA2 knockdown was measured by qPCR in (A) K562, (B) THP1 (n = 3), western blot in (C) THP1 cells, and intracellular FACS (D) for 2 clones in THP1 (n = 1). GAPDH was used as a housekeeping gene in the qPCR. **Western blot performed by Ms. Maria Konstantinou (Cardiff University)**.

In order to study the role of *GATA2* in the context of overexpression in MLL-AF9 driven AML, we transduced THP1 cells with shRNA 31, 34 or scramble control vector. 5 days later, transduced GFP$^+$ cells were sorted and plated in a 72-hour assay (**Figure 5.5A**). Proliferation of *GATA2* KD THP1 cells was severely compromised compared to control THP1 cells, showing a 3-to-4-fold reduction in the number of live cells at 48 and 72-hour time points (**Figure 5.5B**). When the cell cycle status of *GATA2* KD THP1 cells was analysed at 72-hour time point, there was a decrease in the frequency of proliferating cells (S+G2/M) compared to control THP1 cells (**Figure 5.5C** and **Figure 5.5D**). Of interest, the cell cycle profile of *GATA2* KD THP1 cells showed an increase in the subG0/G1 population, which is indicative of cell death (**Figure 5.5C**). We also plated transduced GFP$^+$ cells into CFC assays, and found a 10-fold reduction in the clonogenic capacity of *GATA2* KD THP1 cells compared with control cells **(Figure 5.5F**). In addition, colonies at day 12 from *GATA2* KD THP1 cells were smaller in size compared to colonies from control THP1 cells (**Figure 5.5E**). Collectively these data indicate that *GATA2* is a critical growth regulator of THP1 AML cells.

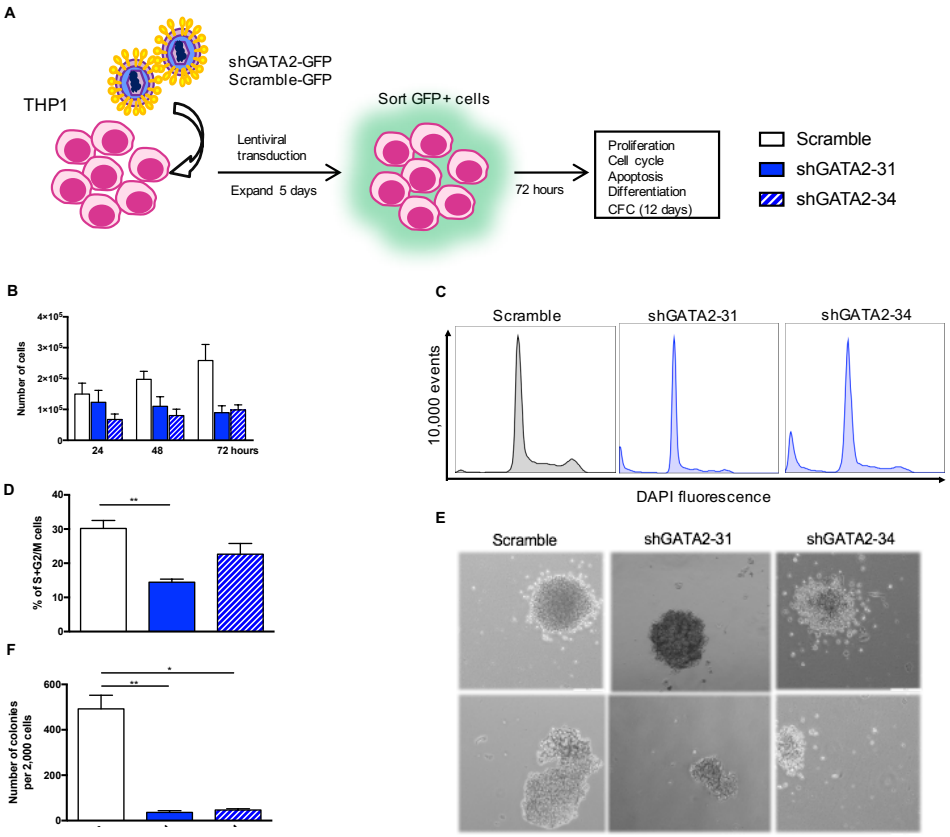


Figure 5.5. GATA2 knockdown impairs the proliferation and clonogenic capacity of THP1 cells. (A) THP1 cells were transduced with lentiviruses encoding a short hairpin against human GATA2 and a GFP reporter. GFP$^+$ cells were FAC-sorted and 150,000 cells plated in a 72 hour assay. (B) Number of cells at 24,48, 72 hour time points (n = 2-7). (C) Representative FACS histogram showing the cell cycle profile. (D) frequency of proliferating cells (S+G2/M) at 72 hours (n = 3-7). (E) Representative CFC pictures at day 12 after plating. (F) Number of colonies at day 12 (n = 3-7). "n" indicates the number of independent experiments. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

5.3.3. *GATA2* knockdown in THP1 AML cells induces apoptosis and differentiation via *CDKN1A*.

To further investigate the apoptotic status of *GATA2* KD THP1 cells, we performed an annexin V assay. We found a significant increase in the number of apoptotic cells at 72-hour time in *GATA2* KD THP1 cells compared to control THP1 cells (**Figure 5.6A** and **Figure 5.6B**), indicating that GATA2 is crucial for the survival of THP1 cells.

Since *GATA2* is important to maintain the undifferentiated status of HSCs (Tipping *et al.* 2009; Persons *et al.* 1999), we investigated whether *GATA2* overexpression was responsible for the differentiation block of THP1 cells: a general feature of AML. Indeed, upon *GATA2* KD we found an increase in the expression of myeloid differentiation markers CD11b and CD14 (**Figure 5.6C**). A significant increase of CD14 was observed in shRNA-31 cells compared to scramble control cells, while a non-significant trend was observed with respect to CD11b expression **(Figure 5.6D** and **Figure 5.6E**). shRNA-34 cells showed an increasing trend of expression of differentiation markers, particularly in CD11b expression, in the two independent experiments performed (**Figure 5.6D** and **Figure 5.6E**).

To explore the molecular mechanisms underpinning GATA-2 mediated control of proliferation, apoptosis and differentiation, gene expression analysis was performed for genes previously related to *GATA2* in regard to its function in the cell-cycle and apoptosis. We observed a significant upregulation of *P21* (*CDKN1A*) in shRNA-31 THP1 cells compared to control THP1 cells (**Figure 5.6F**). *P21* upregulation could explain the decrease of proliferating cells in the cell-cycle and increase of apoptosis upon *GATA2* knockdown. In addition, it has been reported that upon DNA damage production, *P21* upregulation induces differentiation of AML cells in the context of *MLL-AF9* leukaemia (Santos *et al.* 2014). Collectively, our results show that *GATA2* protects AML cells from apoptosis and possibly sustains their differentiation block, at least in part, via *P21* expression.

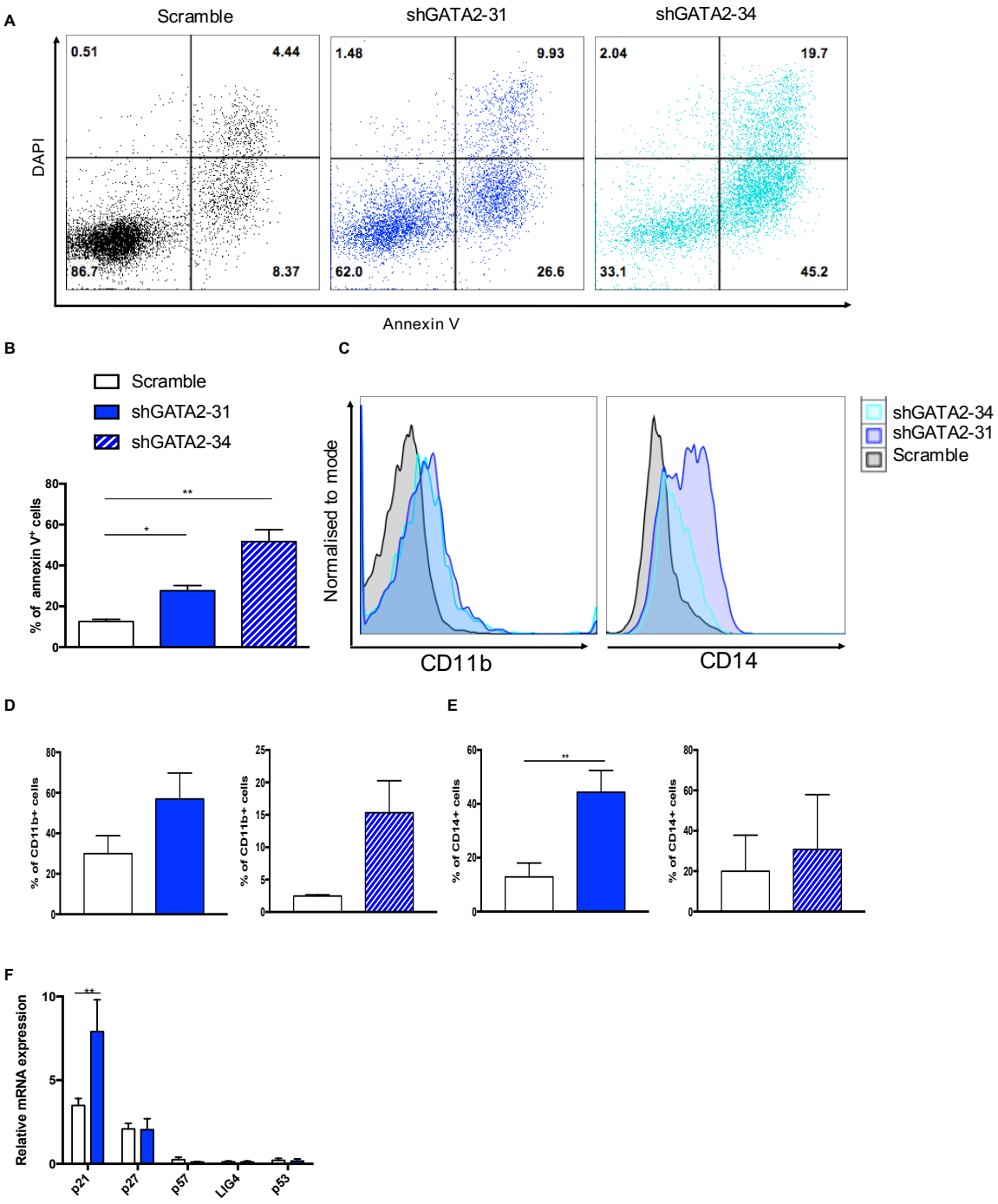


Figure 5.6. GATA2 knockdown induces apoptosis and differentiation of THP1 AML cells via *P21*. (from 5.5A) THP1 cells were transduced with lentiviruses encoding a short hairpin against human GATA2 and a GFP reporter. GFP$^+$ cells were FAC-sorted and 150,000 cells plated for 72 hours prior to assessing their apoptotic and differentiation status. (A) Representative FACS plots showing apoptotic status. (B) Frequency of annexin V$^+$ cells at 72 hour time point (n = 4). (C) Representative FACS histogram showing CD11b (left) and CD14 (right) expression. (D) Frequency of CD11b$^+$ cells at 72 hour time point (n = 6 for shGATA2-31; n = 2 for shGATA2-34). (E) Frequency of CD14$^+$ cells at 72 hour time point (n = 6 for shGATA2-31; n = 2 for shGATA2-34). (F) Relative mRNA expression of *P21*, *P27*, *P57*, *LIG4*, *P53* from GFP$^+$ sorted cells. GAPDH was used as a housekeeping gene (n = 4). "n" indicates the number of independent experiments. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

5.3.4. Pharmacological inhibition of *GATA2* induces apoptosis in AML cells.

In order to corroborate the results obtained with lentiviral shRNA, we pharmacologically inhibited *GATA2* by employing a *GATA2*-specific small molecular inhibitor (Majik *et al.* 2012), K-7174, in THP1 cells. To confirm *GATA2* inhibition, we incubated THP1 cells with increasing concentrations of K-7174 and measured the *GATA2* protein levels by flow cytometry (**Figure 5.7B**). As expected, the MFI of *GATA2* in THP1 cells decreased upon incubation with increasing doses of K-7174, although we saw a plateau effect in the highest dose (**Figure 5.7C**). We then performed a 6-day assay to measure the impact of K-7174 on proliferation and apoptosis status in THP1 cells (**Figure 5.7A**). In line with our shRNA approach, we observed a reduction in the number of live cells and induction of apoptosis in a dose-dependent manner in K-7174 treated THP1 cells (**Figure 5.7F**, **Figure 5.7J**, and **Figure 5.7K**).

To validate that these effects were not THP1 cell-specific, we inhibited *GATA2* using K7174 in a range of leukaemic cell lines harbouring different genetic/epigenetic aberrations and *GATA2* levels (**Figure 5.3**). We further validated *GATA2* inhibition in NOMO1 and KG1 cells (**Figure 5.7D** and **Figure 5.7E**). We were able to replicate these results, including the dose-dependent reduction of proliferation and induction of apoptosis, in NOMO1, KG1, and K562 treated cells (**Figure 5.7G-I** and **Figure 5.7L-N**). Thus, *GATA2* inhibition appears to induce apoptosis in AML cells regardless of their (epi) genetic background and level of *GATA2* expression.

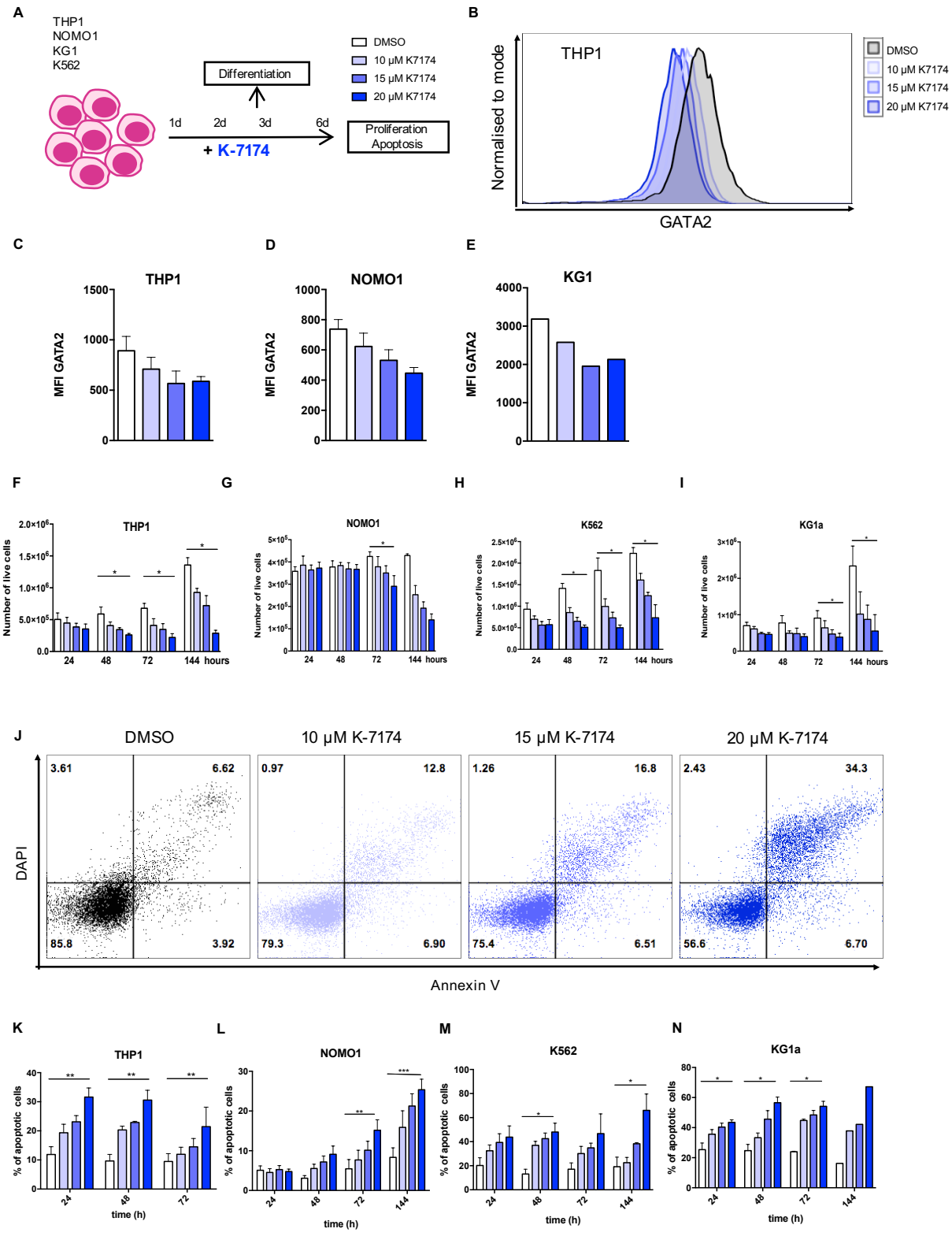


Figure 5.7. Pharmacological inhibition of GATA2 induces apoptosis of leukaemia cells. (A) 400,000 cells from THP1, NOMO1, K562 or KG1, were treated with K-7174 for 6 days. (B) FACS histogram showing intracellular *GATA2* protein levels in THP1 treated cells. (C) MFI of *GATA2* in THP1 (n = 3). (D) MFI of *GATA2* in NOMO1 (n = 2). (E) MFI of *GATA2* in KG1 (n =1). (F-to-I) Number of live cells at each time point in (F) THP1 cells (n = 4), (G) NOMO1 (n = 3-4), (H) K562 (n = 3), (I) KG1 (n =2-3). (J) Representative FACS plots showing apoptotic status of THP1 cells at 48 hour time point. (K-to-N) Frequency of apoptotic cells at each time point in (K) THP1 cells (n = 4), (L) NOMO1 (n = 3-4), (M) K562 (n = 3), (N) KG1 (n = 1-3). "n" indicates the number of independent experiments. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

5.3.5. *GATA2* is not required before or after DNA damage response (DDR) pathway activation in THP1 AML cells.

DNA damage induction in AML cells has been shown to activate *P21* (Viale *et al.* 2009; Santos *et al.* 2014). In order to investigate whether the induction of *P21* and subsequent apoptosis and differentiation was driven by the induction of DNA damage after *GATA2* KD in THP1 cells, we incubated *GATA2* KD THP1 cells with etoposide (VP16) for 30 minutes to induce DSBs. At the indicated time points we collected the treated cells and measured the levels of γ-H2AX, an early marker of the DDR pathway activation (Paull *et al.* 2000; Kuo and Yang 2008), along with the expression of *P21* and other *GATA2* related genes (**Figure 5.8A**). *GATA2* expression was unaltered in *wild-type* THP1 cells after treatment with etoposide in comparison to DMSO control (**Figure 5.8B**). There were no changes in the MFI of γ-H2AX at any of the indicated time point (**Figure 5.8C**). As seen in untreated THP1 cells, *P21* was upregulated in *GATA2* KD THP1 cells at every time point (**Figure 5.8D**), in agreement with the increase in *P21* levels observed in shRNA-31 THP1 cells. Interestingly, we also detected an increase in the expression of *P57* at the 24-and-72-hour time point (**Figure 5.8F**). *P57* is a cell-cycle inhibitor important for maintaining HSC quiescence (Matsumoto *et al.* 2011) and it has been shown that *Gata2* binds and activates *p57* transcription in HSPCs (Billing *et al.* 2016). Taken together, these results indicate that *GATA2* is not required for DDR pathways activation in THP1 cells.

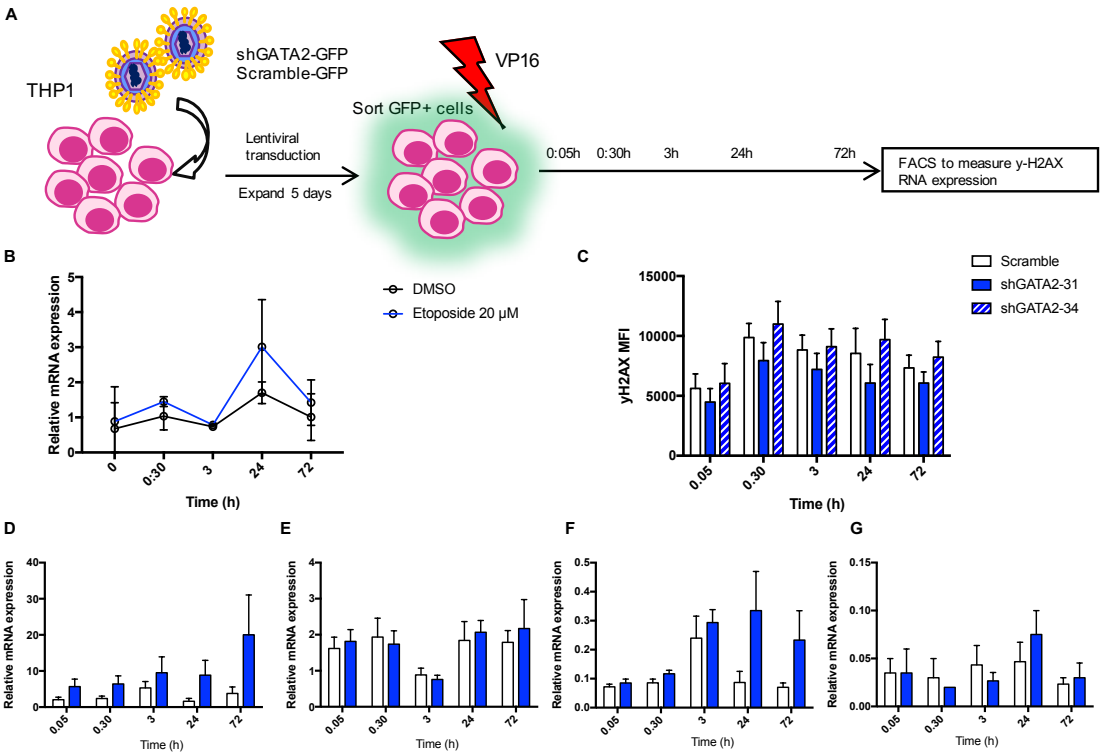


Figure 5.8. GATA2 knockdown does not alter DDR response after DSBs in THP1 cells. (A) THP1 cells were transduced with lentiviruses encoding a short hairpin against human GATA2 and a GFP reporter. GFP⁺ cells were FACS-sorted and DSBs induced by 30 minutes treatment with 20 μM VP16. (B) GATA2 RNA expression from wild-type THP1 cells at the indicated time points (n = 2). (C) MFI of γ-H2AX (n = 4). (D) p21 RNA expression (n = 4). (E) p27 RNA expression (n = 3). (F) p57 RNA expression (n = 3). (G) p53 RNA expression (n = 3). GAPDH was used as a housekeeping gene. Data from 4 independent experiments. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

Next, we assessed whether *GATA2* would affect the DDR pathway response after DSBs induction. Since lentiviral transduction of VP16 treated cells would lead to toxicity and low transduction efficiency, we treated THP1 cells with VP16 for 30 minutes to induce DSBs, and then added K-7174 to inhibit *GATA2*. At the indicated time points, we measured the levels of γ-H2AX and RNA expression of treated THP1 cells with or without K-7174 (**Figure 5.9A**). We found no changes in the MFI of γ-H2AX of K-7174-treated THP1 cells compared to control THP1 cells (**Figure 5.9B**). Therefore, we concluded that *GATA2* is not required for the DDR pathway activation and maintenance in THP1 cells.

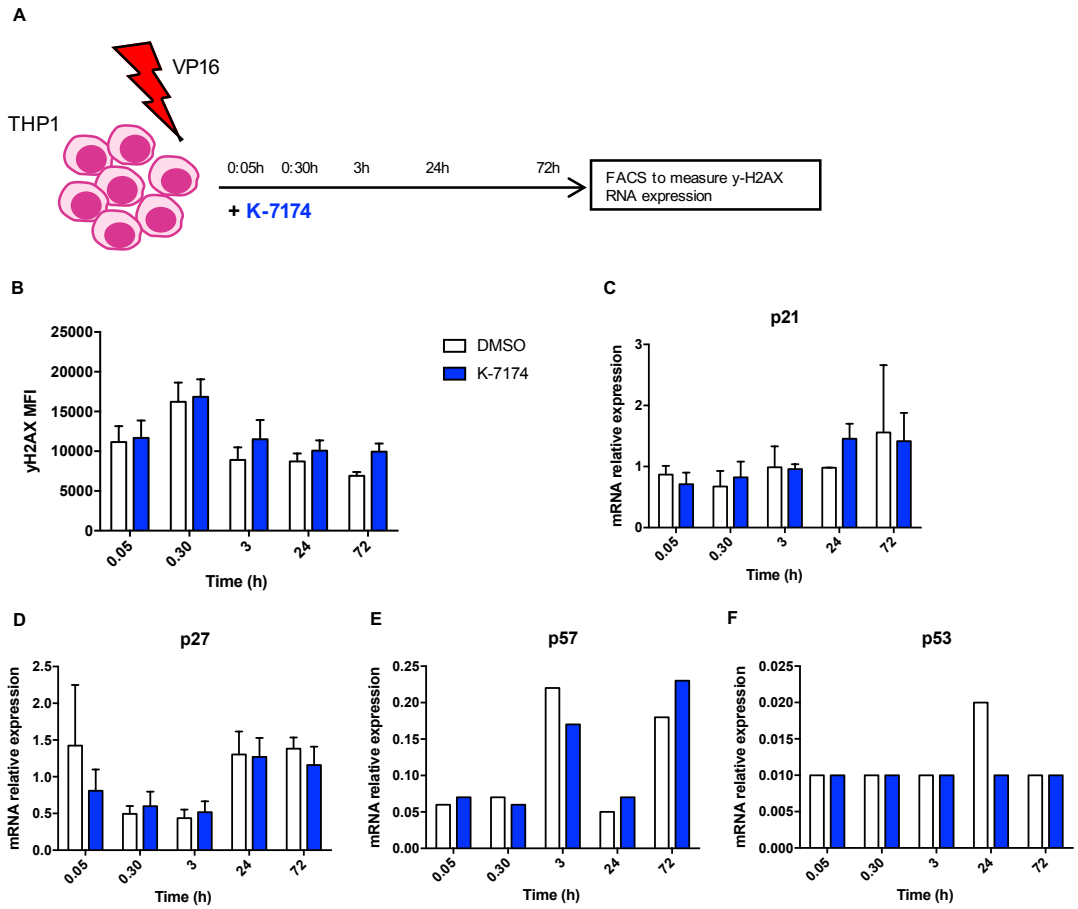


Figure 5.9. K-7174 treatment does not alter DDR response after DSBs in THP1 cells. (A) THP1 cells were treated with 20 μM VP16 or DMSO control for 30 minutes. THP1 cells were then plated with K-7174 or DMSO control. (B) MFI of γ-H2AX (n = 4). (C) p21 RNA expression (n = 2). (D) p27 RNA expression (n = 2). (E) p57 RNA expression (n = 1). (F) p53 RNA expression (n = 1). GAPDH was used as a housekeeping gene. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

5.3.6. K-7174 synergises with Ara-C or VP16 to eradicate THP1 AML cells.

To date, single treatment of specific inhibitors of cancer cells, with the exception of APL, are not yet a first line treatment option in AML (Stein and Tallman 2015). Recently, IDH1/2 inhibitors have shown great potential in phase 2 clinical trials (Levis 2013; Shafer and Grant 2016). Moreover, inhibition of the enzyme dihydroorotate dehydrogenase (DHODH) showed therapeutic potential by inducing differentiation in mouse and human AML models (Sykes *et al.* 2016). Combination of innovative small molecule inhibitors with standard chemotherapeutics is a more approachable treatment option, especially if this allows a calibrated reduction of standard chemotherapeutics and its related toxicity. To assess whether *GATA2* inhibition could improve the killing activity of standard chemotherapeutics such Ara-C and VP16, we incubated THP1 cells with K-7174 and VP16 or Ara-C in a 48-hour assay (**Figure**

5.10A). As expected, treatment with K-7174, VP16, and Ara-C alone decreased the number of live THP1 cells compared to control THP1 cells (**Figure 5.10C**). Interestingly, combination of K-7174 and VP16 further reduced the number of live THP1 cells at 24-and-48-hour time points (**Figure 5.10C**). Combination of K-7174 and Ara-C reduced the number of live THP1 cells at the 24-hour time point, however cells appeared to recover at the 48-hour time point (**Figure 5.10C**), although variation was observed in K-7174 and Ara-C treated THP1 cells. Additional experiments would be required to confirm these results. When we assessed apoptotic status, we found an increase in the number of apoptotic cells in THP1 cells treated with K-7174, VP16, or Ara-C (**Figure 5.10D** and **Figure 5.10E**). Notably, addition of K-7174 to VP16 or Ara-C increased their ability to induce apoptosis in THP1 cells (**Figure 5.10D** and **Figure 5.10E**). Therefore, pharmacological inhibition of *GATA2* enhanced the killing activity of VP16 and Ara-C in AML cells.

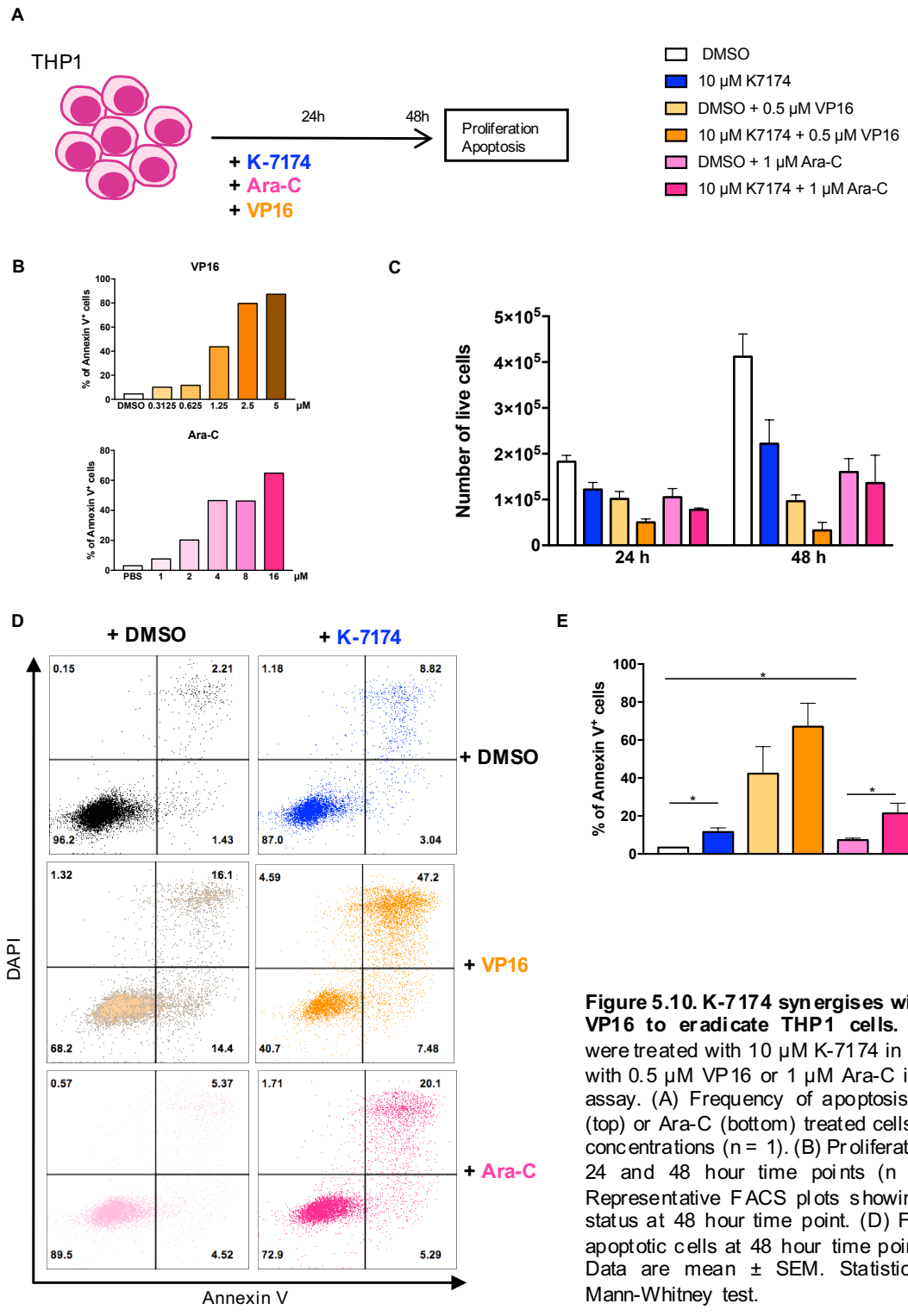


Figure 5.10. K-7174 synergises with Ara-C or VP16 to eradicate THP1 cells. THP1 cells were treated with 10 µM K-7174 in combination with 0.5 µM VP16 or 1 µM Ara-C in a 48 hour assay. (A) Frequency of apoptosis from VP16 (top) or Ara-C (bottom) treated cells at different concentrations (n = 1). (B) Proliferation assay at 24 and 48 hour time points (n = 2-3). (C) Representative FACS plots showing apoptotic status at 48 hour time point. (D) Frequency of apoptotic cells at 48 hour time point (n = 2-4). Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

5.4. Discussion

GATA2 expression studies in AML and MDS have indicated that *GATA2* is (over)expressed in a wide range of patients (Luesink *et al.* 2012; Nandakumar *et al.* 2015; Vicente *et al.* 2012; Shimamoto *et al.* 1994). However, variability in the method used to quantify (over)expression and percentage of *GATA2* (over)expression between studies is present. In addition, other reports have also highlighted lower *GATA2* expression in AML as a consequence of somatic mutations or epigenetic silencing (Bonadies *et al.* 2011; Hyde and Liu 2011; Celton *et al.* 2014). This disparity has likely arisen due to the heterogeneous nature of AML and the relatively small sample size of patients analysed in the expression analysis studies. In this chapter, we addressed the issue of GATA-2 expression in human AML by performing large-scale bioinformatics analysis of public AML databases and found overexpression of *GATA2* in multiple subtypes of AML patients compared to healthy controls, including normal karyotype. Although stronger evidence supports the (over)expression of *GATA2* driving poor prognosis in AML, further characterisation of the role of *GATA2* in AML is required. To this end, we also explored the requirement of GATA2 in human AML by RNAi mediated and pharmacological targeting, and found that targeting *GATA2* induces apoptosis and potentially differentiation, in a variety of human leukaemia cell lines.

When considering the role of GATA2 expression in AML, it is essential to distinguish between its involvement in leukaemia initiation versus its role in leukaemia progression. Both up and downregulation of *GATA2* levels appear to play distinct roles at different stages of AML, a hypothesis reinforced by the fact that studies in normal HSCs have highlighted that both up and downregulation of *Gata2* affect haematopoiesis in a very distinctive manner (de Pater *et al.* 2013; Persons *et al.* 1999). This idea is further reinforced by a report that used sequential analysis to show that the original *GATA2* loss of function mutation might be lost during disease progression, while patients without such *GATA2* mutations can acquire them after relapse (Hou *et al.* 2015). Importantly, patients that acquire *GATA2* mutations during disease progression have better prognosis (Hou *et al.* 2015; Greif *et al.* 2012; Fasan *et al.* 2012). Conversely, *GATA2* expression driving aggressiveness and metastasis in established CSCs has already been documented in different cancer types (Kumar *et*

al. 2012; Vidal *et al.* 2015; Yilmaz *et al.* 2006; Wang *et al.* 2015; Xu *et al.* 2016). Therefore, we posit that lower *GATA2* levels in normal HSC/HPCs leads to leukaemia/MDS transformation, while upregulation of *GATA2* during disease progression confers aggressiveness to established CSCs (LSCs).

Knockdown of *GATA2* on established human THP1 *MLL-AF9* AML cells dramatically reduced proliferative and clonogenic potential due to apoptosis and differentiation induction. *GATA2* has been demonstrated to activate anti-apoptotic genes of the Bcl family (Rodrigues *et al.* 2005) and inhibit *PU.1*, a key myeloid differentiation TF (Walsh *et al.* 2002), which could explain the phenotype observed after *GATA2* KD in THP1 cells. We also found *P21* upregulation after *GATA2* KD, which has been previously linked with *GATA2* in controlling HSC fate (Tipping *et al.* 2009). Interestingly, *P21* is able to link all the effects observed in the context of *MLL-AF9* leukaemia. A study led by Santos MA *et al*., described that upon induction of DNA damage in murine *Mll-af9* leukaemic cells, *p21* is responsible to exit the cell cycle and induce differentiation of leukaemic cells (Santos *et al.* 2014). It is possible that *GATA2* KD follows a similar mechanism in THP1 cells, yet we found no DNA damage induction in our experiments. One possible explanation is that *P53*-mediated activation of *P21*. Co-deletion of *p53* rescued the differentiation potential in liquid culture and colony assays of YS cells from *Gata2*$^{-/-}$ mice (Tsai and Orkin 1997). Despite that we found no changes in mRNA expression of *P53* in our experiments, it is still possible that active *P53* protein levels could be increased, as reported previously (Harris and Levine 2005). Alternatively, the *TFGβ* pathway has been shown to activate *P21* expression through *SMAD3* and *SMAD4* (Ijichi *et al.* 2004). *GATA2* is known to interact with *SMAD4* and inhibit its DNA binding activity (Dong *et al.* 2014), suggesting that *GATA2* KD could lead to *SMAD4* upregulation and therefore *P21* expression. Further experiments decreasing *P21* expression would shed light on the phenotype observed.

In this study, attenuation of GATA2 appears to induce differentiation though some variability was observed. Our knockdown approach demonstrates enhanced CD14 and CD11b expression, however more replicates are needed for shRNA-34. In addition, preliminary experiments inhibiting *GATA2* using a small molecule inhibitor, demonstrated a slight trend towards an increase of CD14 in THP1 and CD71

expression in K562 cells, suggesting that inhibiting *GATA2* could release the characteristic differentiation block of AML cells (data not shown). Thus, these data highlight a promising role for *GATA2* as a novel differentiation agent. To further support this notion, in initial experiments we observed that some shRNA-32 THP1 cells, but not scramble THP1 cells, changed morphology to fibroblast-like cells, reminiscent of macrophages (**Figure 5.11B**). Indeed, *GATA2* KD THP1 cells resembled macrophages similar to those observed in previously published differentiation protocols using THP1 lines (Santos *et al.* 2014). Immunophenotypic analysis showed an approximate 6-fold increase of CD14 expression in shRNA-32 compared to scramble THP1 cells (**Figure 5.11C**). Strikingly, when we looked at the CFC colonies at day 12, we observed macrophage-like clusters in the shRNA-32 plates (**Figure 5.11D**). Positive GFP expression in these macrophage-like cells argues against cross-contamination from other cell lines (**Figure 5.11D**). Unfortunately, we were not able to recapitulate this remarkable morphological transformation with the protocol that was ultimately used in the knockdown experiments presented in this chapter. The initial experiments were performed on transduced THP1 cells, which were expanded and then frozen. The lack of morphological differentiation changes in the experiments presented in this chapter in comparison to this initial experiment could be due the stress suffered during the freeze/thawing procedures, and/or for the longer period that THP1 cells were in culture after knockdown. Long term monitoring of transduced THP1 cells in culture will help to elucidate this result.

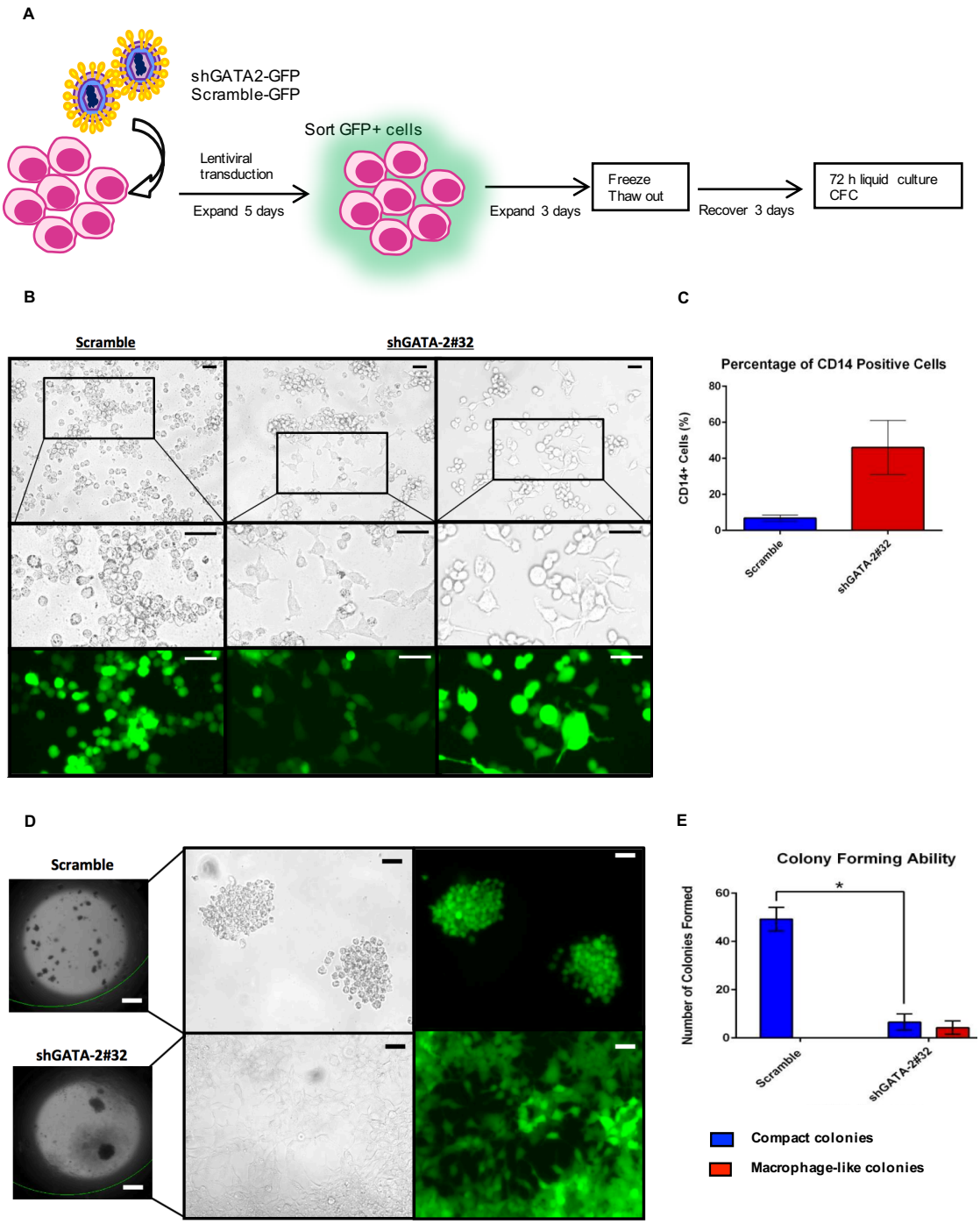


Figure 5.11. *GATA2* KD induces a macrophage-like phenotype in THP1 cells. (A) THP1 cells were transduced with lentiviruses encoding a short hairpin against human GATA2 and a GFP reporter. GFP⁺ cells were FAC-sorted, expanded for 3 days in culture and froze down. Thawed THP1 transduced cells were recovered for 3 additional days before plating into liquid culture or CFC assays. (B) Representative microscope pictures of THP1 shGATA2-32 and scramble control at 72 hour time point in liquid culture (scale bars 10 µm). (C) Frequency of CD14+ cells at 72 hour time point in liquid culture (n = 2). (D) Representative microscopic pictures of colonies at day 12 of CFC (scale bars 100 µm for left panel, and 10 µm for right panel). (E) Number of colonies at day 12 (n = 4). "n" indicates the number of independent experiments. Data are mean ± SEM. Statistical analysis: Mann-Whitney test.

A complementary GATA2 targeting approach used here employed K-7174, a small *GATA2* inhibitor, that has proven to be very effective in multiple leukaemic cell lines. K-7174 was originally designed as a *GATA* specific inhibitor (Majik *et al.* 2012), therefore it can also target other members of the *GATA* family. It would therefore be of interest to assess the impact of *GATA1* inhibition in these leukaemic cell lines. *GATA3* and more recently *GATA4* have been associated in lymphoid, but not myeloid, leukaemia (Han *et al.* 2017). In addition, K-7174 is able to target the proteasome by a unique mechanism to that of the well-known proteasome inhibitor bortezomib, since K-7174 is able to kill bortezomib-resistant myeloma cells (Kikuchi *et al.* 2013). Future studies in our lab will address the development of new and more specific *GATA2* inhibitors in the context of AML.

Transient, pharmacological inhibition of GATA2 used in collaboration with standard chemotherapeutics may prove to be a novel therapeutic strategy in AML. A recent study already showed that treatment of KG1 cells with a wide range chemotherapeutics increased the expression of *GATA2,* whilst also increasing expression of the $CD34^{+}$ population (Yang *et al.* 2017). They also showed that the expression of *GATA2* was higher in AML patients 3 months after chemotherapy when compared to levels prior to chemotherapy (Yang *et al.* 2017). Finally, they showed that K-7174 in combination with chemotherapeutics were able to inhibit *GATA2* expression in AML samples, yet they did not formally test the functional impact of K-7174 treatment in AML samples or KG1 cells (Yang *et al.* 2017). Taken together, if *GATA2* expression is increased after chemotherapy treatment, it would provide a plausible explanation as to why K-7174 enhances the killing activity of VP16 and Ara-C in our study. Future studies using human AML patient samples should assess the efficacy of K-7174 alone and in combination with chemotherapeutics to target leukaemia cells, and in particular LSCs. Inducing cell death and/or differentiation of LSCs are attractive targets for therapy, as they would eliminate the source of leukaemia cell growth/accumulation.

Given that *GATA2* is essential for adult HSCs/HPCs (see Section 3.3), toxicity in the normal haematopoietic system needs to be taken into account when considering pharmacologically targeting the GATA2 axis in AML patients. K-7174 has been tested *in vivo* in the context of multiple myeloma (Kikuchi *et al.* 2013). The authors injected

human multiple myeloma lines into NOD/SCID mice and treated them with K-7174. Some weight loss was reported at higher doses of K-7174, yet lower doses were well-tolerated and able to reduce tumour growth without affecting the weight or white blood cell (WBC) count of the mice during the 28-day experiment (Kikuchi *et al.* 2013). Promisingly, K-7174 has been shown to rescue anaemia *in vivo* (Imagawa *et al.* 2003), which would ameliorate the anaemic status of AML patients (De Kouchkovsky and Abdul-Hay 2016). However, future experiments will need to directly assess the toxicity of K-7174 in human haematopoietic cells, specifically HSC/HPCs.

Bearing in mind that GATA2 is widely expressed across different AML subtypes and that overexpression has been documented in up to 40% and 65% of adult and paediatric AML patients (Vicente *et al.* 2012; Luesink *et al.* 2012), we propose that GATA2 or GATA2 related pathways are a promising, generic therapeutic target in AML. We observed that K-7174 was able to target not only high expressing *GATA2* leukaemic cells such as K562, KG1, and THP1, but also low or normal expressing *GATA2* leukaemic cells like NOMO1. Interestingly, the killing activity of K-7174 in the high expressing *GATA2* cells was present at the 24-hour time point, while NOMO1 cells showed no clear effect until the 72-hour time point, indicating oncogenic addiction to *GATA2* in leukaemia cells. This therefore suggests that *GATA2* inhibition could be a valid therapeutic strategy for both high expressing *GATA2* AML patients, and low or normal *GATA2* expressing AML patients.

Thus, to conclude, GATA-2 is required to regulate key aspects of leukaemic cell fate in human AML. To date, the characteristic differentiation block observed in AML has been successfully targeted therapeutically in a subtype of AML, acute promyelocytic leukaemia (APL), where a combination of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) has achieved cure in up to 80% in APL patients (Long *et al.* 2014). However, attempts to reproduce this induction of differentiation across the broad, genetically and clinically diverse AML subtypes has met with limited success (Ryningen *et al.* 2008; Johnson and Redner 2015). Recently, David Scadden's lab has developed a DHODH inhibitor that overcomes the differentiation blockade in preclinical models of AML (Sykes *et al.* 2016). Thus, a better understanding of GATA-2 mediated mechanisms regulating the cell fate, including the differentiation block,

could assist with new therapeutic approaches to broadly improve AML patient outcome in this otherwise poor prognosis disease.

Chapter 6

Discussion

In this three-year thesis, using different genetic or pharmacologic approaches to alter *GATA2* levels, we have studied the role of *GATA2* in normal and malignant haematopoiesis. This work has demonstrated the critical role of *Gata2* in the maintenance of mouse HSCs, in the delay of leukaemia progression in a mouse model of *Meis1a/Hoxa9* AML driven by pre-LSCs/LSCs, and the potential for *GATA2* pharmacological inhibition in human AML, which induces cell death in concert with standard AML chemotherapeutics. This sets the basis for future work aiming to identify downstream molecular mechanisms mediating *Gata2* in normal HSCs and leukaemia stem cells.

6.1. *Gata2* is a critical regulator of adult HSCs.

In this thesis, it is shown that *Gata2* is a critical regulator of adult HSC maintenance, consistent with previous reports that pinpointed a role for *Gata2* in foetal and adult HSC regulation (de Pater *et al.* 2013). Acute deletion of *Gata2* leads to a rapid loss of HSCs in a cell autonomous fashion, suggesting a survival defect in *Gata2* deficient HSCs. This phenotype is reminiscent of the one observed in *Cited2* (Kranc *et al.* 2009)*, Mcl1* (Opferman 2005)*, Etv6* (Hock *et al.* 2004)*, Nkap* (Pajerowski *et al.* 2010)*,* and *Notchless* (Le Bouteiller *et al.* 2013) deficient HSCs using the same genetic deletion approach. Thus, *Gata2* joins a select group of TFs that are essential for adult HSCs. In studies where the mechanism was interrogated (in *Cited2, Nkap, Notchless*), a common pathway implicating the activation of the *p53-p21* axis and *Ink4/Arf* leads to programmed cell death, or apoptosis, of HSCs (**Figure 6.1A**) (Kranc *et al.* 2009; Pajerowski *et al.* 2010; Le Bouteiller *et al.* 2013). With this in mind, future studies will be directed at identifying *Gata2* target genes in HSCs. Several strategies can be used to advance these aims - *in vitro* deletion of *Gata2* in HSCs (or LSK cells) employing IFNα stimulation, followed by assessment of apoptosis and expression analysis of candidate genes (*p21, p53, Bcl2 family*); *in vivo* deletion of *Gata2* in HSCs using a single dose of pIpC and analysis four days later; genetic rescue experiments by crossing *Gata2* floxed mice with potential candidate genes (i.e. *p53* or *Ink4/Arf* floxed mice).

Our preliminary analysis of the impact of *Gata2* on the haematopoietic niche suggests that *Gata2* deletion in the BM niche has no impact on survival or multilineage maintenance. As the function of *Gata2* in the niche remains ill-defined, efforts will be directed at comprehensively elucidating the role of *Gata2* in the BM niche in different contexts (steady-state, regeneration after HSC transplant, homing, self-renewal of HSCs post contact with a *Gata2*-deficient niche) and other cell types which *Gata2* regulates in different settings, specifically endothelial (*Vec-CreERT*2 or *Tie2-Cre*), and adipocytes (*Adipoq-Cre*) (Tsai *et al.* 2005; Kamata *et al.* 2014; Kawana *et al.* 1995). Given that niche interactions with normal haematopoietic cells appear to be deregulated in the context of AML or that a mutant niche can induce haematopoietic malignancy (Dong *et al.* 2016; Walkley *et al.* 2007; Calvi *et al.* 2003), study of the *Gata2*-deficient niche in the initiation and progression of AML merits further investigation.

6.2. Modelling *GATA2* deficiency syndromes using mouse models

Since the discovery of *GATA2* mutations in hereditary AML in 2011, over 100 different mutations in the *GATA2* gene have been described in more than 50 publications. Importantly, 90% of patients harbouring *GATA2* germline mutations will develop high risk MDS/AML moreover, 30-50% of these patients are at the MDS/AML stage of disease at diagnosis (Dickinson *et al.* 2014; Collin *et al.* 2015; Hyde and Liu 2011). Therefore, a better understanding of *GATA2* syndromes biology is an area of unmet research and clinical need.

Gata2 germline mutations occur in all somatic cells. Therefore, the initiation of *GATA2* deficiency syndromes may be determined by either the niche or cell-autonomously, or through a combination of both mechanisms. Germline deletion of a single *Gata2* allele in mice results in reduced foetal and adult HSCs numbers and functionality (Ling *et al.* 2004; Rodrigues *et al.* 2005), in agreement with our short and long term Mx1-Cre-mediated *Gata2* haploinsufficiency in adult HSC and the BM microenvironment. Interestingly, unpublished observations from a collaborator at Edinburgh University found that haematopoietic *Gata2*-deletion after formation of HSCs in the embryo using the Vav-iCre system has no impact on HSC function. Characterising mouse models that faithfully recapitulate *GATA2* syndromes from a pre-leukaemic state (i.e. *Gata2*

haploinsufficiency alone) to MDS/AML (i.e. by addition of secondary mutation frequently observed in *GATA2*-MDS/AML such *Asxl1*, *Flt3*^{ITD} or chromosome 7 deletion) will offer a tractable platform to screen and test specific inhibitors or treatment combinations to benefit *GATA2* syndrome patients, and as a preventive therapy to non-symptomatic family members carrying the *GATA2* mutation that are in a pre-leukaemic phase. Finally, information obtained from a well-defined stem cell AML progression model will expand our knowledge of leukaemia stem cell biology in this setting, and in particular of pre-LSCs intrinsic functions with regard to chemotherapy resistance (observed in these patients) and clonal evolution of the disease.

6.3. *Gata2* delays LSC development in the *Meis1a/Hoxa9*-mediated AML model but is dispensable for AML driven by the *Mll-af9* translocation.

We employed a step-wise model of AML, driven by *Mll-af9* or its downstream targets, *Meis1a/Hoxa9*, that permitted testing of the requirement for *Gata2* in LSC development and maintenance. A delay in the onset of AML upon acute *Gata2* deletion in recipients transplanted with *Meis1a/Hoxa9* Pre-LSCs was observed, while the development of AML in the *Mll-af9* model appears to be *Gata2*-independent. However, the results gained from these models was complex and contradictory in some respects; namely, *Gata2* levels in pre-LSCs and LSCs were severely depleted in both models, not all the recipients induced for *Gata2* deletion showed complete *Gata2* deletion when they succumbed to AML, and a disparity in *Gata2* function was observed in two closely related AML models.

As outlined in Chapter 4 discussion (see **section 4.6**), the working hypothesis is that deletion of *Gata2* triggers apoptosis of *Gata2*-dependent *Meis1a/Hoxa9* LSCs. Future work will quantify the levels of *Gata2* mRNA in *Meis1a/Hoxa9* (and *Mll-af9*) pre-LSCs/LSCs using droplet digital PCR (ddPCR) technology that is capable of quantifying absolute numbers of low-abundance and rare mRNA (Hindson *et al.* 2013; Hindson *et al.* 2011). Our peripheral blood (PB) analysis of *Meis1a/Hoxa9* leukaemia cells just after pIpC induction indicates a massive drop of *Gata2* KO leukaemia cells suggestive of a survival defect, and either complete or incomplete deletion was observed in the BM of moribund mice, eliminating the idea of a selective pressure phenomenon. We postulate that a subset of *Gata2*-dependent *Meis1a/Hoxa9* pre-

LSCs/LSCs undergo apoptosis, delaying the onset of AML development. This would explain that even when there is incomplete deletion of *Gata2* there is a delay in the onset of AML, and why complete *Gata2* deletion does not prevent AML development; ultimately *Gata2*-independent *Meis1a/Hoxa9* pre-LSCs/LSCs are then capable of driving disease progression (**Figure 6.1B**).

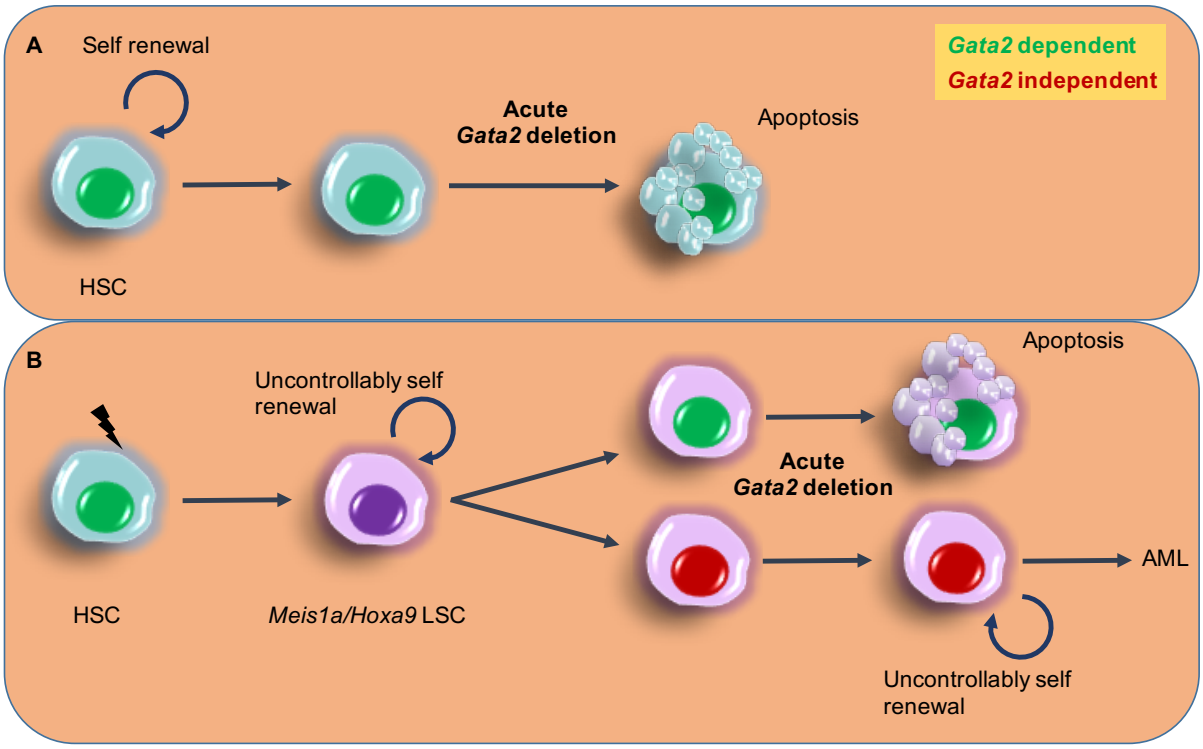


Figure 6.1. Working hypothesis for *Gata2* deletion effects in HSCs and *Meis1a/Hoxa9* LSCs. (A) Adult BM HSCs express high levels of *Gata2*. Acute *Gata2* deletion with the *Mx1-Cre* system produces a rapid HSC loss, consistent with a survival defect. (B) Introduction of *Meis1a/Hoxa9* oncogenes in HSCs initiates the leukaemia transformation alongside a reduction in *Gata2* levels. Upon acute *Gata2* deletion with the *Mx1-Cre* system, *Gata2*-dependent *Meis1a/Hoxa9* LSCs quickly disappear suggesting a survival defect. However, *Gata2*-independent *Meis1a/Hoxa9* LSCs are insensitive to *Gata2* deletion and are able to drive AML progression.

Human AML studies suggest that the standard or normal HSC hierarchy cannot entirely capture the clonal complexity that characterise LSCs (Levine *et al.* 2015; Alexandrov *et al.* 2013). Consistent with this, Stuart Orkin's (Guo *et al.* 2013) laboratory found clonal variability within the "classical" GMP-like LSC population described in these AML models (Krivtsov *et al.* 2006). Single cell clustering of *Mll-af9* GMP-like cells showed no correlation with the surface-marker hierarchy of these cells, and for instance, $CD24^{+}$ GMP-like LSCs developed AML slower than $CD24^{-}$ GMP-like LSCs (Guo *et al.* 2013). Use of recently described *Gata2*-GFP knock-in mice (Kaimakis *et al.* 2016) to monitor *Gata2* expression during the leukaemogenic

transformation, to test the LSC activity in *Gata2*$^{+}$ and *Gata2*$^{-}$ *Meis1a/Hoxa9* pre-LSC/LSCs would allow validation of this hypothesis.

The dichotomy between the results observed in the *Meis1a/Hoxa9* versus the *Mll-af9* model could be explained by the cell of origin used in this study. While *Meis1a/Hoxa9* is able to transform LSK cells, the *Mll-af9* can transform LSK, CMP and GMP progenitor cells (Krivtsov *et al.* 2013). We used a heterogeneous HSPCs (c-kit^{+}) population as starting material indicating that we do not have control over the origin of which clone is selected through the serial replating assays. This could be affecting the results observed in *Mll-af9*-mediated AML since it has been shown that LSK or GMP derived LSC behave differentially *in vivo* and have distinctive expression profiles. In addition, specific deletion of some genes (i.e. β-catenin) show differential cell of origin and oncogene-dependent requirement (Wang *et al.* 2010; Yeung *et al.* 2010). Future work will specifically initiate the leukaemic program in specific populations to investigate the requirement for *Gata2* in specific subsets of HSPCs.

Finally, we will attempt to elucidate the molecular mechanisms downstream *Gata2* in pre-LSCs/LSCs. This can be approached using single-dose pIpC pulse experiments followed by flow cytometry and genome-wide gene expression analysis, which have been described in these AML models (Nguyen *et al.* 2011; Sykes *et al.* 2011). In addition, in order to circumvent the low efficiency of deletion with the *Mx1-Cre* model in this setting, we may employ alternative and more effective genetic deletion that has been observed using tamoxifen-dependent Cre-mediated (i.e. lentiviral vectors, or *Rosa26-CreERT2* mouse models) deletion, allowing specific deletion of *Gata2 in vitro* or *in vivo* at stage-specific time-points in the leukaemogenic assay.

6.4. Can we target *GATA2* in AML?

Targeting TF expression is an attractive option for therapy in haematopoietic malignancy, including AML, where overexpression is often observed. Yet, such an approach is also considered difficult due to the lack of specificity in targeting AML cells at both the cellular and molecular level. TFs regulate normal blood cell functionality, which could be affected directly. Moreover, specific TFs function as part of multi-

protein complexes and efforts to interfere with particular transcription factors of interest, through targeting of protein-protein or protein – DNA interactions, will likely inadvertently impact other transcription factors in a transcriptional complex in the normal haematopoietic setting. During this research, the *GATA2* inhibitor, K-7174 was able to induce apoptosis of human AML cells and murine pre-LSC cell lines. Furthermore, K-7174 targets the leukaemia initiating population (c-kit^{+}) in pre-LSC cell lines and enhances the killing activity of standard chemotherapeutics. Notably, pre-LSCs/LSCs are known to expand after treatment because of their inherent chemo-resistant characteristics (Shlush *et al.* 2014; Shlush and Mitchell 2015).Our results are in agreement with a recent study showing an increase in the stem cell marker CD34 and *GATA2* expression in treated AML patients (Yang *et al.* 2017), suggesting that pre-LSCs/LSCs might become more sensitive to *GATA2* treatment in combination or after treatment with chemotherapy. K-7174 not only targets *GATA2* but can also target other *GATA* factors and components of the proteasome (Fujiwara *et al.* 2013; Kikuchi *et al.* 2013; Majik *et al.* 2012). Proteasome inhibitors are widely used in multiple myeloma and are currently being tested in AML clinical trials (van Dijk *et al.* 2011; Larrue *et al.* 2016; Orlowski *et al.* 2002). Whereas proteasome inhibitor (Bortezomib) single treatment does not seem to improve AML survival, Bortezomib (and other proteasome inhibitors) show positive effects when combined with AML chemotherapy (Csizmar *et al.* 2017). However, proteasome-related toxic effects, such peripheral neuropathy, hypoxia and liver damage, are some of the limiting factors seen in clinical trials (Csizmar *et al.* 2017). In addition, bortezomib-mediated resistance has also been described (Csizmar *et al.* 2017). In order to prospectively assess the effects of *GATA2* blockade in AML, we are currently employing drug modelling techniques to develop *GATA2* specific small molecule inhibitors (*GATA2i*) that will be independent of targeting the proteasome. We postulate that *GATA2i* could be integrated using two different treatment strategies: combination chemotherapy with *GATA2i* followed by BMT, or inductive chemotherapy plus *GATA2i* consolidation treatment to target chemoresistant pre-LSCs or LSC.

As GATA-2 is required for normal HSC maintenance in mouse, haematopoietic-related toxicity derived from *GATA2i* in AML could induce deleterious effects in residual human HSCs or progenitor cells like MLP or GMP. With GATA2 loss of function observed in pre-leukaemic GATA2 haploinsufficiency syndromes, the risk of inducing

a pre-leukaemia state should be also considered. However, short term, transient *GATA2* inhibition may not be sufficient to generate a pre-leukaemic state, since *GATA2* syndromes harbour a germline mutation and often present with MDS/AML after a relatively long-latency. Thus, it is crucial to assess the short and long-term effects of acute-transient *GATA2* blockade in human HSPCs in xenograft mouse models.

Overexpression of a non-haematopoietic *GATA* factor, *GATA4,* has been observed in acute lymphoblastic leukaemia (ALL) (Han *et al.* 2017). Considering the redundancy, regulation, and interactions among *GATA* factors, and especially the relationship between *GATA2* and *GATA3* in many stem cell systems (MSCs (Tong *et al.* 2000; Okitsu *et al.* 2007), neural stem cells (Haugas *et al.* 2016; Tsarovina 2004), prostate (Xiao *et al.* 2016; Nguyen *et al.* 2013; Vidal *et al.* 2015)), it would be worth investigating whether *GATA2*-related AML could benefit from pleiotropic targeting or synthetic lethality of two or more *GATA* factors. Perhaps targeting another *GATA* factor that is not *GATA2* could overlap with some of the *GATA2* related pathways important for LSC, but not HSCs-intrinsic functions. Hence, measuring the expression levels and genetic interactions of *GATA* factors in AML and other *GATA* related cancers will be of interest. This analysis could be extended to normal HSCs, especially the study of a potential interaction, synergistic or antagonist functions, of *GATA2* and *GATA3,* both of which are highly expressed in HSCs.

6.5. *GATA2* in the initiation versus progression in AML.

Gene expression data from retroviral mouse models and *GATA2* syndromes patients suggest that *GATA2* downregulation is a recurrent, necessary event for disease initiation. *GATA2* knockdown experiments in human HSPCs are not yet reported, however we can infer the role of *GATA2* in human HSCs from immunophenotypic analysis of BM progenitor and stem cells performed in *GATA2* syndrome patients with immunodeficiency (DCML, MonoMAC or Emberger's syndromes). The MLP and GMP population are absent in immunodeficient patients, whereas reduced HSCs and MPP population are still present, suggesting that *GATA2* might be more relevant for MLP progenitors, which would be the closest in function to murine LMPPs (Doulatov *et al.* 2012; Kondo *et al.* 1997), in human haematopoiesis. A role for *GATA2* in MLPs is clear

owing to the fact MLPs give rise to DCs, NK cells, B cells, T cells, and monocytes, the blood cell types affected in *GATA2* syndromes, while granulocytes, megakaryocytes, and erythrocytes are neither produced by MLPs nor affected in *GATA2* immunodeficient syndromes (Doulatov *et al.* 2012). In humans, LSCs have been described as MPP-like, LMPP-like, and GMP-like (Goardon *et al.* 2011; Thomas and Majeti 2017). No reports have characterised the LSCs in *GATA2* AML patients, but consistent with their preleukaemic status, it could be theorised that LSCs in these patients arise from the MPP-like compartment, although it cannot be discarded that upon acquisition of secondary mutations required for disease progression, this preleukaemic MPP could differentiate into LMPP-like or GMP-like LSCs.

Patients with AML and cell lines derived from established AML patients demonstrate a predominance of overexpression in *GATA2*, and an associated link with poorer outcome (Vicente *et al.* 2012). This association is also described in a variety of solid cancers (lung, breast, glioblastoma, prostate, colorectal) where *GATA2* expression appears to be a marker of aggressiveness. Nevertheless, it remains a possibility that *GATA2* levels are increased in leukaemic blasts but decreased in LSCs, since in these studies, *GATA2* expression was assessed in the total bulk of AML cells. Bearing in mind that the well-established CSC model, at least in AML, dictates that LSCs are responsible for AML progression and relapse, the incorporation of *GATA2* gene expression, as well as other prognostic factors, including LSC signatures in AML diagnostics, would be warranted (Ng *et al.* 2016; Jung *et al.* 2015; Richard-Carpentier and Sauvageau 2017). This will answer whether *GATA2* is a poor prognosis biomarker of AML and also address the issue of up-regulation in disease-driven LSCs.

Together, our data, combined with our analysis of GATA2 gene expression analysis in human AML (Chapter 5) and published data in *GATA2* syndromes, argue for a cooperative step-wise model (**Figure 6.2)** where *GATA2* downregulation, but not overexpression, perturbs HSCs to a clonal-preleukaemic state characterised by BM failure. Acquisition of secondary hits mediated through *GATA2* in pre-LSCs drives progression to MDS and AML. Thereafter, established LSCs (or AML blasts) that gain *GATA2* expression show a more aggressive phenotype. Therefore, it seems that there is a tipping point where *GATA2* downregulation may not be beneficial for LSCs progression. This is supported by other reports demonstrating that acquired mutations

of *GATA2* relate to better prognosis in non-*GATA2* related AMLs (Fasan *et al.* 2012; Hyde and Liu 2011; Green *et al.* 2013). Whether the proposed model involves fluctuation of *GATA2* levels as a key event during AML initiation and progression is unknown. Importantly, *GATA2*-AMLs never present with loss of heterozygosity (LOH), a recurrent event in tumour suppressor genes that is employed by cancer cells as a disease-progression mechanism. This suggests that *GATA2* expression is required for the maintenance for LSCs, at least, in *GATA2*-AMLs.

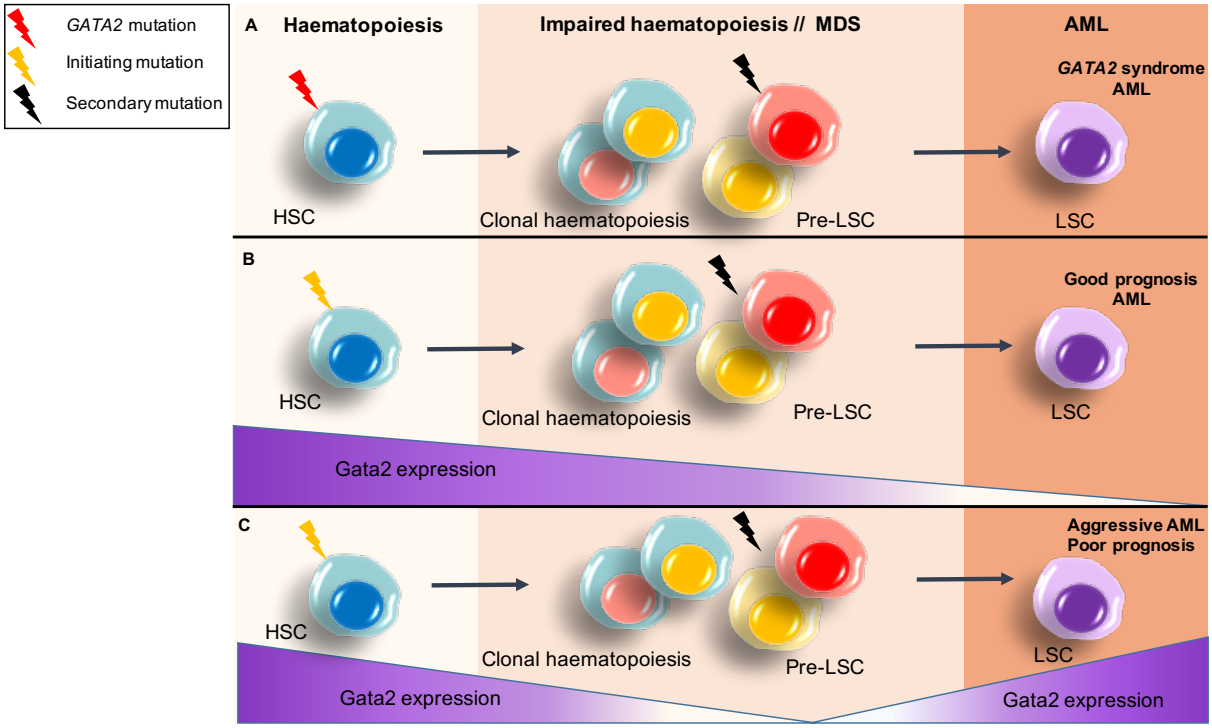


Figure 6.2. Dual requirement for *GATA2* during initiation and progression of AML. (A) *GATA2* mutations induce the formation of clonal haematopoieisis and pre-LSCs, which after acquisition of secondary mutations will become LSCs and develop AML. (B and C) Non-GATA2 initiating mutations lower *GATA2* expression required for pre-LSCs formation. (B) Secondary mutations/events that maintain low *GATA2* expression in LSCs (or AML blasts) will present with a better AML prognosis, whereas (C) secondary mutations/event (i.e. *FLT3ITD*, high *EVI1*) that overexpress *GATA2* will correlate with more aggressive and poor prognosis AML.

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