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The Role Of Jmjd6 In *HoxA9-Meis1*-Driven Acute Myeloid Leukaemia

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Abstract

Acute myeloid leukaemia is the single most common form of leukaemia in adults, with a 27% disease-free survival for five years after diagnosis. With a grim prognosis and low rates of survival, mostly due to relapse following chemotherapy-induced remission, current therapeutic approaches often prove futile and devastating in older patients.

Research indicates that genetic and epigenetic modifications come together to originate cells with an aberrant overproliferative profile and high rates of self-renewal. Such cells, termed leukaemic stem cells, are believed to evolve from haematopoietic stem cells upon dysregulation of normal haematopoiesis. Leukaemic stem cells seem to be responsible for originating the bulk of the tumour, in the form of non-functional, poorly differentiated myeloid cells that quickly accumulate. Deprived from space and nutrients, the remaining blood components become scarce, resulting in a bone marrow failure of lethal consequences.

The presented project aimed to study the role of Jmjd6 in a previously described Hoxa9-Meis1 retroviral model of acute myeloid leukaemia, by analysing its effects on survival, leukaemic cell proliferation, differentiation, apoptosis and cell cycle. BET bromodomain inhibitors I-BET 151 and (+)-JQ1 were used to investigate if Jmjd6 showed any Brd4-dependency in its mechanisms.

The results point to a role in disease initiation, by delaying disease onset. A decrease in colony forming potential in the target cells could mean that Jmjd6 acts by suppressing tumour differentiation. No evidence of a role in sustaining the disease was evident, which could mean that other factors are at play. Jmjd6 shows promise as a therapeutic target in this disease, with the potential to prevent disease initiation and, therefore, reoccurrence, which is the most frequent cause of treatment failure.

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Resumo

A leucemia mielóide aguda é a forma de leucemia mais comum em adultos, com uma taxa de sobrevivência livre de doença por cinco anos após diagnóstico de 27%. Com um prognóstico desanimador e baixas taxas de sobrevivência, maioritariamente devidos a recidiva após remissão por quimioterapia, as atuais abordagens terapêuticas revelam-se frequentemente fúteis e devastadoras em pacientes de idades mais avançadas.

Estudos indicam que modificações genéticas e epigenéticas se aliam para originar células com um perfil anormal de sobreproliferação e elevadas taxas de auto-renovação. Acredita-se que estas células estaminais leucémicas se desenvolvem a partir de células estaminais hematopoiéticas aquando de desregulação dos normais mecanismos de hematopoiese. Células estaminais leucémicas parecem ser responsáveis por originar o maior volume do tumor, na forma de células mielóides não-funcionais e fracamente diferenciadas, que rapidamente se acumulam. Privados de espaço e nutrientes, os restantes componentes do sangue tornam-se escassos, resultando numa falência da medúla óssea de consequências letais.

O presente projeto teve como objetivo estudar o papel de Jmjd6 num modelo retroviral Hoxa9-Meis1 de leucemia mielóide aguda previamente descrito, analisando os seus efeitos em sobrevivência, e na proliferação, diferenciação, apoptose e ciclo celular das células leucémicas. Os inibidores de bromodomínios BET I-BET 151 e (+)-JQ1 foram utilizados para determinar se Jmjd6 mostra ter alguma dependência em relação a Brd4 nos seus mecanismos de atuação.

Os resultados indicam para um papel na iniciação da doença, ao adiar o seu início. Uma diminuição do potencial de formação de colónias nas células alvo pode significar que Jmjd6 atua por supressão de diferenciação tumoural. Nenhuma indicação de um papel na manutenção da doença foi evidente, o que pode significar que outros fatores estão envolvidos. Jmjd6 aparenta ser promissor enquanto alvo terapêutico nesta doença, com potencial para prevenir o início da doença e, assim, a sua reocorrência, a mais frequente causa de falha no tratamento.

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Definitions and Abbreviations

2OG	2-oxoglutarate
AML	Acute myeloid leukaemia
BET	Bromodomain and extraterminal
BM	Bone marrow
Brd4	Bromodomain-containing protein 4
cDNA	Complementary DNA
CC	Cell cycle
CFC	Colony forming cell
CSC	Cancer stem cell
DFS	Disease free survival
DMSO	Dimethylsufoxide
dpc	Days post-coitum
FIH	Factor-inhibiting HIF
GM-CSF	Granulocyte-macrophage colony stimulating factor
HIF	Hypoxia inducible factor
HoxA9	Homeobox A9
HSC	Haematopoietic stem cell
HSPCs	Haematopoietic stem and progenitor cells
IMDM	Iscove's Modified Dulbecco's Medium
IL-3/IL-6	Interleukin 3/6
Jmjd6	Jumonji C-domain containing protein 6
KO	Knockout
LSC	Leukaemic stem cell
Meis1	Meis homeobox 1
MPP	Multipotent progenitor
PB	Peripheral blood
PBS	Phosphate-buffered saline
PRMT	Protein arginine methyltransferases
RNAi	RNA interference
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
SCF	Stem cell factor
WT	Wild type

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Chapter 1

Introduction

The haematopoietic system is a carefully organized hierarchy, where HSCs are the apex entity [1]. These cells reside in a specialized niche in the BM, termed the haematopoietic stem cell niche [2-4]. Here, this rare population is exposed to intrinsic factors and extrinsic cues, in a tightly regulated balance that is believed to modulate cell fate decisions such as survival, self-renewal, quiescence and differentiation [3,4].

HSCs are mostly quiescent, and capable of undergoing self-renewal as well as of differentiating into all blood lineages [5]. Their self-renewal potential allows them to be subdivided into long-term self-renewing HSCs, short-term self-renewing HSCs, and MPPs [6,7]. As HSCs mature, they will lose their self-renewal potential, with long-term HSCs originating short-term HSCs, which in turn give rise to MPPs [8]. According to the cues they are exposed to, MPPs will then differentiate into common lymphoid progenitors or common myeloid progenitors, thus replenishing any necessary cell components [8]. As such, it is this tightly orchestrated balance of cell fate decisions that allows HSCs to carry out life-long haematopoiesis [4,9-11].

However, upon dysregulation of said decisions in HSCs, most significantly that of self-renewal, these cells undergo leukaemogenesis, transforming into LSCs [12]. These cells will, in turn, establish themselves in the organism through overproliferation, giving rise to a full-blown blood cancer, a disease known as leukaemia [13].

For this reason, the understanding of how HSCs undergo this transformation, and how LSCs are then maintained, could prove vital in identifying potential molecular targets for cancer treatment.

Relapse in AML: the great challenge

AML is the most common form of leukaemia in adults, with incidence sharply increasing in those aged older than 60 [14,15]. In this disorder, an aberrant haematopoiesis produces LSCs, which overproliferate and fail to adequately differentiate, giving rise to large numbers of

abnormal, non-functional myeloid precursors [8,16]. The accumulation of these cells leaves little space for the remaining blood components, which consequently reach deficient levels, resulting in BM failure [16,17].

The vast majority of symptoms appear as a consequence of this haematopoietic insufficiency [15,16]. The drop in neutrophils elicits a higher susceptibility to infections, whereas platelet-deficiency causes the patient to bruise and bleed more easily. Similarly, the lack of erythrocytes or haemoglobin result in a decreased ability of the blood to carry oxygen, leading to fatigue and shortness of breath. Overall, the symptoms lead to low quality of life, proving fatal within weeks or months of onset when no therapeutic measures are taken [16]. As a matter of fact, AML is associated with a poor prognosis, with only 27% of all patients surviving for five years or more after they are diagnosed, with older age groups (>65 years of age) facing the worst odds [14,18,19].

While efforts have been made to increase survivability, disease-free survival is still rare, as the patient landscape is marked by high rates of relapse following chemotherapy-achieved remission [20]. Indeed, treatment failure is most frequently caused by relapse, rather than primary resistance or treatment complications [16]. Research has, thus, been focused on finding new therapeutic targets for AML, but the realization that both genetic and epigenetic factors are at play in establishing the disease has presented yet another obstacle in uncovering viable therapeutic opportunities [21,22].

Motivation and Objectives

Evidence that self-renewal is a key feature of HSCs and LSCs led to investigation into its underlying mechanism, with studies suggesting that the responsible pathways are distinct for each of these entities [8,23-26]. The identification of pathways crucial for LSC self-renewal but not for HSCs could prove crucial in pinpointing potential therapeutic targets for more effective and less toxic treatments.

AML is initiated by oncogenes promoting the reprogramming of epigenetic pathways and core cellular processes such as cell-fate decisions [27, 28]. In disrupting normal haematopoiesis, these mechanisms lead the mutated cells to self-renew at extraordinary rates, thus giving them the tools to sustain and spread the disease [17].

Ultimately, the complex underlying mechanisms behind AML set this condition as a paradigm for how multiple genetic and epigenetic modifications can contribute to tumourigenesis [21,22]. This means that a deeper understanding of the molecular pathways involved in this disorder could prove useful in developing a more effective treatment for AML, while also finding applicability in a multitude of other cancers.

Recent work has sought to identify the pathways behind this leukaemic transformation, identifying Brd4 as critical for AML maintenance [29]. A member of the BET protein family, *Brd4* suppression was shown to cause cell-cycle arrest and apoptosis in leukaemia cells, and proved sufficient to produce the same effects in two human AML lines, while having little to no deleterious effect in normal cells [29,30]. Most recently, Brd4 was suggested to physically and functionally interact with JMJD6. Having already been tied to lung, breast and colon tumourigenesis, this connection sparked interest in a potential role for JMJD6 in AML [32-35].

Given the hypoxic character of the stem cell microenvironment, and its critical role in balancing cell proliferation, self-renewal and quiescence, it is believed that pathways responsible for regulating hypoxic mechanisms could be key in tackling leukaemic disorders [3,5,36-39]. Indeed, Jmjd6 is part of a family of oxygen sensing enzymes, which regulate the hypoxia-inducible factor system through the HIFα subunits, further establishing its potential role in AML [40,41].

As the "Haematopoietic stem cells and leukaemia group" in the MRC Centre for Regenerative Medicine, where this project took place, has focused on the effects of hypoxic mechanisms in leukaemia, this project was embraced with a clear objective in mind: to clarify the role of Jmjd6 in a previously characterized disease model, *HoxA9-Meis1*-driven AML, in the hopes that it will shed light on the key mechanisms behind disease initiation and maintenance [3].

To determine if Jmjd6 does is relevant in the context of AML, and if this role is connected to its theorized interaction with Brd4, the following questions must be answered:

* Does Jmjd6 show indications of playing a role in disease initiation and maintenance?
* Does Jmjd6 knockout affect cell differentiation, proliferation, apoptosis, and cell cycle progression?
* Do BET bromodomain inhibitors show a significant effect in AML, in a manner dependent on Jmjd6?

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Chapter 2

Literature Review

Genetic and epigenetic pathways in AML – an intricate web

In recent years, AML has been suggested to result from a cooperation of genetic and epigenetic factors alike, setting this condition as a paradigm for dissecting these complex mechanisms and their contribution to oncogenesis [20,21]. Particularly, finding mechanisms necessary for LSCs but not HSCs could open the path for new therapeutic approaches with a potential to prove less toxic to normal haematopoietic processes. Given that self-renewal plays a role in ensuring life-long haematopoiesis as well as in leukaemia initiation and maintenance, a search for LSC-specific pathways ensued [8]. The key initiating events in AML are known to involve the oncogene-induced reprogramming of epigenetic pathways, which will then corrupt cell-fate regulation, and in this manner induce an abnormal self-renewal that allows LSCs to sustain and spread the disease [44].

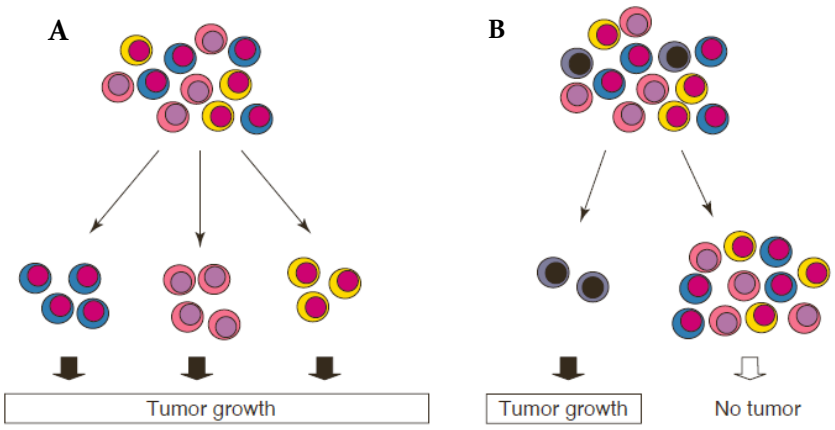

Figure 1. Schematic representation of the **A:** stochastic model; and **B:** stem cell model [12].

AML functional heterogeneity inspired the proposition of two models of tumour stem cell proliferation: the stochastic model (Fig.1A), and the stem cell model (Fig.1B) [12,45,46]. The first considers that self-renewal and differentiation are randomly carried out by single cells within the tumour population, with those randomly selected cells alone retaining self-renewal potential and thus initiating the neoplastic process [12]. The second, on the other hand,

considers that tumours are composed of multiple cell types with different biological and functional profiles, where a yet unidentified CSC is capable of both initiating and sustaining the growth of the neoplastic clone in vivo [12]. Upon placing both models to the test, it was found that it is possible to isolate the cells responsible for initiating and maintaining AML from the bulk tumour cell population, thus ruling out the first model [17,47].

It has therefore been hypothesized that leukaemia, like a variety of solid tumours, could see its beginning in a CSC [12]. In fact, HSCs and primitive progenitors have long been suspected to be the initiating cell, accumulating genetic mutations that make these cells behave abnormally, and giving rise to haematological tumours [8]. Among the strongest arguments is the fact that long-term self-renewal uniquely positions HSCs to undergo such mutations, as they divide over longer periods of time than the vast majority of cell types such as committed progenitors [8]. Indeed, HSCs and LSCs share a similar immunophenotype, with both cells expressing CD34 but not CD38 [17]. According to studies conducted in some types of solid tumours, there is indication that CSCs are at the apex of systems overcome by tumour cells, creating yet another similarity with HSCs in the haematopoietic hierarchy [1,12,43]. Furthermore, serial transplantation assays showed that there is a heterogeneous ability to repopulate secondary and tertiary recipients, suggesting that, much like normal haematopoiesis, leukaemogenesis contemplates the existence of distinct cell types with varying degrees of self-renewal potential [48]. These similarities not only further corroborate that AML key initiating events occur in stem cells rather than in more mature progenitors, but also point towards the possibility that the pathways involved in cell-fate decisions in HSCs are not completely disrupted in LSCs, still displaying certain common aspects. As such, it becomes key to understand their biology, and what pathways are common and completely separate between these two entities, if we are to potentially employ that knowledge in developing therapies capable of targeting vulnerabilities exclusive to LSCs.

A vital step in characterizing these cells is the identification of their driver mutations and resulting passenger mutations of biological significance, while also assessing their role in encoding epigenetic modifiers [49]. Large-scale analysis has suggested that these mutations can be mutually exclusive amongst each other, or assume cooperating parts in initiating the disease [50]. This creates another interesting aspect: that co-occurring mutations and respective pathways could engage in complex cross-talk, converging functionally to deregulate other downstream pathways, while mutually exclusive alterations could allow the identification of pathways directly engaged in initiating AML [49].

Uncovering Jmjd6 *potential*

Accumulating mutations lead AML leukaemic cells to become characterized by epigenetic alterations, which was the basis for the analysis of potential epigenetic vulnerabilities in AML through RNAi screening [19,51,52]. This technique allowed the identification of Brd4 as critical in sustaining the disease [19]. A member of the BET protein family, Brd4 is known to influence transcription [29]. It has previously been associated with cancer, being identified as a proto-oncogene that suffers mutations in a type of carcinoma, and that plays a role in other cancers [19,53-57]. Zuber *et al*. have shown that, in the context of AML, *Brd4* knockdown inhibited tumour growth, but did not have consequences in the growth of non-transformed erythroblast cells [19]. *Brd4* suppression also resulted in a higher rate of apoptosis, as well as in cell-cycle arrest in leukaemic cells, while in murine embryonic fibroblasts it displayed no cytotoxic effects and poor cell-cycle inhibition. In two human *MLL-AF9*$^{+}$ lines, *BRD4* inhibition was sufficient to induce cell-cycle arrest. Altogether, these results suggest a critical role for Brd4 in *MLL-AF9*-driven AML, and a promising non-toxic drug target [19].

Recently, Brd4 was suggested to physically and functionally interact with the Jmjd6, sparking interest in a potential role in AML tumourigenesis [31]. The Brd4-Jmjd6 interaction appears to be related to the P-TEFb complexes, a heterodimer made up of Cdk9 and a cyclin component [58,59]. Brd4 is able to release an inactive P-TEFb complex from its inhibitory factors by interacting with Cyclin T1, which is believed to be vital for Brd4 function [58,60-64]. The P-TEFb complex is thus activated, and Brd4 and Jmjd6 are suggested to retain P-TEFb through a physical interaction with Cyclin T1 and CDK9, respectively [31]. This link is made stronger by the evidence that Brd4 is required for the transcriptional activation of multiple genes which also require Jmjd6 for gene activation [31].

Since its identification, Jmjd6, member of the Fe(II) and 2OG-dependent oxygenase family, has been intriguing researchers [65]. While under its original name of phosphatidylserine receptor, it was wrongly thought to mediate the engulfment of apoptotic cells [66]. Jmjd6 has since been suggested to be involved in a multitude of other processes, from organ differentiation during embryogenesis, to RNA splicing [40,67]. It is possible, however, that this enzyme might play its biggest role yet revealed in tumour progression.

Thoughts of a potential involvement of Jmjd6 in tumourigenesis arose from its arginine demethylase activity on both histone and non-histone substract [31,68]. Arginine methylation, a post translational modification carried out by a family of protein arginine methyltransferases (PRMT), has been linked to tumour activity, as these enzymes are found to be overexpressed in

various cancers [69]. In fact, in breast cancer, as well as in lung and colon adenocarcinomas, a high expression of Jmjd6 appears to promote tumourigenesis by increasing proliferation, migration, and invasion [33,70]. As a consequence, Jmjd6 is being considered as a potential biomarker for tumour aggressiveness and poor prognosis. The mechanisms behind Jmjd6 effects in tumourigenesis are, however, incompletely understood: in contrast to the findings made by Lee *et al.*, where Jmjd6 knockout in breast cancer samples resulted in tumour growth inhibition, Poulard *et al.* saw Jmjd6 silencing result in increased proliferation, in vivo migration and tumour engraftment, although both groups demonstrated that Jmjd6 overexpression is associated with a poor prognosis and low disease-free survival rates [34,35]. While the different results may merely result from differences in technical approach or cell origin, this raises the need to seek a deeper understanding of the role played by Jmjd6 in tumourigenesis.

Nevertheless, Jmjd6 function seems to be associated with its demethylase activity and RNA splicing hydroxylation function, as catalytically dead mutants failed to produce the same effects [34]. It is also its enzymatic activity as a 2-OG-dependent oxygenase that seems to allow Jmjd6 to reduce HIF transcriptional activity by keeping it from binding to transcriptional co-activators CBP/p300 [33,72]. Sequence analyses suggests that Jmjd6 is homologous to the factor-inhibiting HIF, meaning that Jmjd6 could affect the tightly orchestrated cell-fate balance by disrupting the hypoxic mechanisms that appear to play a role in regulating them [40,41].

Hoxa9-Meis1-driven AML

In order to study Jmjd6 function in AML, a *Hoxa9*/*Meis1* retroviral mouse model was employed. *Hox* genes have been shown to regulate haematopoietic proliferation and differentiation, and to be expressed only in early haematopoietic progenitors [73]. While *Hox* genes appear to be critical targets in leukaemia, research seems to indicate that loss of any individual Hox protein can be balanced out by expression of other Hox proteins, under favourable conditions [74]. This has created speculation as to whether therapy targeting a single Hox protein could ever prove successful [75]. On the other hand, *Meis1* has been isolated as a common site of viral integration in certain AML cases, often containing proviral integrations that led to overexpression of Hoxa7 and Hoxa9 [42,76].

Studies have shown that *Meis1* and *Hoxa9* collaborate in leukaemogenesis: even though neither are acutely transforming on their own, co-overexpression results in a quick onset of AML [42]. More specifically, *Hoxa9* induces leukaemia, whereas *Meis1* makes it acute by dramatically accelerating disease progression [42,77]. These proto-oncogenes are, furthermore,

sufficient to induce acute leukaemic transformation [75,78]. *Hoxa9-Meis1* AML is characterized by partial myeloid differentiation, with a clear predominance of poorly differentiated cells of the myeloid lineage over functional myeloid cells [17]. In this model, leukaemic cells were 100% Mac-1$^+$ (a receptor found on macrophages, NK cells, and polymorphonuclear leukocytes) and 70% Gr-1$^+$ (a marker found in monocytes and neutrophils), but were not recognized by lymphoid mature-lineage marker antibodies such as CD4 and CD8, indicating that while these cells are of the myeloid lineage, they are not fully differentiated [42]. Organ infiltration was verified in all mice, and infiltrated spleen, BM, lymph nodes and thymus were detected at the time of sacrifice, and clonogenic progenitor assays showed that the leukaemic cells had higher plating efficiency than normal cells [42].

The drugs: I-BET 151 and (+)-JQ1

To aid in the characterization of Jmjd6 influence in AML, two distinct drugs were employed, both of which have a role as BET bromodomain inhibitors, blocking BET recruitment to chromatin [57,79]. Having previously been demonstrated to have promising results in leukaemic cell lines *in vitro*, we selected I-BET 151 dihydrochloride and (+)-JQ1 [29,79]. Because these drugs can inhibit Brd4, it is possible that Brd4 inhibition and Jmjd6 knockout will lead to a greater effect on AML, which we aim to assess.

I-BET 151 hydrochloride has shown effectiveness in tackling aggressive *MLL*-mediated leukaemias, which have been shown to directly up-regulate *Hox* expression [75,79]. Given that MLL fusion proteins led to persistent expression of *Hox* genes, which was accompanied by an up-regulation of *Meis1*, it is possible that there is a correlation with the *Hoxa9-Meis1* model being employed in this project [75,77,78,80]. By displacing Brd4 and other components from chromatin, I-BET inhibited transcription of key genes, and induced early cell cycle arrest and apoptosis in both murine and human *MLL*-fusion leukaemic cell lines, with significant efficiency [79]. Results showed that I-BET was effective in improving *in vitro* and *in vivo* survival, both in murine and in human leukaemia cell lines [79].

(+)-JQ1, similarly, is an inhibitor of BET bromodomains, with most affinity to Brd4 [29]. Very recently, JQ1 was demonstrated to have anti-tumoural properties in triple negative breast cancer, the most aggressive form of breast cancer [81]. JQ1 produced such effects by impairing tumour response to hypoxia, suppressing hypoxia-induced genes, and downregulating angiogenesis, ultimately impairing tumour growth and metastasis [81]. Zuber *et al*., in *MLL*-fusion leukaemia models, verified that sub-micromolar concentrations of JQ1, similarly to Brd4

knockdown, significantly impacted malignant cell proliferation, while showing little effect in fibroblasts and other non-transformed cells. In human leukaemic cell lines, JQ1 treatment and showed great growth-suppression activity, while triggering cell-cycle arrest and apoptosis. Regarding *in vivo* results, JQ1 administration in diseased mice caused a marked delay in disease progression, increasing survival, without compromising normal haematopoiesis. It showed similar effects in an independent mouse model known to resist conventional chemotherapy. Additionally, JQ1 treatment increased surface expression of Mac-1, a myeloid differentiation marker, and decreased expression of Kit, a marker associated with LSCs in mice models of *MLL* leukaemia, seemingly inducing morphological signs of maturation phenotypes. In all tests, *Brd4* knockdown displayed identical effects [82,83]. This concordance between the phenotypes induced by *Brd4* knockdown and JQ1 treatment suggests a critical requirement for Brd4 in leukaemia initiation and maintenance, as well as in preventing terminal myeloid differentiation, that can be effectively inhibited by JQ1 [29].

Chapter 3

Materials and Methods

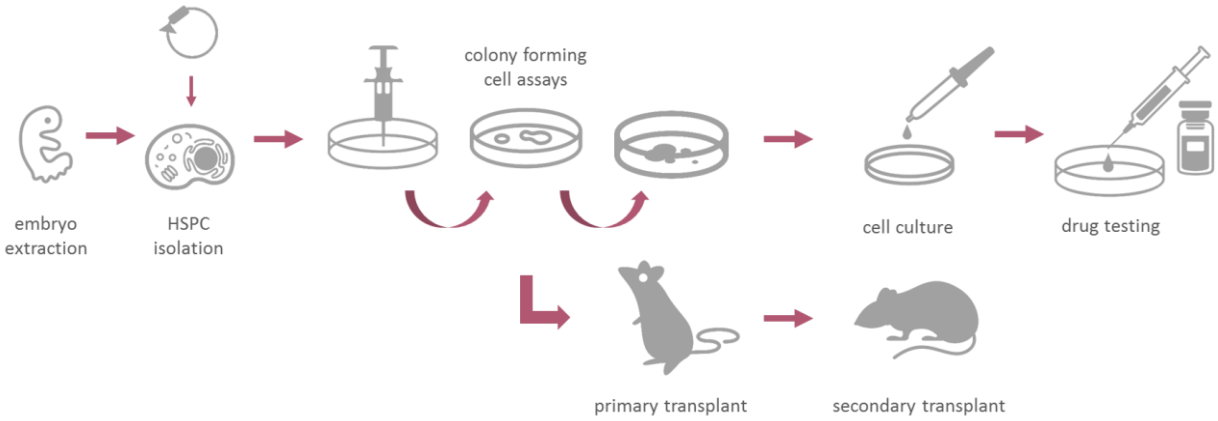

Figure 2. Schematic representation of the procedural sequence [84]. HSPCs isolated from foetal liver were retrovirally transduced with Hoxa9 and Meis1. They were then plated in methylcellulose for CFC assays, with CFC2 cells being used for primary transplant. Secondary transplant was performed using cells from sick primary recipients. CFC3 cells were placed in culture and ultimately used in drug testing.

Mouse husbandry

Mice were donated by Dr. Andreas Lengeling from the Roslin Institute, University of Edinburgh. 14.5 dpc, female mice were dissected and their embryos used for c-Kit$^+$ cell (which comprise haematopoietic stem and progenitor cells) extraction. 8- to 12-week-old mice were used for all analyses. All animal experiments were approved by the Animal Welfare and Ethical Review Body of the University of Edinburgh, and authorized by the UK Home Office.

Primary murine cells

Time-mated mice were culled and dissected to extract their uteri. The embryos were then removed from the uterus, and the foetal liver was dissected and placed in a sterile cell strainer with a 70µm nylon mesh (Fisherbrand®, Fisher Scientific, Cat. #22363548), inside a 35mm petri dish (Sterilin, Cat. #121V). The tissue was disrupted, after which 10mL PBS (Sigma-Aldrich®, Cat. #E8537) with 2%FCS were added to the extracted HSPCs.

Retroviral transduction of murine HSPCs with Meis1 and Hoxa9

In order to accomplish gene delivery, murine HSPCs were retrovirally transduced with Hoxa9 and Meis1. RetroNectin® (Takara, Cat. T100A/B), was then used to aid in this transduction. Cells expressing both viral strains were then selected for a period of three days, employing double resistance selection against antibiotics neomycin for Hoxa9 proviruses (1.0mg/mL; Sigma-Aldrich®) and puromycin for Meis1 (1.5µg/mL; Sigma-Aldrich®).

Colony forming assay

The cells selected through antibiotic resistance were counted using trypan blue viability staining (Sigma-Aldrich®, Cat. #T8154), and live cells were then plated in methylcellulose-based medium (MethoCult™ M3231, Stem Cell Technologies, Cat. #03231), supplemented with 20mL IMDM (gibco®, ThermoFisher Scientific, Cat. #12440-053), 100 I.U./mL penicillin, 100 mg/mL streptomycin, 10ng/mL GM-CSF (Biolegend, Cat. #576302), 20ng/mL SCF, 10ng/mL IL-3 (Biolegend, Cat. #575506), and 10ng/mL IL-6 (Biolegend, Cat. #575706). HSPCs transduced with Hoxa9/Meis1 were seeded at a concentration of 1.000 cells/mL, and the selection antibiotics were added in the same concentrations used in the previous section. PBS was used to fill all empty wells to minimize sample evaporation. The cells were kept in culture for five days, corresponding to CFC1, at which point colonies were scored, counted and once again plated under the same concentration and culture conditions, for CFC2. The same procedure was repeated for CFC3.

Colony analysis

Microscopy imaging was performed using an Olympus IX50 microscope (Olympus®), at 20x magnification. Images of colonies in CFC1, CFC2 and CFC3 were captured, with resource to the QCapture software (QImaging®). CFC1, CFC2 and CFC3 samples were more thoroughly analysed using the Operetta High-Content Imaging System® (Perkin Elmer Inc., Cat. #HH12000000). For this effect, one 96 well plate was assembled for each of the CFC assays. For the CFC1 plate, 800 cells of each of the six samples were plated in 100µL methylcellulose per well (for a total of 10 replicates per sample), leaving the outermost rows and columns filled only with H_2O to avoid evaporation. For CFC2 and CFC3, the number of cells per well was of 700. Five days after plating, the Operetta System was employed for the analysis of the total number of colonies per well, mean colony area, and ratio of colonies per number of cells plated.

DNA extraction

DNA was isolated from each sample using cells from CFC2. The Isolate II Genomic DNA Kit (Bioline, Cat. #BIO-52067) was employed for this procedure, following manufacturer protocol.

PCR and gel electrophoresis

The isolated genomic DNA from each sample was amplified through PCR, using TProfessional Basic Thermocycler (Biometra). For *Vav-iCre*, the program was as follows: (5 minutes at 95ºC, 40 seconds at 94ºC, 40 seconds at 64ºC, 30 seconds at 72ºC) for 30 cycles, 5 minutes at 72ºC, and 10ºC until removed. For Jmjd6: (5 minutes and 30 seconds at 95ºC, 30 seconds at 65ºC,30 seconds at 72ºC) for 30 cycles, 10 minutes at 72ºC, and 10ºC until removed.

An agarose gel (2.5%) was prepared and both the amplified genomic DNA and the controls (for *Vav-iCre*, +/+, -/-, and H_2O; for Jmjd6, +/+, +/fl, fl/fl, fl excised, and H_2O) were loaded. Gel electrophoresis was then performed to allow for the separation of DNA according to its size and charge, thus making it possible to analyse the results and determine which samples are WT and which are KO. Gel capture was performed using G:BOX EF2 (Syngene), with the assistance of GeneSys automatic control software (Syngene).

RNA extraction

RNA from each sample was extracted and purified using the RNeasy® Mini Kit (Qiagen, Cat. #74104), according to the manufacturer protocol guidelines. RNA concentration for each resulting sample was measured using the NanoDrop® 1000 spectrophotometer (ThermoFisher Scientific), which was the basis for the dilutions to normalize the concentration at 200ng/µL for all samples.

RT-PCR

RT-PCR was performed, to transcribe the RNA into cDNA through reverse transcriptase. For this effect, a master mix was prepared using 2.0µL 10x RT buffer, 0.8µL dNTP mix (100 mM), 2.0µL 10x RT random primers, 1.0µL reverse transcriptase, 0.5µL RNase inhibitor, all from the same High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™; Cat. #4368813), and 3.7µL distilled water (DNase and RNase free; gibco®, ThermoFisher Scientific, Cat. #10977-035) per sample. The samples were processed using TProfessional Basic Thermocycler, where a cDNA

synthesis program ran at 25ºC for 10 minutes, 37ºC for 120 minutes, 85ºC for 5 minutes, and 4ºC until use.

qPCR

In order to quantify Hoxa9 and Meis1 expression in each sample and, in this way, confirm that both viruses were expressed in the same proportion, qPCR analysis was employed. To ensure that the sample volume was normalized, β-actin was added to the samples as a housekeeping gene. Technical triplicates of each sample were analysed, and each well was filled with 2.5µL TaqMan® Universal PCR Master Mix (Applied Biosystems™, ThermoFisher Scientific, Cat. #4304437), 0.25µL of the TaqMan ® probe each gene - β-actin, HoxA9, and Meis1 - at a time (Applied Biosystems™ Cat. #4331182), 1.25µL distilled water, and 1µL of the cDNA sample. The samples were analysed using LightCycler® 480, programmed for 40 cycles. All signals were quantified using the ΔΔCt method, based on the number of cycles it takes for the sample to fluoresce.

Hoxa9/Meis1-driven AML mouse model

Harvesting and counting of pre-LSCs from CFC2 was carried out. 100.000 live pre-LSCs, $CD45.2^+$ and 200.000 WT $CD45.1^+$ unfractioned BM cells (which served as support) were mixed and transplanted into $CD45.1^+/CD45.2^+$ recipient mice, previously irradiated at a lethal dose of 11Gy. In order to accomplish this, the cells were stained with CD45.1 (Biolegend, Cat. #110706) for BM support cells, CD45.2 (Biolegend, Cat. #109820) for the cells of interest ($CD45.1^+/CD45.2^+$ cells correspond to the remaining haematopoietic components), followed by cell sorting.

Monitoring for disease development was performed through blood sampling through the tail vein, at a frequency of 2-6 weeks following transplantation. For the secondary transplant, 500 $CD45.2^+$c-Kit^{high} cells sorted from dying primary recipients and mixed with 200.000 WT CD45.1+ unfractioned BM cells, and injected into secondary $CD45.1^+/CD45.2^+$ recipients.

Monitoring of AML development

AML development in the mouse model was monitored through regular blood sampling (every 2-6 weeks post-transplant). Leukocytes were stained for all blood lineages in each of the samples, to allow confirmation that the condition developed by the mice was indeed AML and not another variant of blood tumour. This staining consisted of CD19 (Biolegend, Cat. #115530) for B cells, CD4/8 (Biolegend, Cat. #130310 and 100712) for T cells, CD11b/Mac-1 (Biolegend, Cat.

#101212) and Gr-1 (Biolegend, Cat. # 108416) for myeloid cells. Flow cytometry analysis of all blood samples confirmed that the mice developed AML. Mice were sacrificed as they developed symptoms, and pathophysiology was consistent with AML.

Blood smears and microscopy

Blood smears were prepared and stained using Shandon™ Kwik-Diff™ Stains (ThermoFisher Scientific, Cat. #999070), and following manufacturer protocol. Once dry, the samples were analysed and captured using a BX61 Motorized Microscope (Olympus®), with the assistance of the Volocity® software (Perkin Elmer Inc.).

Cell suspension

At the end of each CFC, the cells were placed in culture. After resuspending the cells in each well, they were washed with supplemented IMDM, plated in 2mL PBS (2% FCS), and stored in an incubator at 37ºC and 5% CO_2.

Proliferation assay

Technical duplicates were prepared by transferring 1.5mL of each cell sample to a well in a 24 well plate, at a concentration of 200.000 cells/mL. Three different plates were used, one for t=0h, another for t=24h, and yet another for t=48h. The live and dead cells in each well were counted at the indicated times, using a trypan blue viability stain.

Apoptosis assay

Cells were washed with ice-cold PBS, after which they were again washed with 500µL solution of Annexin V binding buffer 1x (BD Pharmingen™, Cat. # 556454), and then again with 1mL of the same solution. They were stained with a 100 µL solution of Annexin V buffer containing 5 µL PE Annexin V (conjugated with FITC, BD Pharmingen™, Cat. #556421), and incubated for 15 minutes, in the dark and at RT. 400µL of Annexin V buffer were then added to stop the reaction, and after centrifuging the cells for 5 minutes at 500 x g, the supernatant was discarded, and the cells resuspended in 100µL Annexin V buffer. Finally, the samples were incubated at RT for one minute in 100µL DAPI, at a final concentration of 10µg/mL, and then acquired using FACSFortessaV (BD Biosciences). Technical duplicates were used for each sample.

Cell cycle assay

The cell cycle assay was performed by staining the cell samples used for the apoptosis assay with DAPI Prep, DNA Staining Solution (5µg/mL DAPI in PBS and Nonidet P40; Sony Biotechnology Inc., Cat. #AE700570), homogenizing them through vortex, and incubating for exactly 1 minute at RT. Acquisition was then conducted using FACSFortessaV.

I-BET 151 calibration curve and IC50 determination

In order to find IC50, a calibration curve was used. A cell mix was prepared using 1mL of each sample. The cells in this mix were counted, and used to prepare the sample with a concentration of 1.000.000 cells/mL and a volume of 2.6mL. 100µL of this sample were transferred to each well of a 96 well plate, with technical duplicates for each concentration considered, and a blank sample to assess the vehicle effect associated with the DMSO (Molecular Probes®, ThermoFisher Scientific, Cat. #D12345). An I-BET 151 (Tocris, Cat. #4650) stock solution at 100mM was prepared by adding 201µL DMSO to 10mg I-BET 151. This solution was used to prepare the drug solutions at each of the considered concentrations: 0, 1, 5, 10, 20, 30, 40, 50, 100, 250, 500, and 1000µM. 100µL of the drug at each concentration were added to a set of technical duplicates, and 100µL of DMSO solutions at the same concentrations were added to the wells destined for the blank samples. IMDM was used to dilute the stock solution to achieve such drug solutions, and to dilute the DMSO to prepare the blank samples. At t=24h, the cells were counted using trypan blue viability staining, and the result indicated an IC50 of 50µM.

I-BET 151 proliferation assay

A drug solution at the calculated IC25 (so that a ratio of live/dead cells of 1:1 would only be reached at t=48h) was prepared. Using a 24 well plate, 1mL of each cell sample was added to each well, with 500.000 cells per well (and creating technical duplicates), and 1mL of the drug solution at IC25 was added to all samples. Live and dead cells were counted at t=0, 24 and 48h, using trypan blue viability staining. The cells used for apoptosis and cell cycle were taken from this plate.

(+)-JQ1 calibration curve and IC50 determination

In order to calculate IC50, a calibration curve was used. A cell mix was prepared using 1mL of each sample. The cells in this mix were counted, and used to create the sample with a

concentration of 250.000 cells/mL and a volume of 7mL. 200µL of this sample were transferred to each well of a 48 well plate, with technical duplicates for each concentration considered, and a blank sample to assess the vehicle effect associated with the DMSO. A (+)-JQ1 (Cayman Chemical, Cat. #11187) stock solution at 22mM was prepared by adding 100µL DMSO to 1mg (+)-JQ1. This solution was used to prepare the drug solutions at each of the considered concentrations: 0, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100µM. 200µL of the drug at each concentration was added to a set of technical duplicates, and 200µL of DMSO solutions at the same concentrations was added to the wells destined for the blank samples. IMDM was used to dilute the stock solution to achieve such drug solutions, and to dilute the DMSO to prepare the blank samples. At t=24h, the cells were counted using trypan blue viability staining, and the concentration at which 50% of cells were dead was determined to be of 0.25µM.

(+)-JQ1 proliferation assay

A drug solution at the calculated IC25 was prepared. Using a 24 well plate, 1mL of each cell sample was added to each well, with 1.000.000 cells per well (and creating technical duplicates), and 1mL of the drug solution at IC25 was added to all samples. Live and dead cells were counted at t=0, 24 and 48h, using trypan blue viability staining. The cells used for apoptosis and cell cycle were taken from this plate.

Data analysis

The data from the apoptosis and cell cycle assays were analysed using the FlowJo software (FlowJo, Inc.). Data from the proliferation assays, drug assays, and qPCR were analysed with GraphPad Prism (GraphPad Software, Inc.). Statistical analysis was performed with GraphPad Prism based on Mann-Whitney U tests, and P value representation is indicated as follows: not indicated for $P > 0.05$, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, and **** for $P \leq 0.0001$.

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Chapter 4

Results and Discussion

After successfully employing retroviral transcription to induce overexpression of *Hoxa9* and *Meis1* in the cells that were to be transplanted into the mice, and thus induce AML, these primary cells were incubated and selected for puromycin and neomycin resistance to purify the samples. While initially other models of AML were to be employed, failure to induce disease in mice led to this project studying only Hoxa9-Meis1-induced leukaemia. Cell samples were collected to perform genotyping through PCR:

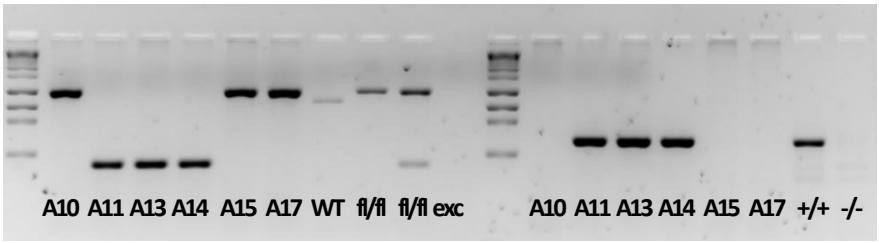

Figure 3. Capture of genotyping PCR gel using GeneSys. The first set of samples corresponds to the genotyping results for *Jmjd6*, whereas the second was prepared to assess *Vav-iCre*.

Three of the samples, A10, A15 and A17, were Jmjd6$^{fl/fl}$;*Vav-iCre*$^{-}$, representing the WTs, whereas A11, A13 and A14 were Jmjd6$^{fl/fl}$;*Vav-iCre*$^{+}$, corresponding to the KOs (Fig.3). To confirm that the induced disorder was AML and not another type of blood malignancy, immunophenotyping at CFC1 was performed, and results showed a clear predominance of cells displaying a myeloid phenotype (CD11b^{+}/Gr-1^{+}) over only vestigial amounts of B (CD19^{+}) and T (CD4^{+}/CD8^{+}) cells (Fig.4), consistent with what is observed in AML *in vivo*.

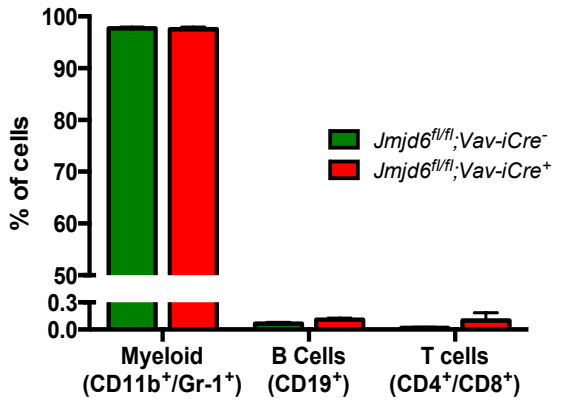

Figure 4. Abundance of each cell type in CFC1 cells, as assessed through immunophenotyping.

Immunophenotyping characterization of CFC2, the transplanted cells, showed that the percentage of myeloid cells and myeloid/c-Kit$^+$ cells are very similar (Fig.5), ensuring that their predominance will not lead to biased results, and that the genotype will be the source of any observed effects.

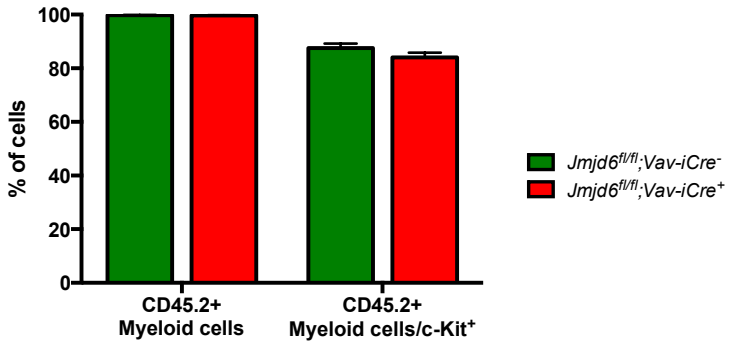

Figure 5. Immunophenotyping results for CFC2 cells.

To assess the colony forming capacity displayed by KO and WT cells alike, a total of three CFC assays were then performed. A plate for each CFC assay was analysed using Operetta, with ten replicates for each biological sample, and a statistically significant increase in the number of colonies formed per number of cells plated was observed in KO samples, in comparison with the WT (Fig.6). These results show that Jmjd6 decreases leukaemic cell colony forming potential, and thus its ability to undergo leukaemic transformation, once again suggesting an anti-tumoural role.

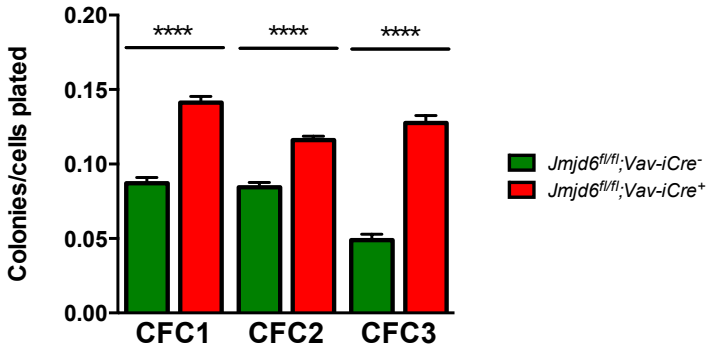

Figure 6. Colonies formed per cells plated for CFC1, CFC2 and CFC3.

These colonies were also observed under the microscope, which allowed to verify an increased transformation and selection from one CFC to another (Fig.7), with cells forming increasingly compact colonies. Upon the end of CFC3, cell suspensions were prepared and plated.

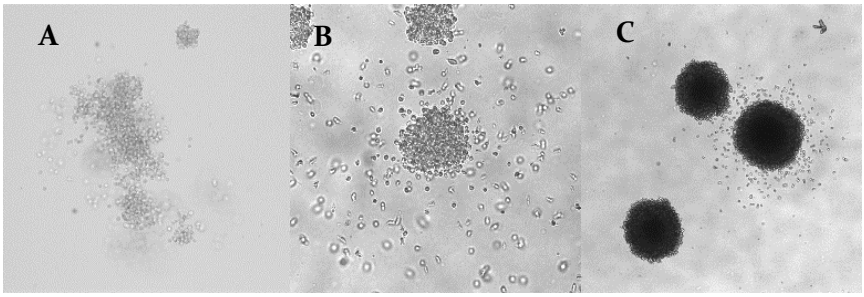

Figure 7. Examples of transformed colonies at 40x magnification in **A:** CFC1; **B:** CFC2; **C:** CFC3.

A qPCR analysis, applying the ΔΔCt method, showed that both WT and KO cells were efficiently transfected, displaying near identical expression levels of Hoxa9 and Meis1 (Fig.8), and in turn ensuring that any effects will be caused by the genotype, and not by unbalanced expression.

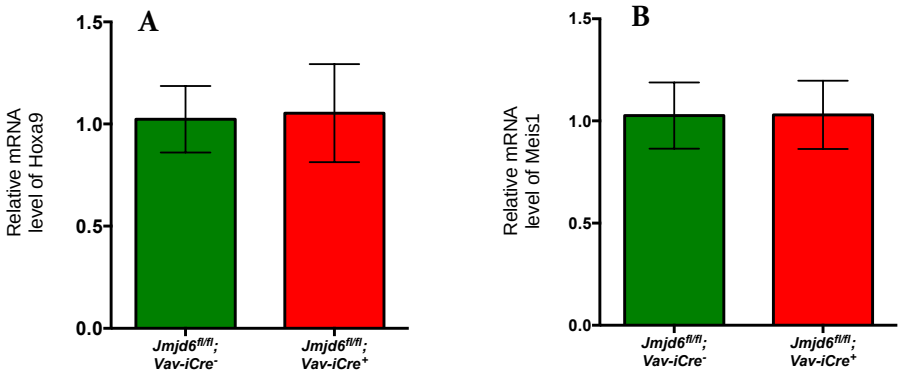

Figure 8. Relative mRNA expression vs. β-actin, in WT and KO cells, of A: Hoxa9; and B: Meis1.

Blood sampling after transplant, on the other hand, showed that the transplant of CD45.2^{+} pre-LSCs was effective (Fig.9A), as they were found in high percentages in peripheral blood, with a successful engraftment being shown through the increasing percentage as weeks passed after transplant. In addition, there was a significant difference in the engraftment between WT and KO cells, with the latter proving more effective, suggesting that Jmjd6 could be implicated in delaying leukaemogenesis in the organism. A great number of the transplanted cells that were engrafted appear to be myeloid (Fig.9B), expressing Mac-1 and Gr-1, markers of myeloid cells, which is crucial in establishing AML. In KO cells, this percentage suffers a decrease as time goes by, which is caused by the death of the sickest mice as translated in the Kaplan-Meyer curves that will be presented further on. Values for KO and WT cells, however, show no significant differences, suggesting that Jmjd6 could have a more direct effect in pre-LSCs than in the poorly differentiated myeloid blasts, and therefore a less relevant role in disease maintenance.

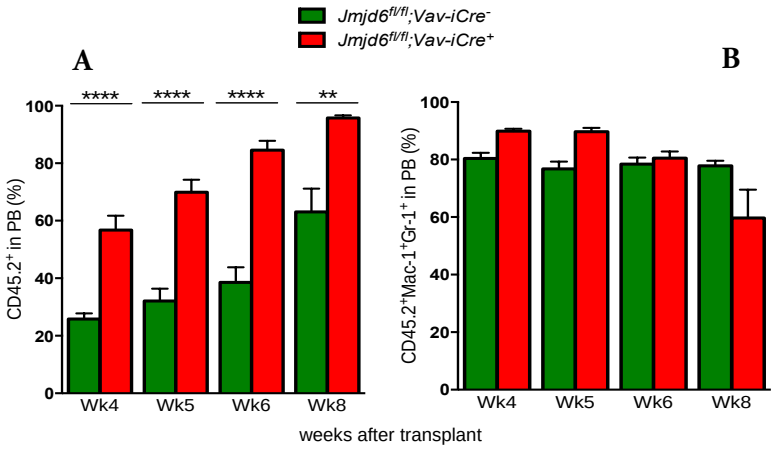

Figure 9. Blood sampling in weeks succeeding transplant. **A**: CD45.2$^+$ cells (%); **B:** CD45.2$^+$, Mac-1$^+$; Gr-1$^+$ cells (%) in PB.

Microscopy analysis of blood smears revealed that there was indeed an accumulation of monocytes in the blood (Fig.10A) in comparison with a what is observed in normal haematopoiesis (Fig.10B), signalling the onset of AML. Similarly, as the mice fell ill, they displayed symptoms such as anaemia (resulting in visibly whiter paws), weight loss, shortness of breath, and fatigue (leading to less active animals). At this stage, the mice showed a high percentage of LSCs in the blood, and dissection allowed to observe organ infiltration, which translated into an enlarged spleen and lymph nodes, consistent with AML pathophysiology.

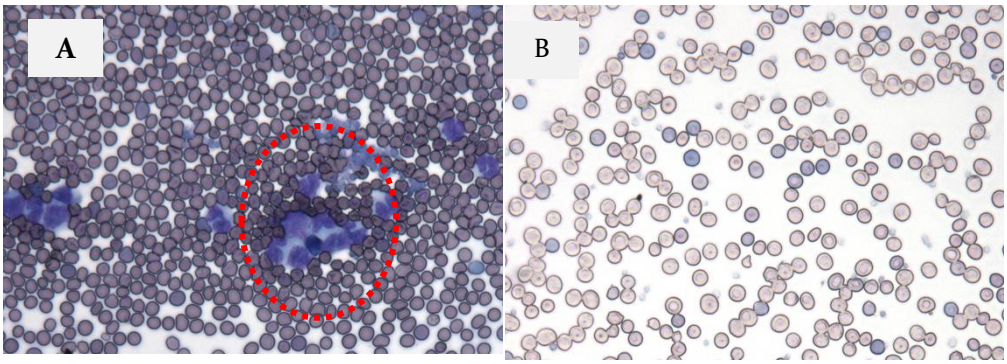

Figure 10. Blood smears at 20x magnification from **A:** leukaemic blood; and **B:** healthy blood.

In accompanying the progress of the mice recipients of the primary transplant (Fig.11A) and of the secondary transplant (Fig.11B), it was possible to determine that mice transplanted with KO cells succumbed to AML faster than those transplanted with WT cells. This implicates Jmjd6 as a tumour suppressor, playing a relevant role in disease initiation by delaying. On the contrary, secondary transplant showed no significant differences between WT and KO recipients, meaning that Jmjd6 seems to be dispensable in AML maintenance.

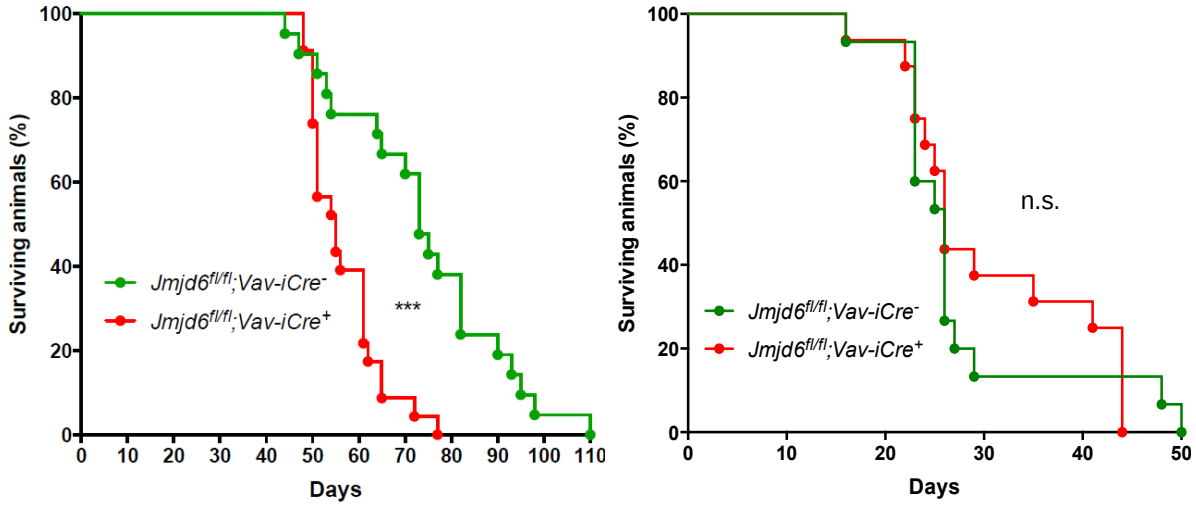

Figure 11. Kaplan-Meier curves of mice survival percentage over time. **A**: Recipients of primary transplant; **B:** Recipients of secondary transplant. Two independent experiments were conducted for each transplant.

Next, we assessed proliferation *in vitro*, counting the live and dead cells at t=0, 24 and 48 hours (Fig.12), to determine if there would be a relevant difference between the proliferative capacity of WTs and KOs. A slight decrease in proliferation was observed for KO cells, although not statistically significant, suggesting that Jmjd6 might not directly regulate leukaemic cell proliferation.

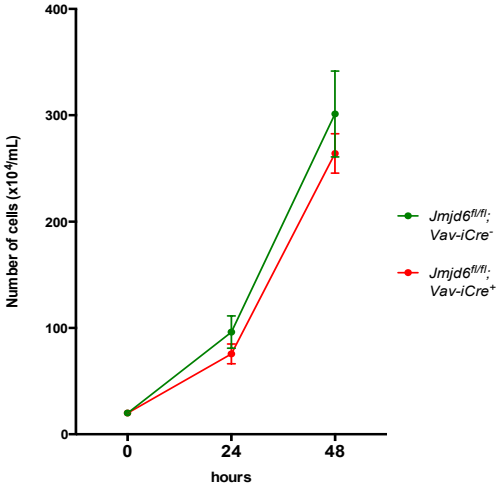

Figure 12. Cell proliferation in WT and KO samples in cell suspension.

Apoptosis and cell cycle were then analysed, with no biologically significant differences across the map (Fig.13, 14) Overall, KO cells displayed a similar behavioural pattern with WT cells. In sum, these results suggest that Jmjd6 on its own lacks an effect in AML maintenance, suggesting that other factors are play.

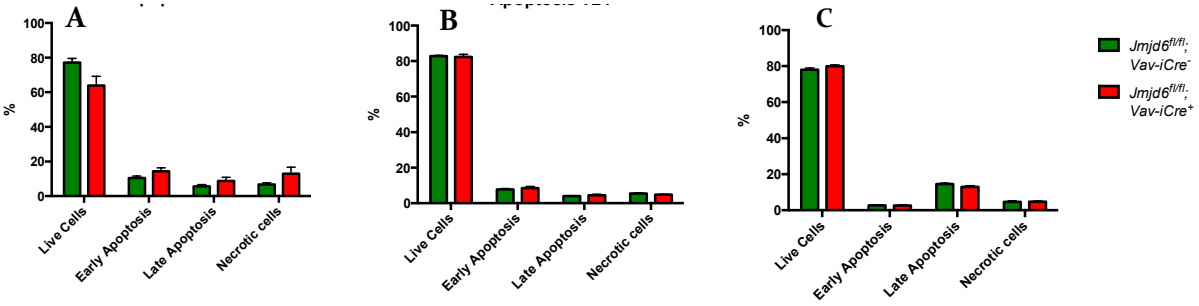

Figure 13. Apoptosis assay results in cell suspension at **A:** t=0; **B:** t=24; and **C:** t=48 hours.

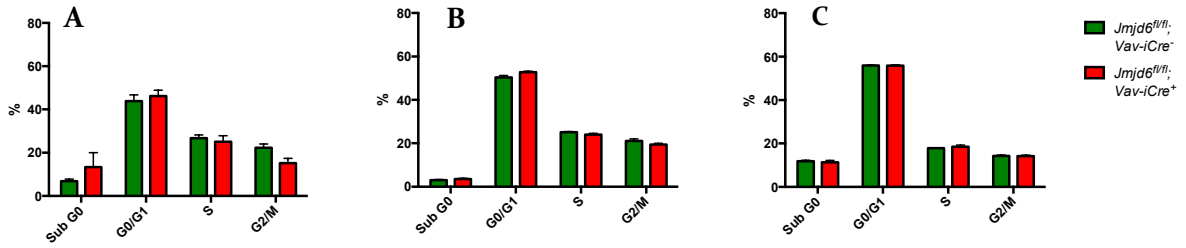

Figure 14. Cell cycle behaviour in suspension at **A:** t=0; **B:** t=24; and **C:** t=48 hours.

The following step was to incubate these cells with I-BET 151, and with (+)-JQ1, to assess potential effects. IC50 determination for each of the drugs was performed, incubating a cell mix in a multitude of drug concentrations. The selected vehicle was DMSO, and vehicle effect was assessed to determine the correct IC50 value for each drug. In the case of I-BET 151, there was no significant vehicle effect. However, in (+)-JQ1, there was some effect caused by DMSO, which would require optimization. Given that cell death was already significant with no DMSO in the blank assay, the issue did not seem to be fully associated with the vehicle, and as such we used the resulting value nonetheless. The fact that literature indicates similar values was also taken into consideration [29]. Ultimately, the IC50 after 24 hours of incubation for I-BET 151 was of 50μM (Fig.15), and that of (+)-JQ1 was of 0.25μM (Fig.16), as these were the concentrations at which 50% of cells were dead.

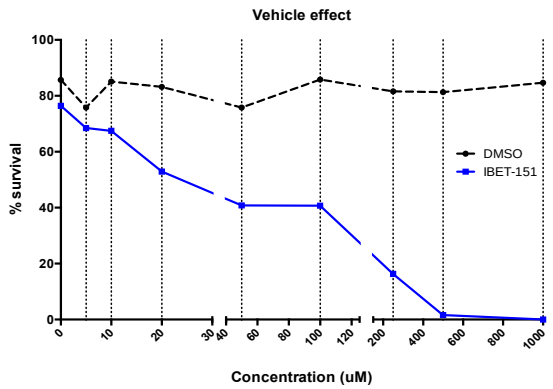

Figure 15. I-BET 151 calibration curve.

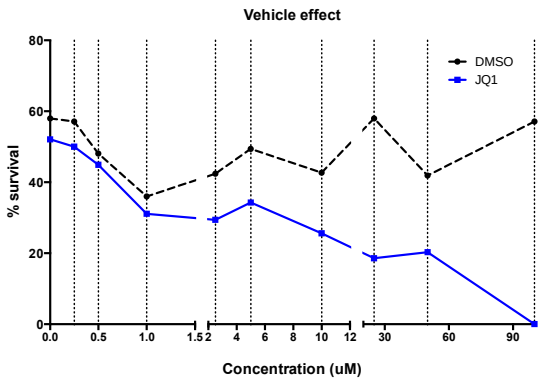

Figure 16. (+)-JQ1 calibration curve.

The cells were then incubated in each drug at IC25, for 48 hours. Proliferation, apoptosis, and cell cycle assays were performed at 0, 24 and 48 hours. In terms of proliferation (Fig.17), no significant differences were observed for either drug between WT and KO cells, although I-BET 151 seems to decrease leukaemic cell proliferation over time, implicating that it could more directly interfere with Jmjd6 function than (+)-JQ1 appears to.

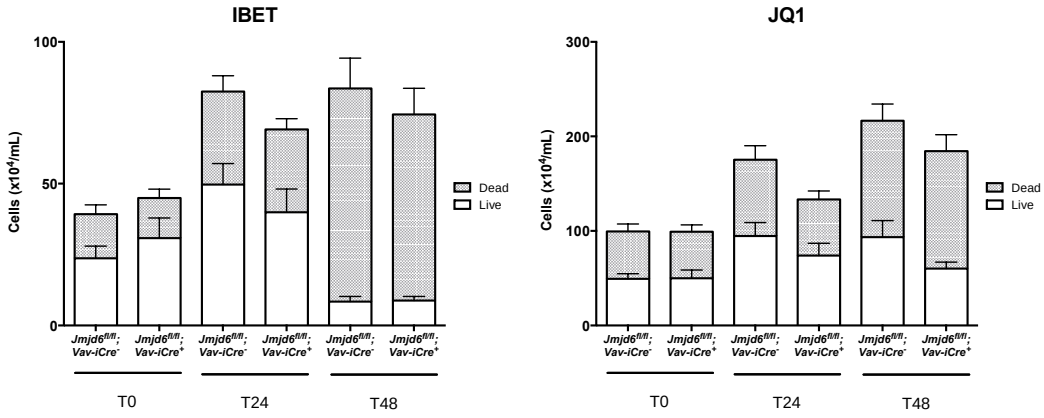

Figure 17. Cell proliferation data for **A:** I-BET 151; and **B:** (+)-JQ1.

In terms of apoptosis (Fig. 18, 19), I-BET 151 seems to induce large scale apoptosis at the used concentration after 48 hours of incubation, but because this is true for both WT and KO, it seems to have no connection to Jmjd6 expression. Similarly, (+)-JQ1 appears to affect WT and KO in the same way, with both cell types displaying identical behaviours. While a slight increase in the number of cells in late apoptosis was detected, with an accompanying decrease in the number of live cells, I-BET 151 still proves much more effective in depleting the tumour cells.

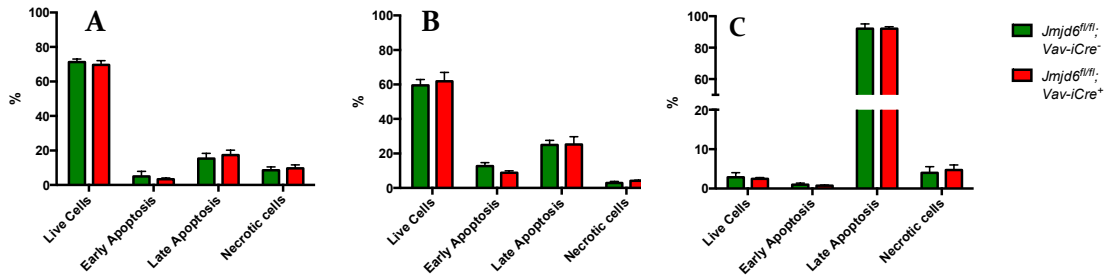

Figure 18. Apoptosis assay for cells incubated in I-BET 151 at **A:** t=0; **B:** t=24; and **C:** t=48 hours.

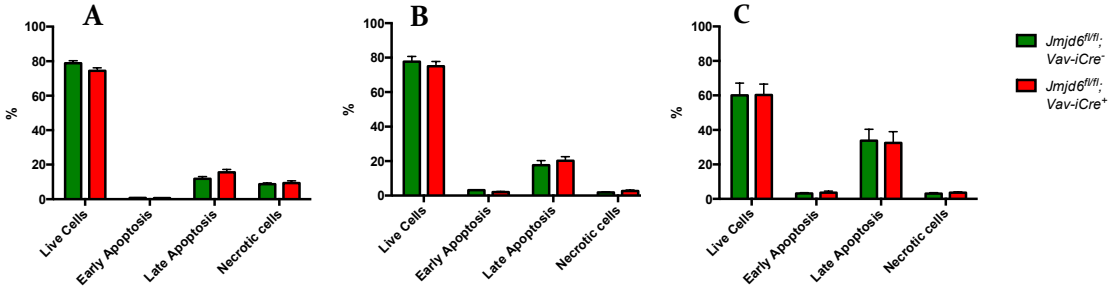

Figure 19. Apoptosis assay for cells incubated in (+)-JQ1 at **A:** t=0; **B:** t=24; and **C:** t=48 hours.

I-BET seems to induce early cell cycle arrest, with most cells remaining in a sub G1 stage, indicative of apoptosis (Fig.20). In this regard, (+)-JQ1 seems to have a much tamer effect over time, but indeed most cells are found in sub G1 (once again a sign of apoptotic phenomena) and G0/G1 stages (Fig.21). This contrasts with what was observed for cells in the absence of either drug, where sub G1 corresponded to the least predominant phase. Once again, no significant differences were observed between WT and KO cells, apparently corroborating that Jmjd6 is not directly affected by either drug.

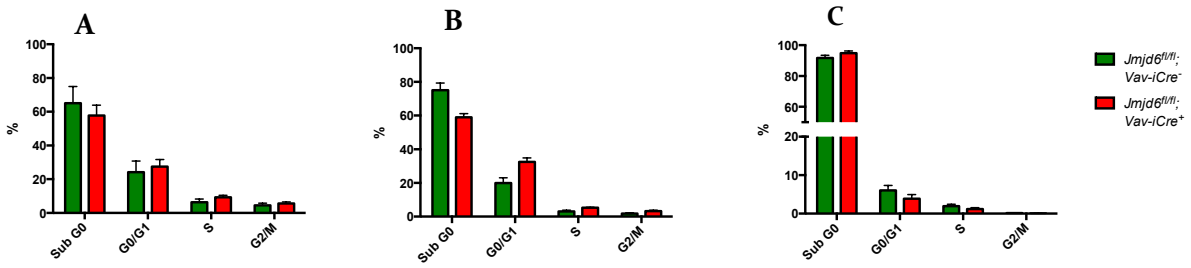

Figure 20. Abundance of cells incubated with I-BET 151 in each cell cycle stage at **A:** t=0; **B:** t=24; and **C:** t=48 hours.

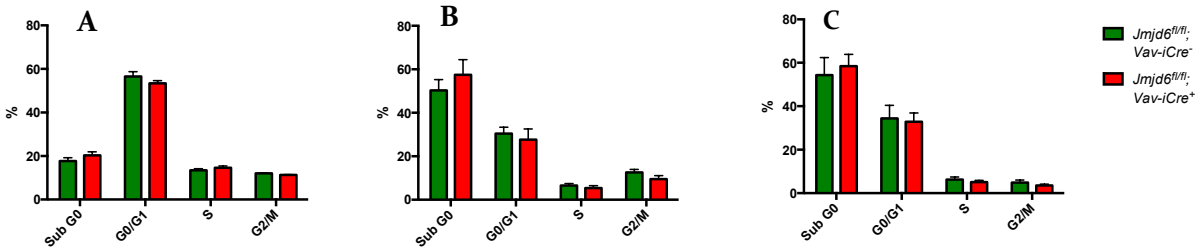

Figure 21. Abundance of cells incubated with JQ1 in each cell cycle stage at **A:** t=0; **B:** t=24; and **C:** t=48 hours.

Ultimately, the results achieved in this project seem to implicate Jmjd6 as a tumour suppressor, much like in the results documented by Poulard *et al*. in breast cancer [34]. In AML, Jmjd6 seems to be relevant only in disease initiation, but not in its maintenance. The use of BET-inhibitors, particularly I-BET 151, showed promising effects in fighting the disease, although these appear to bear no correlation with Jmjd6 expression or lack thereof, which could imply that Jmjd6 functions as tumour suppressor could be Brd4-independent. Jmjd6 upstream and downstream factors could as such be investigated as promising targets in AML therapeutics, when in tandem with other complementary approaches such as chemotherapy. Finally, given its role in leukaemogenesis, Jmjd6 could be key in preventing relapse, although this would require further investigation.

Chapter 5

Final Remarks

In assessing Jmjd6 role *in vitro* and *in vivo,* and in studying the effect of BET-bromodomain inhibitors that act over Brd4 in *Jmjd6* KO mice, we obtained results that suggest that Jmjd6 plays a relevant anti-tumour role in delaying AML initiation. This role could be performed acting upon primary leukaemic cells, thought to be responsible for the overproliferative profile of AML. Jmjd6 alone had no visible effect over proliferation, apoptosis and cell cycle of AML leukaemic cells, which could implicate that other factors are also at play. Jmjd6 seemed, however, to decrease leukaemic cell colony forming potential, possibly attributing it a role in suppressing pre-LSC differentiation into the non-functional myeloblasts that constitute the bulk of the tumour. Additionally, the suppressive effect of Jmjd6 seems to be independent of Brd4 expression, indicating that Jmjd6 and its enzymatic substrates might be sufficient for this tumour suppression to be observed. We also verified that Jmjd6 appears to have little to no involvement in disease maintenance. Overall, this implicates Jmjd6 upstream and downstream factors as attractive targets for AML therapeutics, with potential to prevent disease initiation in those most susceptible to it, which could be vital in fighting the grim prognosis associated with the condition.

Future Work

Further research should seek to optimize the procedures contemplated in this dissertation, as well as delve into better understanding the landscape Jmjd6 inserts itself into in the mechanisms behind AML.

Other disease models should be tested, namely mixed-lineage leukaemia eleven nineteen (MLL-ENL) and MLL-AF9, to study the role of Jmjd6 in AML when caused by other oncogenes. Given the potential role played by Jmjd6 in disease initiation, it would be of interest to seek to identify Jmjd6 enzymatic substrates, considering its involvement in hydroxylation of RNA splicing factors. With a growing body of evidence that epigenetics can be vital to

leukaemia, identifying such substrates could uncover potential therapeutic targets that play a critical role in LCS biology.

Regarding the *in vitro* drug testing, (+)-JQ1 assays should be optimized, in order to lessen the number of dead cells present at t=0, allowing a better assessment of the effects caused by the drug itself. The use of a density separation medium could be of use in achieving a sample with a higher percentage of live cells. This was indeed attempted in the ambit of this dissertation, but the inability to completely remove the separation medium once the procedure was completed led to reservations regarding its effects on the results, and was consequently set aside for the present project.

Comparing the affinity of the target cells to I-BET and (+)-JQ1 could be of interest in determining if this is a key factor in treatment success. Similarly, employing drugs that could inhibit each of the components of the proposed active P-TEFb complex, as well as Brd4 and Jmjd6, in alternating patterns would help clarify the role each of them plays, and whether they are essential or not.

Colony forming assays would be of use in determining the true potential leukaemic cells carry to form colonies when exposed to these drugs, as well as to assess their effect in different media, for a fuller comprehension of their inner workings.

Finally, the clearest necessary step would be to proceed to *in vivo* testing, to determine if *in vivo* behaviour is distinct from what was observed *in vitro*. This would consist of injecting two sets of mice with either I-BET 151 or (+)-JQ1 immediately after being transplanted with Hoxa9-Meis1 cells, to assess the effect of each of these drugs in disease initiation, and on another set as the mice fell ill from the transplant, to study their effect in disease maintenance. This would be important in helping identifying which molecules are at play in each of these steps, and if Brd4 and Jmjd6 act cooperatively in maintenance, even though the AML initiation pathway seems to find Jmjd6 acting in a Brd4-independent manner *in vitro*.

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84. Schematic representation made using http://thenounproject.com icons.

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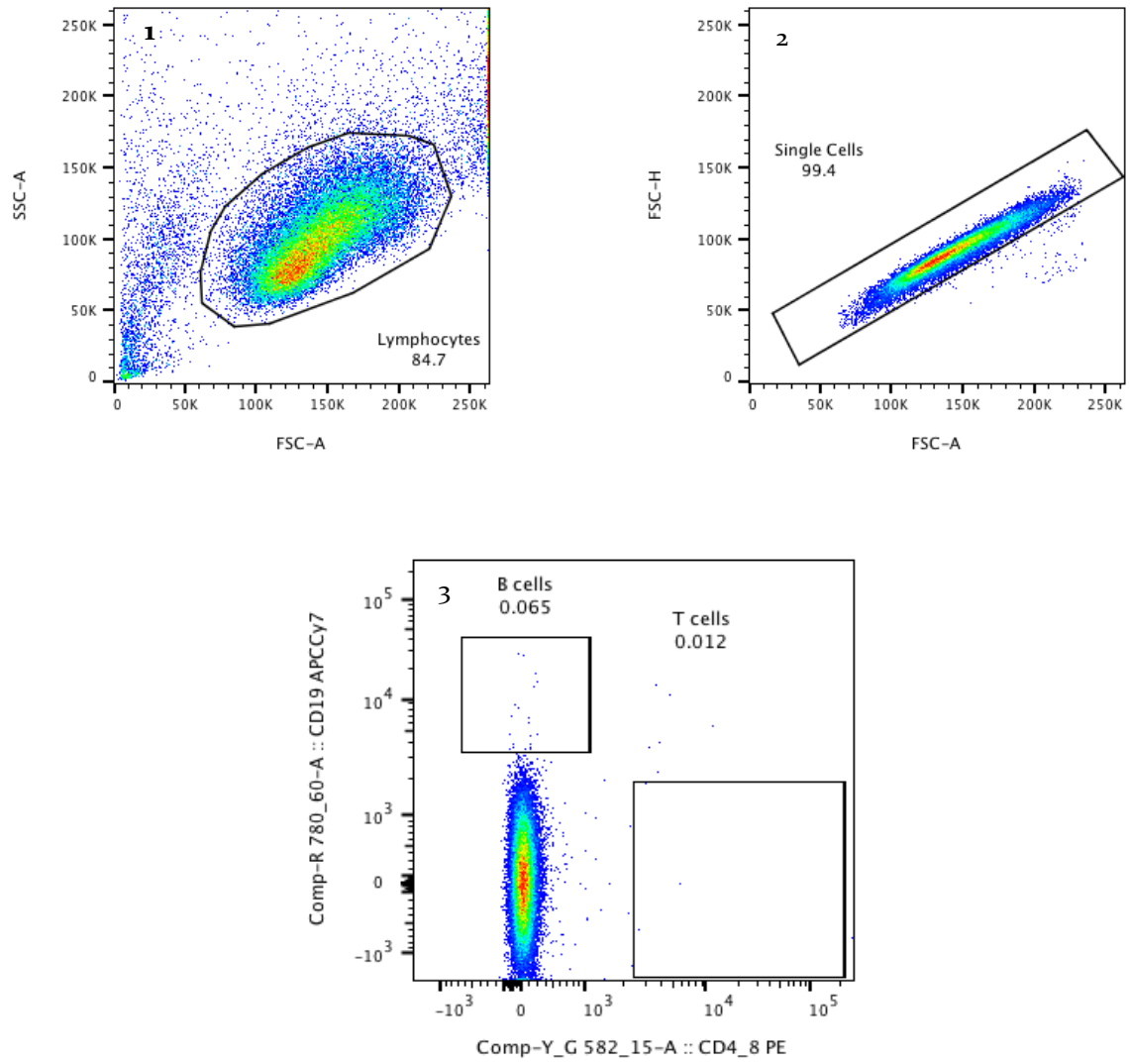
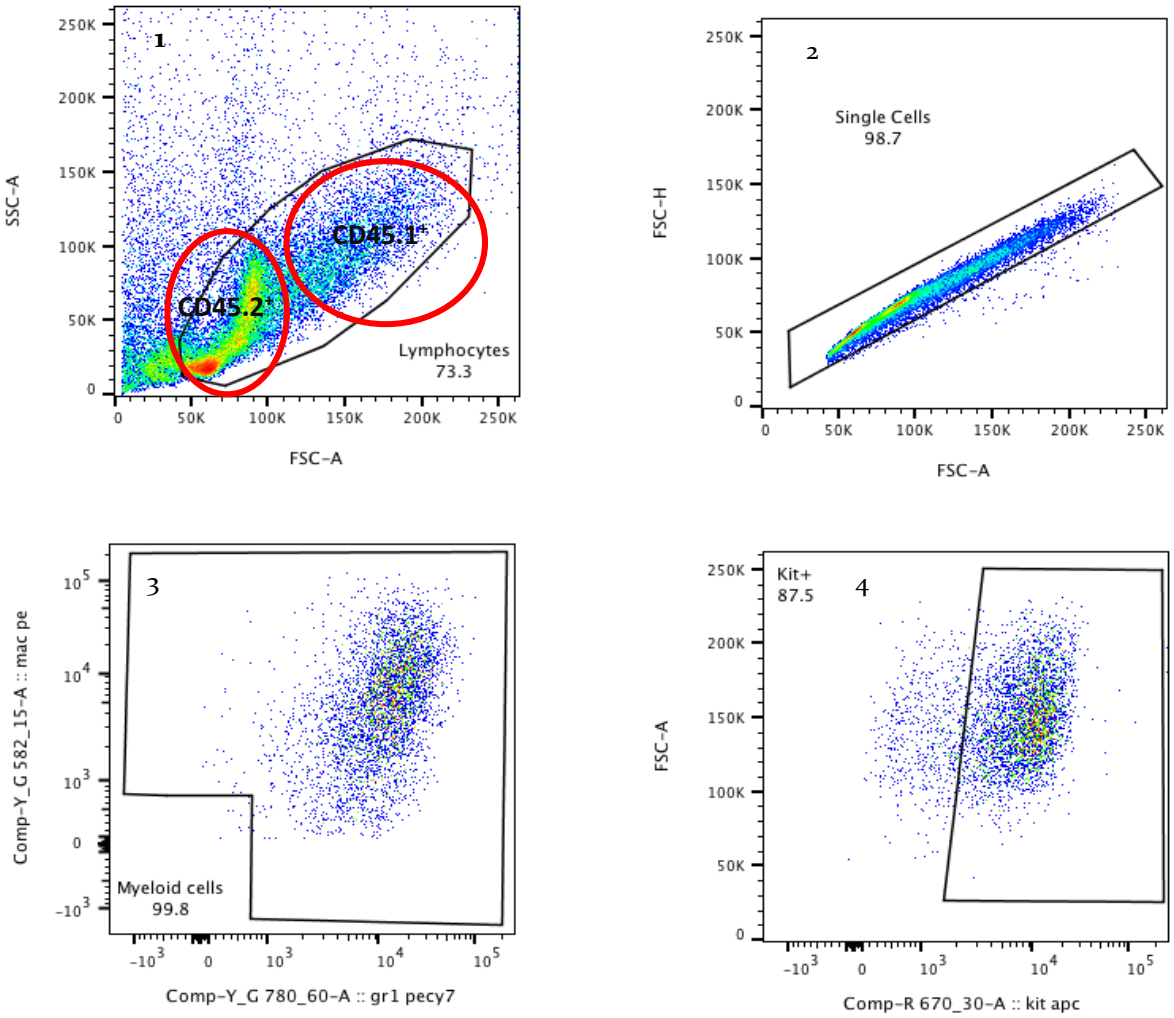
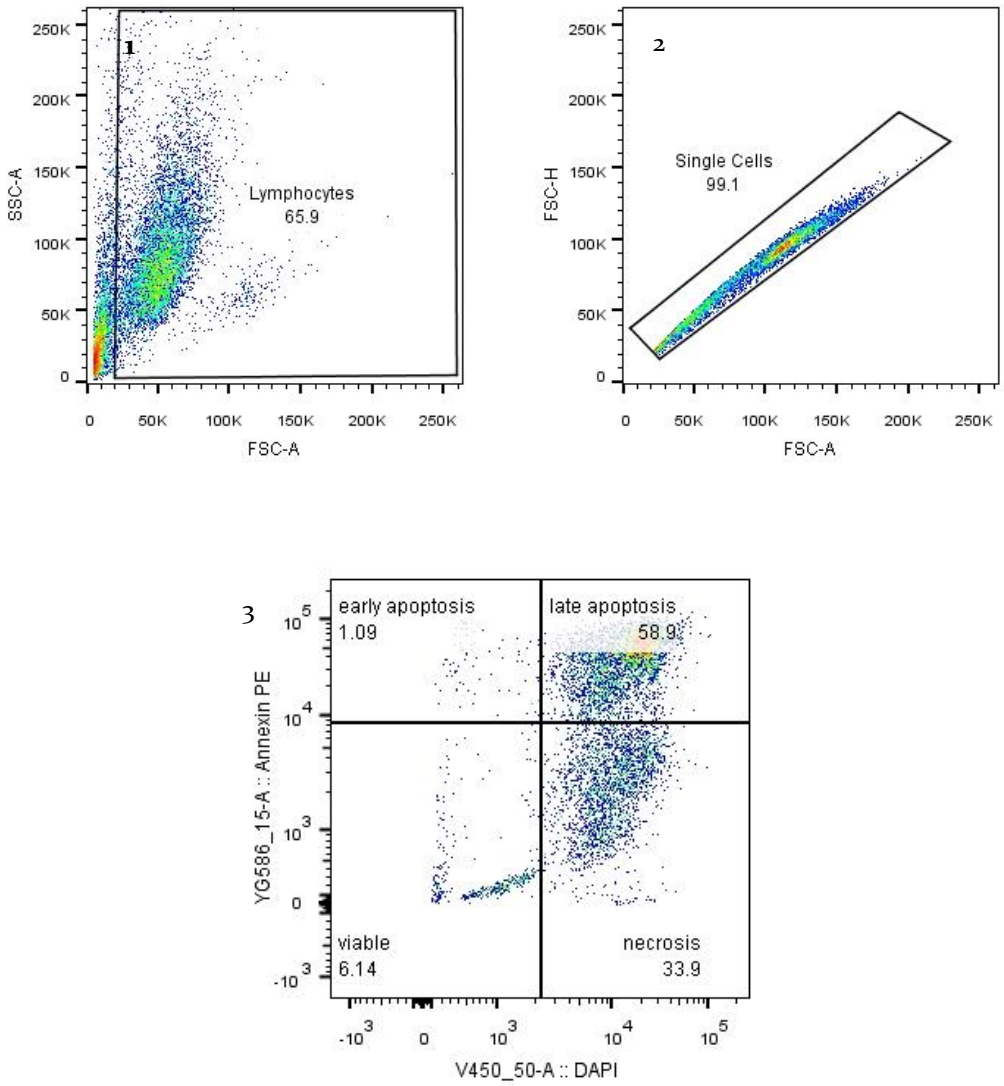
Supplements



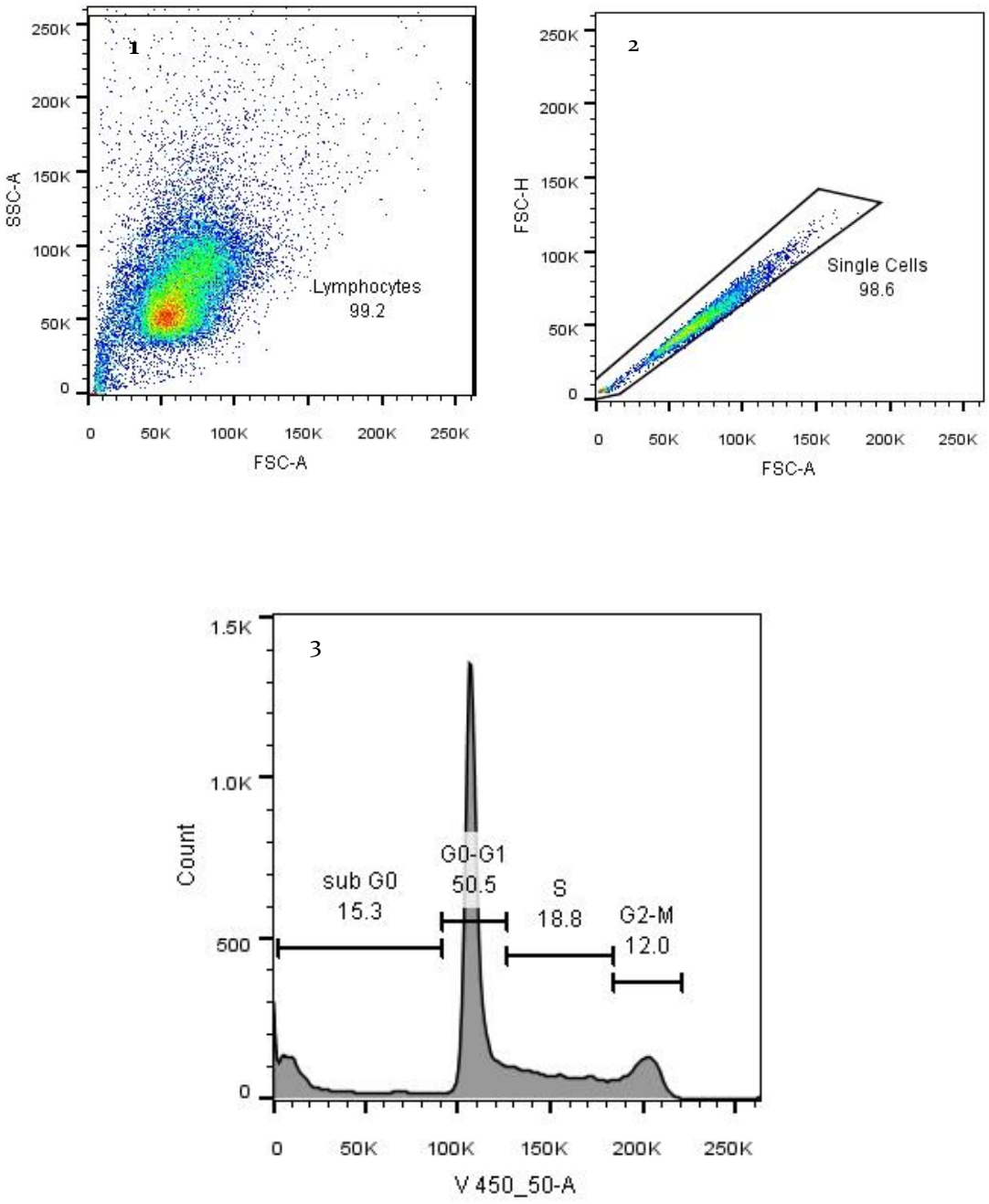
Supplement 1. FACS plotting representative of gating strategy for immunophenotypic characterization of CFC1 cells. The first plot excludes dead cells. The second plot discards any doublets to eliminate false results. The third plot allows us to assess the percentage of myeloid cells vs. B cells and T cells.



Supplement 2. FACS plotting representative of gating strategy for immunophenotypic characterization of CFC2 cells. The first plot excludes dead cells and gates lymphocytes. The second plot discards any doublets to gate only single cells. The third plot selects myeloid cells, and the last selects myeloid c-Kit$^+$ cells (HSPCs).



Supplement 3. FACS plotting representative of gating strategy for apoptosis assay. The first plot excludes debris and gates lymphocytes. The second plot discards any doublets to gate only single cells. The third plot divides cells in viable, early apoptotic, late apoptotic, or necrotic.



Supplement 4. FACS plotting representative of gating strategy for cell cycle assay. The first plot gates lymphocytes (all cells). The second plot discards any doublets to gate only single cells. The results are represented using a histogram, where each cell cycle phase is distinguished.