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Identifying and Targeting Dormant Cells in Acute Myeloid Leukaemia

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Abstract

Relapse in AML is thought to arise from dormant leukaemic cells that are characterised by low RNA synthesis activity, protected by the bone marrow (BM) niche, and may evade the effects of chemotherapeutic drugs. Our aim was to investigate agents which might be able to overcome chemoprotection by targeting the intrinsic apoptosis pathway. We developed *in vitro* assays to identify and characterise the dormant AML cells using combinations of markers, including the cell-division marker PKH26, leukaemia-associated phenotypes (LAPs), and dormancy markers. In a dormancy model based on 12-day AML/stroma co-culture, we have shown that the expression of some aberrant phenotypes can persist for several days. Also, after 12 days, some of the $CD34^{+}$, $PKH26^{high}$ (dormant), and LAP^{+} (leukaemic) cells maintained their primitiveness and were still clonogenic. Furthermore, our chemosensitivity data showed that novel agents TG02, and BH3 mimetics ABT-737 and ABT-199, which inhibit the B-cell lymphoma 2 (BCL-2) family of anti-apoptotic molecules, could efficiently target BM niche-mediated chemoresistance, which is thought to be one of the main obstacles to traditional chemotherapy. We explored various candidate dormancy markers based on the low RNA, non-proliferative profile of dormant cells. Among those tested, the RNA synthesis marker Pyronin Y (PY), and an antibody to the transferrin receptor CD71 were the most reproducible in terms of marker expression and stability. We endeavoured to characterise cell dormancy on the molecular level by investigating gene expression in the PY^{low} (dormant) and PY^{high} (proliferating) subsets and have obtained limited results. In summary, this study has identified and partly characterised dormant AML cells by development of *in vitro* assays, and has shown chemosensitivity to novel agents TG02, ABT-737 and ABT-199 in dormant leukaemia cells.

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List of Publications

Articles

Jawad, M., **Yu, N.**, Seedhouse, C., Tandon, K., Russell, N. H. & Pallis, M. Targeting of CD34+CD38- cells using Gemtuzumab ozogamicin (Mylotarg) in combination with tipifarnib (Zarnestra) in Acute Myeloid Leukaemia. BMC Cancer, 2012, 12**,** 431-454.

Yu, N., Seedhouse, C., Russell, N., Pallis, M. Dormant AML cells are sensitive to ABT-199 and ABT-737. (Manuscript in preparation)

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Yu, N., Abdul-Aziz, A., Burrows, F., N., R., Seedhouse, C. & Pallis, M. Combined targeting of MCL1 with TG02 and BCL2 with ABT-737/ABT-199 strategy for eliminating AML cells. British Journal of Haematology, 2013, 161 (supp 1), p58, abstract 144. (Poster presentation at the British Society of Haematology Conference, Liverpool, UK, 2013)

Burrows F., **Yu N.**, Abdul-Aziz A., Hitosugi S., Seedhouse C.H., Russell N., Boise L.H., Pallis M. Combined targeting of Mcl-1 with TG02 and Bcl-2 with

ABT-199/ABT-737 for treatment of hematologic malignancies. Clinical Lymphoma, Myeloma & Leukemia, 2014, 14, S152

Abdul-Aziz, A., Burrows, F., **Yu, N.,** Russell, N.H., Seedhouse, C., Pallis, M. ABT-737 and ABT-199 complement the multikinase inhibitor TG02 to induce apoptosis in acute myeloid leukemia cells. Cancer Research, 2014, 74(8; Supp1): Abstract 4536. (Poster presentation at the American Association for Cancer Research conference, San Diego, USA, 2014)

Pallis, M., Burrows, F., **Yu, N.,** Jawad, M., Seedhouse, C., Russell, N.H. The Multi-kinase inhibitor TG02 downregulates MCL-1 in AML cells and preferentially targets CD34CD38-CD123 cells from samples with an internal tandem duplication of FLT3. Haematologica, 2011, 96(S2: 16th Congress of EHA): Abstract 0209. (Poster presentation at the European Hematology Association 16th Congress, London, UK, 2011)

List of Abbreviations

5-Aza	5-aza-2'-deoxcytidine
5-FU	5-Fluorouracil
7-AAD	7-Amino-Actinomycin D
α-MEM	Minimum Essential Medium Eagle, Alpha
ADCC	Antibody-dependent Cellular Cytotoxicity
aDNA	antisense-DNA
aRNA	antisense-RNA
ALDH	Aldehyde Dehydrogenase
AML	Acute Myeloid Leukaemia
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Ang-1	Angiopoietin-1
ALL	Acute Lymphoblastic Leukaemia
APAF-1	Apoptotic protease activating factor 1
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cyanine 7
APL	Acute Promyelocytic Leukemia
ATM	Ataxia telangiectasia-mutated
ATP	Adenosine triphosphate
β2M	Beta-2-Microglobulin
BAK	BCL-2-antagonist/Killer
BAX	BCL-2-Associated X protein
BCL-2	B-cell lymphoma-2
BFL-1	Bcl-2-related protein A1
BID	BH3 Interacting Domain
BIM	Bcl-2-like protein 11
BM	Bone Marrow
BTG	B Cell Translocation Gene
CD	Cluster of Differentiation
CDC	Complement-dependent Cytotoxicity
CDK	Cyclin-dependent Kinase

cDNA	Complementary Deoxyribonucleic Acid
CEBPA	CCAAT/enhancer-binding protein-α
CFU	Colony-forming Unit
CHK2	Checkpoint Kinase 2
CI	Confidence Interval
CIP	CDK Interacting Protein
CITED2	CBP/p300-interacting-transactivator-with-an ED-rich-tail 2
CKI	Cyclin-dependent Kinase Inhibitor
CLL	Chronic Lymphocytic Leukaemia
CLP	Common Lymphoid Progenitor
CML	Chronic Myeloid Leukaemia
CMML	Chronic Myelomonocytic Leukaemia
CMP	Common Myeloid Progenitor
CO_2	Carbon Dioxide
Con A	Concanavalin A
CR	Complete Remission
Ct	Threshold Cycle
C-terminal (terminus)	The Carboxy-(COOH)-Terminal (Terminus) of a Protein
Cv	Coefficient of Variation
CXCR	C-X-C chemokine receptor type 4
DC	Dendritic Cell
DECR1	2,4-dienoyl CoA reductase 1
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotides
DSB	Double Strand Break
dsDNA	double stranded DNA
EDTA	Ethylenediamine Tetra-Acetic Acid
EPO	Erythropoietin
ETS	E-twenty-six

FAB	French-American-British
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal Calf Serum
FcR	Fc Receptor
FISH	Fluorescence In Situ Hybridization
FITC	Fluorescein Isothiocyanate
FLT3	FMS-like Tyrosine Kinase 3
FoxO	Forkhead O
FPGS	folylpolyglutamate synthase
FSC	Fixed Stained Cell
G0 Phase	Gap 0 Phase of the Cell Cycle
G1 Phase	Gap 1 Phase of the Cell Cycle
G2 Phase	Gap 2 Phase of the Cell Cycle
G-6-PD	Glucose-6-phosphate Dehydrogenase
GATA2	GATA Binding Protein 2
G-CSF	Granulocyte Colony Stimulating Factor
GF	Growth Factor
Gfi-1	Growth Factor Independent-1
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Granulocyte-monocyte Progenitor
HBSS	Hanks Buffered Saline Solution
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
Hes1	Hairy and Enhancer of Split-1
HIF	Hypoxia-inducible factor
HoxA9	Homeobox protein Hox-A9
HSC	Haematopoiesis Stem Cell
Hsc70	Heat Shock Cognate 70
IC50	The concentration that induced 50% reduction in cell number
Ig	Immunoglobulin
IL-3	Interleukin-3
IL-6	Interleukin-6

IMDM	Iscove's Modified Dulbecco's Media
ITD	Internal Tandem Duplications
IVT	In vitro transcription
JAK2	Janus kinase 2
KIP	Kinase Inhibitory Protein
LAP	Leukaemia-associated Phenotype
LIC	Leukaemia-initiating Cell
LKB1	Liver Kinase B1
LMPP	Lymphoid-primed Multipotent Progenitor
LP	Leukapheresis Product
LSC	Leukaemic Stem Cell
LT-HSC	Long-term haematopoietic stem cell
M Phase	Mitosis Phase of the Cell Cycle
mAb	Monoclonal Antibody
MAPK	Mitogen-activated Protein Kinase
MCL-1	Myeloid Cell Leukaemia-1
MCM	Minichromosome Maintenance
Mdm2	Mouse double minute 2 homolog
MEF	Myeloid ELF-1-like Factor
MEP	Megakaryocyte-erythroid Progenitor
MM	Multiple Myeloma
MMRV	Moloney Murine Leukaemia Virus
MOMP	Mitochondrial Outer Membrane Permeabilisation
MPD	Myelo-proliferative Disorder
MPP	Multipotent Progenitor
MPFC	Multi-parameter Flow Cytometry
MRC	Medical Research Council
MRD	Minimal Residual Disease
mRNA	Messenger Ribonucleic Acid
MSC	Mesenchymal Stem Cell
mTOR	Mammalian Target of Rapamycin
NK	Natural Killer

NOD/SCID	Non-Obese Diabetic with Severe Combined Immunodeficiency
NOS	Not Otherwise Specified
NPM	Nucleophosmin
NSG	NOD/SCID/IL2R?null
ORC	Origin Replication Complex
OS	Overall Survival
P-value	Probability Value
PB	Peripheral Blood
PBS	Phosphate-Buffered Saline
PBSAA	Phosphate-Buffered Saline Albumin Azide
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PHA	Phytohaemagglutinin
PS	Phosphatidylserine
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PKB	Protein Kinase B
PML	Promyelocytic Leukaemia
PMT	Photomultiplier
PP	Proliferative Potential
PPIB	Peptidylprolyl isomerase B
Pten	Phosphatase and Tension Homologue
PY	Pyronin Y
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
R Point	Restriction Point
Rb	Retinoblastoma Tumour Suppressor Protein
R-CHOP	Rituxibam-Cyclophosphamide-doxorubicin-vincristine-prednisone
ROS	Reactive Oxygen Species
RNA	Ribonucleic Acid

RNase	Ribonuclease
RNasin	Ribonuclease Inhibitor
RP2	RNA Polymerase ii
RPMI	Roswell Park Memorial Institute Medium
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S Phase	Synthesis Phase of the Cell Cycle
SAHF	Senescence-associated Heterochromatin foci
SA-β-Gal	Senescence-associated β-galactosidase
SCF	Stem Cell Factor
SCH	Stem Cell Harvest
SD	Standard Deviation
SDF-1	Stroma-derived Factor-1
SEER	Surveillance, Epidemiology, and End Results Program
SFEM	Serum-Free Expansion Medium
STAT5	Signal Transducer and Activator of Transcription-5
ST-HSC	Short-term Haematopoietic Stem Cell
STR	Short Tandem Repeat
TGF-β	Transforming Growth Factor-beta
TKI	Tyrosin Kinase Inhibitor
TPO	Thrombopoietin
Tr	Treatment-related
TSC	Tuberous Sclerosis Complex
TRAP1	TNF receptor-associated protein 1
VCAM-1	Vascular Cell Adhesion Molecule 1
VLA-4	Very Late Antigen-4
WCC	White blood cell count
WHO	World Health Organisation
WT	Wild-type
XIAP	X-linked Inhibitor of Apoptosis Protein

Table Of Contents

List of Figures

CHAPTER 1. INTRODUCTION

CHAPTER 2. MATERIALS AND METHODS

CHAPTER 3. TARGETING BM NICHE-MEDIATED CHEMORESISTANCE IN AML

CHAPTER 4. IDENTIFYING AND TARGETING DORMANT AML SUBSETS *IN VITRO*

CHAPTER 5. EXPLORING DORMANCY MARKERS

CHAPTER 6. MOLECULAR CHARACTERISATION OF DORMANCY IN AML

List of Tables

CHAPTER 1. INTRODUCTION

CHAPTER 2. MATERIALS AND METHODS

CHAPTER 4. IDENTIFYING AND TARGETING DORMANT AML SUBSETS *IN VITRO*

CHAPTER 5. EXPLORING DORMANCY MARKERS

CHAPTER 6. MOLECULAR CHARACTERISATION OF DORMANCY IN AML

CHAPTER 1

INTRODUCTION

1.1 Overview of AML

1.1.1 Risk Factors and Genetic Disorders

Acute myeloid leukaemia (AML), also known as acute myelogenous leukaemia, acute myelocytic leukaemia, or acute non-lymphocytic leukaemia (ANLL), is a heterogeneous haematologic malignancy characterised by clonal expansion of myeloid blasts in the peripheral blood (PB), bone marrow (BM), and/or other tissues (O'Donnell et al., 2012, Estey and Dohner, 2006). The incidence of AML is strongly related to age. As the population ages, the incidence rises gradually from around 40-44, and more steeply in older patients, from around 60-64, with the highest rates in the 80^{+} age group (Figure 1.1). The median age at diagnosis is 67, with most patients diagnosed at 65 years or older (O'Donnell et al., 2012).

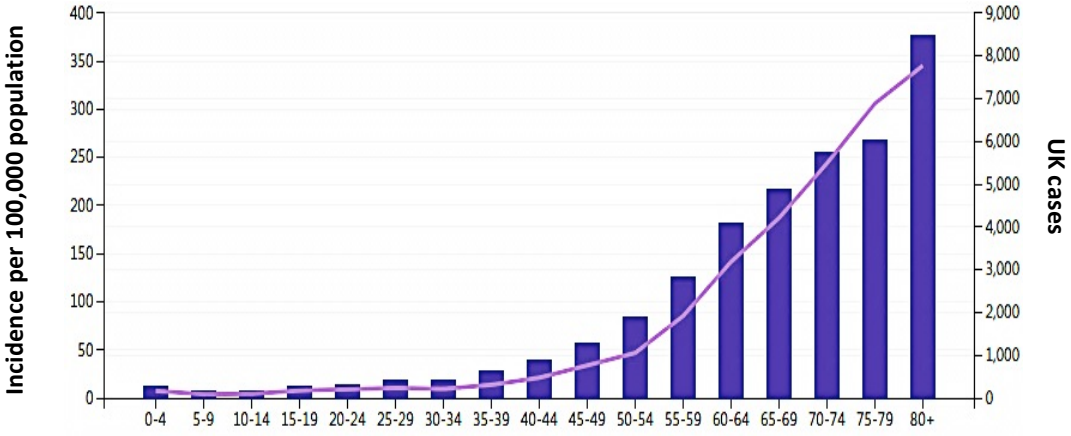


Figure 1.1. The age-specific incidence of AML (2004-2014, UK). All races, both sexes. Source: the Haematological Malignancy Research Network (HMRN), https://www.hmrn.org/Statistics/incidence; accessed May 2016.

Besides age, the development of AML has been linked to several other risk factors including antecedent haematologic disease, genetic disorders (e.g.

Down syndrome, Klinefelter syndrome, Patau syndrome, Li-Fraumeni syndrome, Ataxia telangiectasia, etc.), physical and chemical exposures to substances (e.g. benzene, pesticides, herbicides, cigarettes, certain drugs, etc.), radiation exposure, and previous chemotherapy (e.g. alkylating agents, topoisomerase-II inhibitors, anthracyclines, taxanes, etc.) (Deschler and Lubbert, 2006). However, known risk factors only account for a limited number of observed cases (Sandler and Collman, 1987, Deschler and Lubbert, 2006). Most cases of AML occur *de novo* without objectifiable leukaemogenic exposure.

Congenital genetic defects are one of the key factors associated with AML (Pui, 1995). Down Syndrome (trisomy 21) children have a 10-20 fold increased likelihood of developing leukaemia (Fong and Brodeur, 1987). Chromosomal and gene rearrangement lesions may lead to leukaemogenesis. Acute promyelocytic leukaemia (APL) is a subtype of AML bearing the hallmark t(15;17) translocation leading to the expression of the leukaemia-inducing PML/RARα fusion protein (Puccetti and Ruthardt, 2004). An increased trend of APL cases is especially recognised in adult patients of Hispanic South American descent, with a unique gene rearrangement of breakpoint lesions (bcr1) in the RARα gene in the PML/RARα transcript in intron 6, which may be due to a predisposition gene (Estey et al., 1997, Douer et al., 2003, Douer, 2003). Acquired (somatic) clonal chromosomal lesions have been found in 50-80% of AML cases. Such abnormalities include loss or deletion of chromosome 5, 7, Y, and 9; translocations t(8;21)(p22;q22), t(15;17)(q22;q11), trisomy 8 and 21, as well as aberrations involving chromosomes 16, 9, and 11.

The incidence increases with secondary AML or older age (Leith et al., 1997, Mauritzson et al., 1999, Rossi et al., 2000).

1.1.2 The "Two Hit Hypothesis" of AML Pathogenesis

The "two hit hypothesis" has been a long-standing model for AML leukaemogenesis (Takahashi, 2011, Conway O'Brien et al., 2014). In this hypothesis, two different types of genetic mutations are required for malignant transformation of a myeloid precursor. Class I mutations, in genes such as BCR-ABL, the FMS-like tyrosine kinases 3 (FLT3), c-KIT, and RAS, are thought to confer constitutive activity to tyrosine kinases or dysregulation of downstream signaling, result in uncontrolled cellular proliferation and evasion of apoptosis (Conway O'Brien et al., 2014). Class II mutations, exemplified by RUNX1/ETO, CBFβ/MYH11, PML/RAR, NPM1, EVI1, and MLL-related fusion genes, primarily serve to impair haematopoietic differentiation (Takahashi, 2011, Conway O'Brien et al., 2014). However, many of the mutations affecting epigenetic regulations, in genes such as DNMT, TET2, IDH1/2, ASXL1, are not classified by the two-hit model. Also, there is evidence that some mutations occur in a particular temporal order during leukaemogenesis (Murati et al., 2012), as is reported in acute promyelocytic leukemia (APL). New models for the development of AML have become increasingly complex (Takahashi, 2011, Murati et al., 2012, Conway O'Brien et al., 2014) with the detection of novel mutations and the understanding of their roles in leukaemogenesis.

1.1.3 AML classification

A conventional, longstanding classification of AML was by the French-American-British (FAB) co-operative group framework (Table 1.1) and it is still widely used today as a clinical reference at diagnosis. The FAB system originally divided AML into eight subtypes (M0 to M7), based on the origin and the degree of maturity of leukaemia cells through morphological and cytogenetic tests (Bennett et al., 1976). A ninth morphologic subtype (M8), namely acute basophilic leukaemia, was introduced subsequently (Duchayne et al., 1999).

Table 1.1 The FAB classification of Acute Myeloid Leukaemia.

Type	Name	Cytogenetics
M0	Acute myeloblastic leukaemia, minimally differentiated	
M1	Acute myeloblastic leukaemia, without maturation	
M2	Acute myeloblastic leukaemia, with granulocytic maturation	t(8;21)(q22;q22), t(6;9)
M3	promyelocytic, or acute promyelocytic leukaemia (APL)	t(15;17)
M4	Acute myelomonocytic leukaemia	inv(16)(p13q22), del(16q)
M4eo	myelomonocytic together with bone marrow eosinophilia	inv(16), t(16;16)
M5	Acute monoblastic leukaemia (M5a) or acute monocytic leukaemia (M5b)	del (11q), t(9;11), t(11;19)
M6	Acute erythroid leukaemias, including erythroleukaemia (M6a) and very rare pure erythroid leukaemia (M6b)	
M7	Acute megakaryoblastic leukaemia	t(1;22)
M8	Acute basophilic leukaemia	

Adapted from (Bennett et al., 1976) and (Duchayne et al., 1999).

However, the FAB classification system does not take into account many of the factors that affect disease outcomes. The World Health Organisation (WHO) updated the classification of AML in 2008 (Table 1.2), based on an increasing

focus on recurrent cytogenetic changes within malignant cells (Dohner et al., 2010). Many genetic subtypes have been determined and cytogenetic analysis revealed that a majority of AML patients carry karyotypic abnormalities. Also many AML cases with a normal karyotype exhibit mutations in genes such as nucleophosmin (NPM), FLT3, and CCAAT/enhancer-binding protein-α (CEBPA).

Table 1.2 WHO 2008 classification of acute myeloid leukaemia and related precursor neoplasms, and acute leukaemias of ambiguous lineage. Adapted from (Dohner et al., 2010).

Categories
1) Acute myeloid leukaemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with 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)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPA
2) Acute myeloid leukaemia with myelodysplasia-related changes
3) Therapy-related myeloid neoplasms
4) Acute myeloid leukaemia, not otherwise specified (NOS)
Acute myeloid leukaemia with minimal differentiation
Acute myeloid leukaemia without maturation
Acute myeloid leukaemia with maturation
Acute myelomonocytic leukaemia
Acute monoblastic/monocytic leukaemia
Acute erythroid leukaemia
Pure erythroid leukaemia
Erythroleukaemia, erythroid/myeloid
Acute megakaryoblastic leukaemia
Acute basophilic leukaemia
Acute panmyelosis with myelofibrosis (syn.: acute myelofibrosis; acute
5) Myeloid sarcoma (syn.: extramedullary myeloid tumour; granulocytic sarcoma; chloroma)
6) Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (syn.: transient myeloproliferative disorder)
Myeloid leukemia associated with Down syndrome
7) Blastic plasmacytoid dendritic cell neoplasm
8) Acute leukemias of ambiguous lineage
Acute undifferentiated leukaemia
Mixed phenotype acute leukaemia with t(9;22)(q34;q11.2); *BCR-ABL1*[1]
Mixed phenotype acute leukaemia with t(v;11q23); *MLL* rearranged
Mixed phenotype acute leukaemia, B/myeloid, NOS
Mixed phenotype acute leukaemia, T/myeloid, NOS
Provisional entity: Natural killer (NK)–cell lymphoblastic leukemia/lymphoma

1.1.4 Diagnosis of AML

Blood tests and BM aspiration are part of a standard procedure leading up to the diagnosis for suspected AML cases. The lineage of blasts and the cellular differentiation stage are defined by morphologic (microscopic), immunophenotypic (flow cytometry), cytogenetic and molecular genetic analyses (Hoffbrand and Moss, 2011). The presence of over 20% of blasts in PB or the BM smear is generally required to define AML, with exception of cases with specific leukaemia-associated cytogenetic or molecular lesions, namely t(15;17), t(8;21), inv(16) or t(16;16). Some rare cases of pure erythroid leukaemia are also included in the diagnosis of AML (Table 1.2) (Dohner et al., 2010). Determination of blast count by flow cytometry is not a substitute for morphologic evaluation (Vardiman et al., 2009). Nonetheless, the diagnosis of certain AML subtypes, such as AML with minimal differentiation, acute megakaryoblastic leukemia, and acute leukaemias of ambiguous lineage, has to be established by immunophenotyping (Swerdlow et al., 2008).

Immunophenotypic evaluation is routinely performed in modern AML diagnosis to confirm and to accurately classify the disease. Multiparameter flow cytometry (MPFC) provides a rapid and effective way to gather this information, and offers a modality for early minimal residual disease (MRD) evaluation that can identify various patient risk groups and predict impending relapses (San Miguel et al., 2001). Multiple unique surface markers, namely leukaemia-associated phenotypes (LAPs), which are absent or present at very low frequencies in normal blood and BM cells, are regularly detected on subsets of blasts in patients at diagnosis (Freeman et al., 2008, Feller et al.,

2013, Ossenkoppele et al., 2011). Multi-colour technology and comprehensive antibody panels allow the construction of LAPs with highest specificity (Paietta, 2012). Antibodies suitable for LAPs and MRD detection include (1) markers that can distinguish leukaemic blasts from normal myeloid precursors; (2) markers that help to identify lineage-infidel antigens, such as the lymphoid-affiliated CD2, CD3, CD5, CD7 or CD19, on myeloid cells; (3) antibodies that detect altered density of myeloid or lineage-uncommitted antigens, such as CD33 and CD11a and (4) antibodies that detect asynchronous antigen expression, such as CD123 and CD34 (Paietta, 2012). The sensitivity for detection of leukaemic cells with MPFC ranges in a majority of AML cases between 0.01% and 0.1% of nucleated BM cells, depending on the panel of antibody combinations (Coustan-Smith et al., 1993, Campana and Coustan-Smith, 1999).

1.1.5 Prognostic Factors

Complete remission (CR), i.e. less than 5% blasts in the BM, can be achieved in the majority of AML patients following chemotherapy. However, relapse is very often observed with resistant disease. Some AML cases become refractory to chemotherapy at disease recurrence, whilst others develop second and even third remissions, with shortened duration which indicates an increase in drug-resistant residual leukaemic cells (Forman and Rowe, 2013). In the past few decades, greater insights into the prognostic factors of AML have provided us with invaluable information to 'forecast' patient outcomes under front-line chemotherapy (Grimwade and Hills, 2009). Pre-treatment parameters such as age, white blood cell count (WCC), performance status, cytogenetic and

molecular lesions of the leukaemic clone, give directions to AML management strategy, in terms of identifying the possibility for tailored targeted therapy or decisions on BM transplantation.

Karyotype analysis offers a powerful independent prognostic factor for CR rates, risk of relapse and overall survival (OS) in multivariable analyses. A majority of AML patients present with an abnormal karyotype, which is deemed as a key determinant of disease outcome (Grimwade and Hills, 2009).

Table 1.3 Medical Research Council (MRC) cytogenetic classification in AML (2010). This update was based on a multivariable analysis of a cohort of 5836 younger adult patients (16-59 years) in the MRC AML10, AML12, and AML15 trials. abn, abnormal; unrel abn, unrelated abnormality.

	MRC cytogenetic classification **(Grimwade et al., 2010)**
Favourable	t(15;17)(q22;q21) t(8;21)(q22;q22) * inv(16)(p13q22)/t(16;16)(p13;q22)
Intermediate	Entities not classified as favourable or adverse
Adverse	abn(3q) [excluding t(3;5)(q21~25;q31~35)], inv(3)(q21q26)/t(3;3)(q21;q26), add(5q), del(5q), -5, -7, add(7q)/del(7q) †, t(6;11)(q27;q23), t(10;11)(p11~13;q23), t(11q23) [excluding t(9;11)(p21~22;q23) and t(11;19)(q23;p13)] t(9;22)(q34;q11), -17/abn(17p), Complex (≥ 4 unrelated abnormalities)

* Irrespective of additional cytogenetic abnormalities; all favourable-risk abnormalities.
† Excluding cases with favourable karyotype; all adverse-risk abnormalities.

The MRC trials had previously stratified diagnostic cytogenetics according to patient outcome into favourable, intermediate, and adverse groups (Grimwade et al., 1998). However, uncertainty later emerged concerning a number of rare cytogenetic aberrations in about 10% of AML cases, which posed a challenge

to their prognostic significance and as a result there was a lack of consensus of risk assessment across international clinical trial groups (Grimwade et al., 2009, Cornelissen et al., 2007). To eventually enable comparison of clinical trial data from these groups, the MRC has revised the classification of cytogenetic risk groups based on the UK AML10, AML12 and AML15 trials (Grimwade et al., 2010). A cohort of 5836 younger adults (16-59 years) was recruited with successful karyotypic examination. Cox regression test did not reveal any additional cytogenetic abnormalities conferring a favourable prognosis after excluding 1462 favourable cases with t(15;17), t(8;21) and inv(16)/t(16;16) and having adjusted for age, WCC, and AML type (i.e. *de novo* or secondary AML). Intriguingly, several abnormalities, abn(3q) [excluding t(3;5)(q21~25;q31~35)], inv(3)(q21q26)/t(3;3)(q21;q26), add(5q), del(5q), −5, −7, add(7q), t(6;11)(q27;q23), t(10;11)(p11~13;q23), t(9;22)(q34;q11), −17 and abn(17p) with other changes, were found to help predicting an adverse outcome (Table 1.3). Patients with complex karyotype, i.e. having 4 or more unrelated abnormalities but not bearing the above aberrations, also presented a markedly poorer prognosis (Grimwade et al., 2010). The most recent, ongoing MRC/NCRI AML17 trial combines cytogenetics with molecular abnormalities (FLT3/NPM mutations) to determine risk groups by the risk score (http://aml17.cardiff.ac.uk/files/aml17_protocolv2.pdf), and has identified the presence of an $FLT3^{+}$/$NPM1c^{-}$ genotype as high risk.

The integration of MRD as a post-treatment prognostic factor has superseded conventional outcome prediction based on pre-treatment factors. MRD cells, often detected after intensive chemotherapy, are thought to cause disease

relapse. Tracking MRD using molecular cytogenetic (Grimwade and Freeman, 2014) or aberrant phenotypic markers (Freeman et al., 2013) may assist in predicting the long-term outcome in terms of remission or relapse. Real time PCR (RT-PCR) provides a powerful tool for monitoring MRD by detecting leukaemia-specific molecular lesions, such as gene fusions, gene mutations and gene overexpression (Grimwade and Freeman, 2014). MRD may also serve as a tool to guide patient-tailored therapy, an approach that has been adopted in childhood AML (Rubnitz et al., 2010). Ongoing studies are designed to evaluate the clinical practice of MRD monitoring in AML bearing certain mutations, such as NPM1 mutations, as promising new therapeutic targets (Schnittger et al., 2009).

1.2 Haematopoiesis and the Perception of Dormancy in AML

A small subpopulation of cells called leukaemic stem cells (LSCs) and their immature progeny, named progenitors, play an important role in the initiation and pathogenesis of AML. LSCs are considered to retain many essential 'stemness' characteristics such as a potent self-renewal capacity, a hierarchical pattern of haematopoiesis, and a predominantly dormant profile. Understanding the mechanisms regulating LSC dormancy, survival and functions as discussed blow may help us to improve the disease treatment and outcome.

1.2.1 Normal and Leukaemic Haemato-poiesis

The hematopoietic system produces blood cells through a strict regulation and hierarchy (Figure 1.2). Haematopoiesis starts with the pluripotential HSCs, which are rare cells (1 in 10^4 to 10^8 nucleated cells in the BM) that can self-renew and give rise to multiple specialised cell lineages (Li and Wu, 2011), HSCs can be functionally defined as either long-term (LT-HSC) or short-term (ST-HSC) by their capacity to provide life-long or transient haematopoiesis (Ho, 2005). LT-HSCs reside in special niche areas of the BM, which provides crucial support for adult HSCs for their maintenance of dormancy and long-standing functions, whereas ST-HSCs may be mobilised and exit the niche, actively undergo expansion and give rise to multipotent progenitors (MPPs) that lack self-renewal potential (Chumsri et al., 2007, Ho, 2005, Wilson and Trumpp, 2006, Zhang et al., 2003, Nilsson et al., 2001). MPPs in turn give rise to lymphoid-primed multipotent progenitors (LMPPs) and common myeloid progenitors (CMPs), either of which can give rise to granulocyte-monocyte progenitors (GMPs). CMP can also differentiate into megakaryocyte-erythroid progenitors (MEPs). Disruption of hematopoiesis, such as mutations or other transforming events in HSCs or the downstream progenitors or both (Figure 1.2) can lead to leukaemic transformation (Horton and Huntly, 2012, Shlush et al., 2014), characterised by increased number of immature myeloid blasts in the BM and PB.

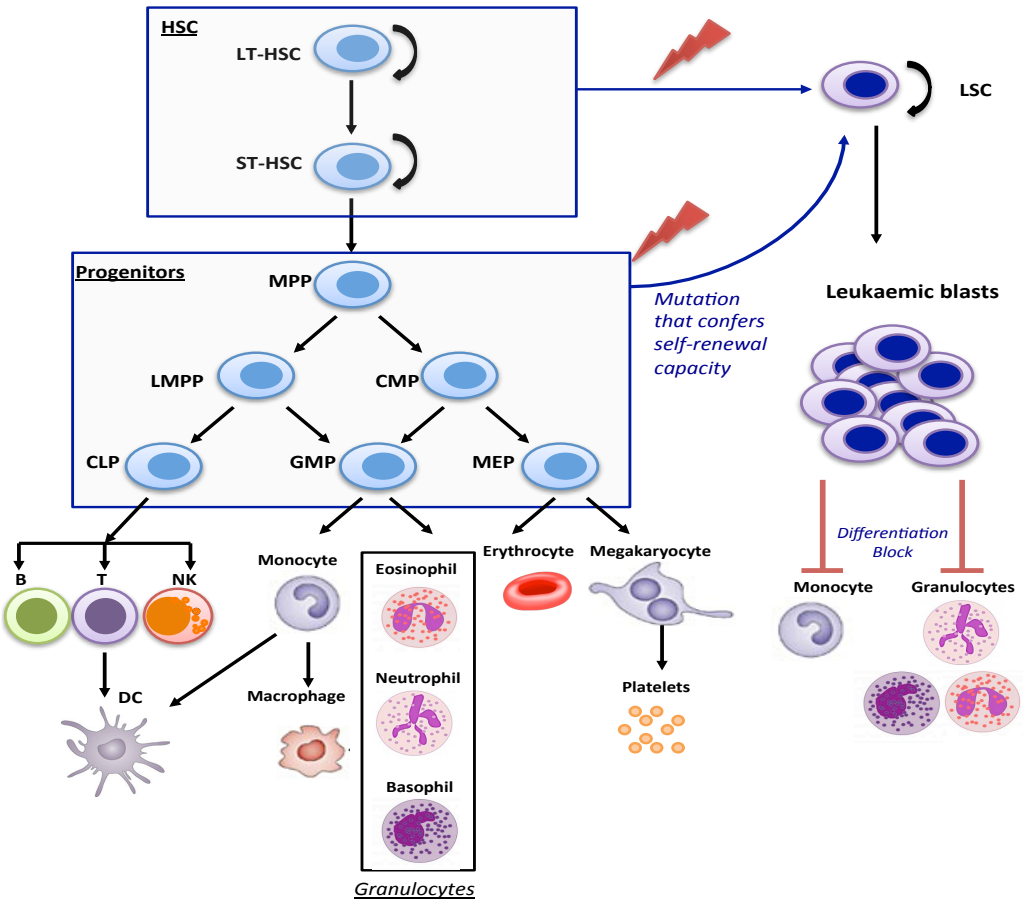


Figure 1.2. Normal haematopoiesis and the cell origin of AML. Normal HSCs display a balanced self-renewal and differentiation process. HSCs differentiate into mature blood cells via progenitor populations in a series of lineage restriction steps. HSCs first differentiate into MPPs that in turn give rise to LMPPs and CMPs. GMPs are formed from either LMPPs or CMPs, and only CMPs give rise to MEP. Mutations of HSCs initiate stem cell malignancy and transform HSCs into LSCs, which retain the self-renewal capacity. Mutations may also occur in the progenitor populations, which inherently lack self-renewal activity. The mutations therefore must confer this capacity to the progenitors in order for the transforming events to be propagated in a self-renewing (pre)-LSC. The maturation process is interrupted and arrested, manifested by the accumulation of immature leukaemic cells (blasts) in the BM. LT-HSC, long-term HSC; ST-HSC, short-term HSC; MPP, multipotent progenitor; LMPP, lymphoid-primed multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, granulocyte/macrophage progenitors; MEP, megakaryocyte/erythroid progenitors; B, B cell; T, T cell; NK, natural killer cell; DC, dendritic cell. Adapted from (Horton and Huntly, 2012).

1.2.2 Leukaemic Stem Cells

AML has emerged a paradigm for studying cancer stem cells. Leukaemia-initiating cells (LICs or SL-ICs) were first described in the 1990s by Lapidot and colleagues, when they observed selective engraftment capacity of $CD34^{+}CD38^{-}$ AML cells in nonobese diabetic/severe combined immunodeficient (NOD/SCID) and CB17-SCID mice (Lapidot et al., 1994). Following studies further confirmed the existence of these AML-initiating cells by engrafting human LSC preparations into immunodeficient xenogeneic mice models (Bonnet and Dick, 1997, Hope et al., 2004). Thus far, the ability to engraft human leukaemia cells in a xenogeneic transplantation model *in vivo* and then to serially transplant the engrafted leukaemia into secondary recipients provides the proof for the LSC concept and self-renewal function (Tan et al., 2006, Ho and Fusenig, 2011, Buss and Ho, 2011). Later mice models, such as NOD/LtSz-Fag1^{null}, NOD/SCID/IL2Rg^{null} (NSG), are engineered with extended immunodeficiency and higher engraftment rates (Shultz et al., 2000, Ishikawa et al., 2005). Later on, the NSG-3GS model was introduced, where NSG mice were engineered with add-on transgenetic expression of human growth factors, including stem cell factor (SCF), *Granulocyte-macrophage colony-stimulating factor* (GM-CSF), and Interleukin-3 (IL-3) to achieve higher permissiveness for human myeloid cells (Wunderlich et al., 2010).

1.2.3 Leukaemic Stem Cell Function in AML

An AML-initiating cell is named leukaemic stem cell, not just because it can arise from a normal stem cell, but also because it functionally satisfies the criteria for defining normal stem cells (Jordan, 2007). LSCs are essential for sustaining AML neoplasms and replenishing the leukaemic hierarchy by their distinct capacity of self-renewal, and the ability to generate clonogenic progenitors which produce large numbers of leukaemic blasts (Bonnet and Dick, 1997, Hope et al., 2004, Jin et al., 2006, Lapidot et al., 1994).

Self-renewal, with the production of at least one daughter cell that has identical stem cell properties to those of the parent cell (Dick, 2003), is often depicted as a 'hallmark' of haematopoietic stem cells. The replication of single HSCs is restricted to a limited number of times (80-200) in a lifetime (Harrison, 1983, Shepherd et al., 2007). Self-renewal and repopulation potential is commonly enhanced during leukaemogenesis (Jordan and Guzman, 2004b) and is virtually unlimited, albeit varying according to the heterogeneity of LSCs. This is surmised from their indefinite capability of supporting leukaemic clonal expansion continuously and to be inexhaustibly propagated in murine serial bone marrow transplantation (Hope et al., 2004, Warner et al., 2004). There is a linkage between mechanisms of self-renewal and genomic stability. Genes with oncogenic self-renewal potential, such as Homeobox protein Hox-A9 (HoxA9), which are expressed in $CD34^+$ marrow cells and often upregulated in AML, have been shown to induce cytogenetic abnormalities when unregulated (Thorsteinsdottir et al., 2002, Lawrence et al., 1997, Lawrence et al., 1999).

Upon activation, HoxA9 reduces genomic stability and subsequently subjects stem cells to oncogenic events which lead to deregulated self-renewal activities.

A distinct degree of cellular heterogeneity is an integral character of primary tumours in many tissues including blood. Various types of cells are generally found in the clonal tumour populations, suggesting that distinct mechanisms, such as genomic instability in the malignant population and intrinsic development processes within stem cell-based hierarchies, may exist to drive the process of cell differentiation from the tumour-initiating cells (Jordan and Guzman, 2004a, Passegue et al., 2003). This explanation is supported by substantial evidence from extensive studies on cancer stem cells, and the earliest evidence indicating the stem cell origin for cancer dates back to the mid-1960s, when parallel studies on a small subset of murine leukaemia cells showed similar results to normal haematopoietic stem and progenitor cells in producing clonally derived colonies *in vitro* and *in vivo* (Bruce and Van Der Gaag, 1963, Wodinsky et al., 1968). Successive studies in leukaemia patients used the X-linked gene glucose-6-phosphate dehydrogenase (G-6-PD) to examine the heterozygosity for the A and B isoenzymes of the haematopoietic populations (Fialkow, 1977, Fialkow et al., 1977). By investigating single-enzyme phenotypes in the multiple haematopoietic lineages, these studies provided further evidence of the clonal origin of leukaemic stem cells and that they are the site of abnormality in myeloid leukaemias.

1.2.4 Stem Cell Dormancy in AML

In contrast to invertebrate germline stem cells which are vastly active in the cell cycle, human HSCs and LSCs predominantly survive in a deep, long-term 'dormant' state (Essers and Trumpp, 2010), accumulate at a 2n DNA content and are arrested outside the cell cycle (G_0 phase), rather than just pausing or passing through at a prolonged, slow G_1 phase (Coller, 2007, Smith and Martin, 1973). Whilst the term 'quiescence' generally refers to a reversible G_0 growth arrest (either transient or long-term), the concept of cellular 'dormancy' represents a stage of long-term quiescence, where quiescent cells retain the capacity to proliferate again (Chomel and Turhan, 2011, Viale and Pelicci, 2009). Although not exactly identical, the two terms ('quiescence' and 'dormancy') are often used synonymously (Chomel and Turhan, 2011). The long-term LSC dormancy is of major importance in AML and is one of the key factors in cancer biology in general. Dormancy is likely a critical mechanism for the observed resistance of LSCs to anti-proliferative and other chemotherapy regimens as they are not only non-cycling, but also require less energy (Trumpp and Wiestler, 2008, Essers and Trumpp, 2010), and are thought to be the culprit of MRD and disease relapse after treatment (Paez et al., 2012). Therefore identifying and characterising the dormant leukaemic populations would help us to further understand chemoresistance and improve therapeutic regimens for AML.

1.2.5 Dormancy And The Cell Cycle

The key control point regulating the cell cycle progress from G_1 to S phase is defined as the 'restriction point (R point)' by Pardee (Pardee, 1974). It is described as a unique point in the G_1 phase of the mammalian cell cycle that nutrient-deprived or inhibited cells come to rest. Traversing the R point is regarded as the critical event in cellular proliferation control (Lundberg and Weinberg, 1999). Studies have provided convincing evidence consistent with this hypothesis and have indicated that dormant cells are not just arrested within a cell cycle. In a study on cultured 3T3 fibroblasts using video-microscopy (Zetterberg and Larsson, 1985), cell cycle of serum-deprived cells was timed from one mitosis to the next. It was shown that the duration of the cell division was closely related to the time point when serum was withdrawn. For early G_1 phase cells that started the cycle for less than 3.5 hours, an elongated G_1 phase was observed in the cell cycle, with an approximate 8-hour delay. Interestingly, those cells that were older than 3.5 hours in the cell cycle did not respond to the serum withdrawal. This finding supports the idea that dormant cells are different from the cycling cells, although they both have 2n DNA contents.

It has been suggested that the most important difference between cycling and dormant cells is whether the proteins liable for the initiation of DNA replication remain chromatin-bound or not (Coller, 2007). One of these dormant markers is CDC6, a specific E2F target that plays critical roles in the replication complex formation. Whether cells will progress from G_1 to S phase or enter dormancy depends on the localisation of CDC6 on chromatin. A

previous study on Xenopus laevis eggs showed that immuno-depletion of CDC6 inhibits replication of dormant nuclei but not their 'growing' counterparts (Madine et al., 2000). Origins of replication, facilitated by licensing molecules including CDC6, the origin replication complex (ORC), the origin-associated protein CDT1, and replicative helicase minichromosome maintenance proteins (MCMs), are exclusively 'licensed' in the G_1 phase, where preparation and firing of genome replication take place only once in the cell cycle and the license is removed after S phase is complete. In S phase, phosphorylation of CDC6 and CDT1 by the cyclin-dependent kinases (CDKs) leads to export of CDC6 from the nucleus, ubiquitination and degradation of CDT1. Down-regulation of CDC6 and CDT1 disrupts loading of MCM proteins and inhibits replication origin relicensing.

1.2.6 Dormancy Is Distinct From Senescence And Terminal Differentiation

Cellular dormancy should be distinguished from cellular senescence and terminal differentiation. Figure 1.3 illustrates the relationship between these non-cycling cell states and the cell cycle. As previously stated (in Section 1.2.4), the term 'dormancy' implies the ability of a cell to re-enter the cell cycle from the G_0 resting state. Unlike dormant cells, senescent cells are permanently withdrawn from the cell cycle, and have irreversibly lost their proliferative potential (PP) - the potential to resume proliferation (Blagosklonny, 2012, Campisi, 2011). In addition to losing the ability to divide, senescent cells are characterised by enlarged cell morphology,

increased intracellular reactive oxygen species (ROS) production, altered intracellular organelles, and the appearance of distinct markers, such as senescence-associated β-galactosidase (SA-β-Gal) and senescence-associated heterochromatin foci (SAHF) (Leontieva et al., 2012, Campisi, 2011, Hwang et al., 2009). Conversion from proliferative arrest to senescence, termed 'geroconversion', is in part driven by growth promoting pathways, such as the mammalian target of rapamycin (mTOR), which is involved in aging (Leontieva et al., 2012). mTOR inhibition by its inhibitors or hypoxic conditions suppresses senescence during cell cycle arrest. As a result, cells stay dormant rather than senescent (Demidenko and Blagosklonny, 2009, Demidenko et al., 2009, Pospelova et al., 2009, Leontieva et al., 2012). Furthermore, cellular senescence, cancer, and aging all have the telomere connection (Campisi et al., 2001). Telomeres are the repetitive DNA sequences and specialised proteins that cap the ends of linear chromosomes, allowing cells to distinguish the chromosome ends from DNA double strand breaks (DSB). Telomere length, structure and organisation, which are strongly associated with cancer and aging, can be influenced by basic cellular processes such as proliferation, differentiation, and DNA damage. It is thought that shortened and unprotected telomeres together with serial cell divisions and DNA damage response are responsible for replicative senescence (Campisi et al., 2001, Hwang et al., 2009). Senescent growth arrest cannot be reversed by known physiological stimuli (Rodier and Campisi, 2011). However, some senescent cells that do not express p16INK4a can resume growth via genetic manipulations which inactivate the tumour suppressor p53 (Beausejour et al.,

2003). But there is no evidence so far, that p53 inactivation occurs spontaneously in senescent cells either *in vitro* or *in vivo*.

Terminally differentiated cells are non-proliferative, mature lineage cells arise from a small number of pluripotent stem cells. They share many characteristics with dormant and senescent cells, including a 2n DNA content, the presence of hypophosphorylated Rb proteins that mediate inhibitory E2F activity, and high cyclin-dependent kinase inhibitor (CKI) activities (Pajalunga et al., 2007). Unlike cell dormancy, terminal differentiation is usually coupled with permanent exit from the cell cycle and represents the most common cellular state in adult mammalian cells (Buttitta and Edgar, 2007). Intriguingly, several non-mammalian vertebrate species possess an ability to naturally regenerate/de-differentiate (Jopling et al., 2011). And an increasing number of regeneration studies shows that in certain mammalian cell types, terminally differentiated cells can be reprogrammed by defined conditions to pluripotency, as observed in amphibian limbs and mature B lymphocytes (Tsonis, 2000, Hanna et al., 2008), although the reversal of differentiation is rather difficult in other cell types such as the skeletal muscle (Camarda et al., 2004). A recent study by Riddell and colleagues from Harvard University successfully reprogrammed committed myeloid and lymphoid progenitors, as well as myeloid effector cells (including monocytes, macrophages, and granulocytes) from mice into HSCs through a cocktail of functionally screened HSC-specific transcription factors, and demonstrated that the induced HSCs have the functional hallmarks of normal HSCs, are able to self-renew, and can give rise to all of the cellular components of the blood (Riddell et al., 2014).

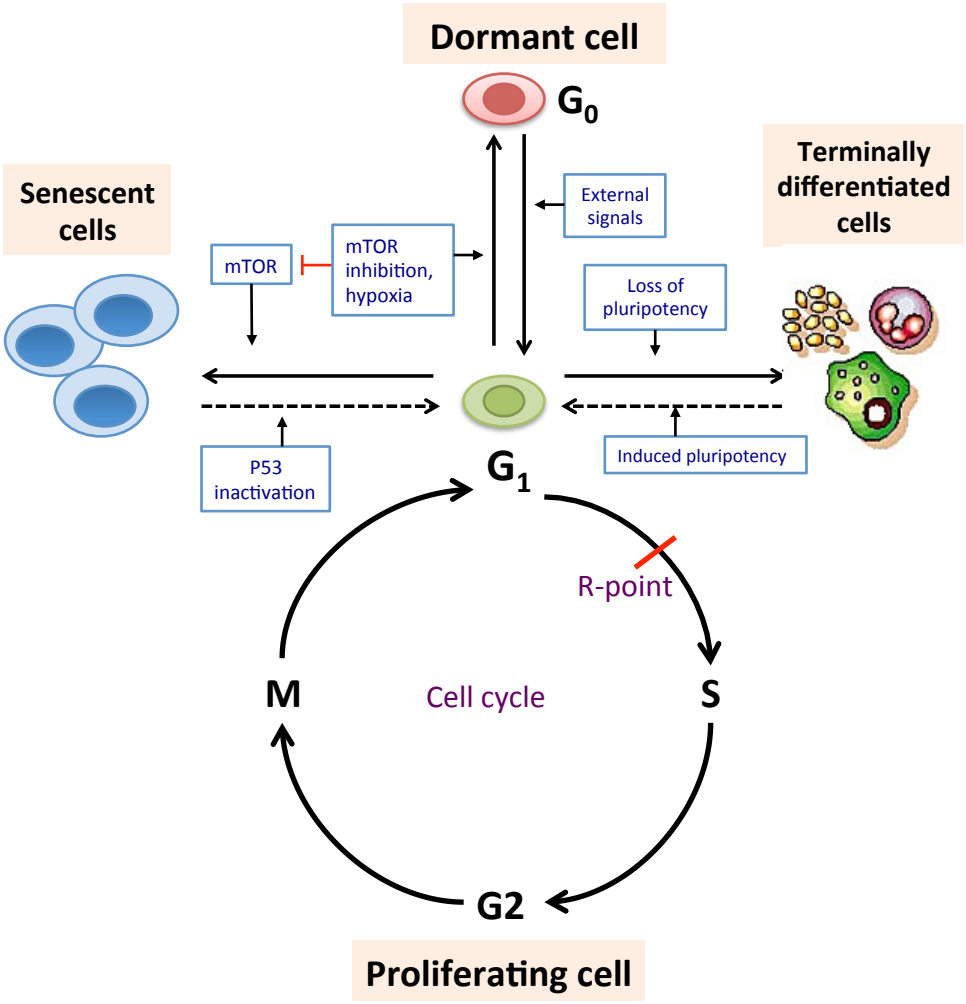


Figure 1.3. Reversibility of dormancy, senescence, and terminal differentiation in blood cells. Cells are able to enter the reversible (dormant) or irreversible (senescent and terminally differentiated) resting states from the G_1 phase of the cell cycle before the R-point. Once reaching the R-point, cells are committed to the whole cell cycle. In response to external signals, dormant cells enter the cell cycle and become activated. The cell fate is determined during G_1, where cells differentiate, become senescent or re-enter dormancy. Essentially, both senescent cells and terminally differentiated cells are permanently withdrawn from the cell cycle. However, recent studies suggest that this exit is reversible (shown in dotted lines) by artificial reprogramming and genetic manipulations.

1.2.7 Regulation of Stem Cell Dormancy and Functions in AML

HSCs and LSCs must stay dormant to maintain their 'stemness' (Chen et al., 2008). But how dormancy and long-term function of the stem cells is associated is poorly understood (Zhang et al., 2006, Cheng et al., 2000, Zhang et al., 2003). Studies have indicated that genetic disruption of stem cell dormancy would invariably inactivate the stem cell function. To date, several intrinsic mechanisms, including cell cycle regulation, pro-survival pathways, pathways regulating oxidative stress, and molecular mechanisms promoting self-renewal, and the activation of extrinsic pathways, such as transforming growth factor-beta (TGF-β), and Notch signaling, are known to be involved in the regulation of leukaemic stem cell survival and dormancy (Pietras et al., 2011, Konopleva and Jordan, 2011). Interactions between stem cells and the BM niche also play critical roles in the long-term maintenance of dormancy (Spradling et al., 2001).

1.2.7.i The Intrinsic Regulatory Mechanisms in LSC Dormancy and Functions

LSC survival may be facilitated by the molecular mechanisms and signalling pathways that are essential for maintaining normal HSC dormancy (Konopleva and Jordan, 2011). Dormancy in haematopoietic cells is strictly regulated by cell cycle regulatory genes, such as the Rb proteins, E2F, p53, p21, and p27, on the protein level (Matsumoto and Nakayama, 2013). The tumour suppressor gene p53 is well known for its importance in cellular response to DNA damage recognition and DNA repair activation. Evidence suggests that p53 also plays

essential roles in HSC dormancy in steady-state haematopoiesis (Lacorazza et al., 2006, Liu et al., 2009). p53 is more preferentially expressed in HSCs than in progenitor cells, and promotes dormancy (Li and Bhatia, 2011, Liu et al., 2009). An important interdependency between E-twenty-six (ETS), transcription factor myeloid ELF-1-like factor (MEF) or ELF4 and p53 on HSC dormancy has been identified (Liu et al., 2009). MEF/ELF4 regulates p53 expression and mediates stem cell entry into the cell cycle from dormancy. MEF-null mouse embryonic fibroblasts exhibit accumulation of p53 protein and premature dormancy, which may be due to modulation of p53 expression and direct inhibition of the oncogene, mouse double minute 2 homolog (Mdm2), an important negative regulator for p53, by MEF/ELF4 (Liu et al., 2009). Similarly, MEF-null HSCs have shown increased p53-dependent dormancy and are resistant to myelosuppressive chemotherapy and radiation (Liu et al., 2009, Lacorazza et al., 2006). p53 is also recognised as an important modulator of the G1/S cell cycle checkpoint. Activation and stabilisation of p53 in turn activates the transcription of the CDK2/Cyclin E inhibitor p21, a major target of p53 (Shiloh, 2003) and a member of the CDK interacting protein/Kinase inhibitory protein (CIP/KIP) family of CKIs, which are known for their roles as negative regulators of G_1-phase cell-cycle progression (Denicourt and Dowdy, 2004). p21 governs stem cell pool size under conditions of stress and prevents HSC exhaustion by restricting HSC entry into the cell cycle (Zhang et al., 2006, Zhang et al., 2003, Cheng et al., 2000). In AML, LSCs over-express p21 to preserve their dormancy. Studies on the p21-/- mouse model provided evidence that increased HSC cycling and proliferation lead to stem cell exhaustion after serial BM transplantation (Cheng et al., 2000).

However, p21 may only play a minimal role in HSC self-renewal under steady-state conditions (van Os et al., 2007). Two other p53 target genes, growth factor independent-1 (Gfi-1) and Necdin have also been identified as modulators for HSC dormancy (Hock et al., 2004, Liu et al., 2009).

The other CIP/KIP family members, p27 and p57, are found to cooperatively maintain haematopoietic stem cell dormancy through interactions with the Hsc70 heat shock protein. An association between p57, p27 and Hsc70 was found to maintain the cytoplasmic localisation of the Hsc70/cyclin D1 complex and regulate the cell cycle entry of HSCs (Zou et al., 2011). The intracellular expression of Hsc70 is also found to be reversely correlated to the susceptibility of AML cells to apoptosis (Chant et al., 1996).

The tuberous sclerosis complex (TSC) - mTOR pathway has been recognised as a key regulator for numerous cellular functions, including protein synthesis, autophagy, endocytosis and nutrient uptake (Wullschleger et al., 2006, Chen et al., 2008). Several lines of work provided evidence for the critical link between this pathway and HSC dormancy. It has been indicated that the negative regulators for mTOR, such as the phosphatase and tension homologue (Pten) (Yilmaz et al., 2006, Zhang et al., 2006, Shen et al., 2007) and promyelocytic leukaemia protein (PML) (Bernardi et al., 2006, Blagosklonny, 2008) may maintain HSC dormancy. Pten is a negative regulator of phosphoinositide 3-kinase (PI3K) and Akt (also known as protein kinase B, PKB). It interrupts the inhibition of these two kinases for TSC, and restricts HSC activation, which results in reduced LT-HSC function (Yilmaz et al., 2006, Zhang et al., 2006).

Pten also plays critical roles in lineage fate determination, and prevention of leukaemogenesis (Zhang et al., 2006). Mice with Pten mutation in the BM present increased myeloid and T-lymphoid lineages, and develop myelo-proliferative disorders (MPD) (Kogan et al., 2002). Moreover, cells expanding in Pten-mutants are found to match those dominant in AML/ALL arising from MPD (Zhang et al., 2006). Although often observed in solid tumours, Pten mutations are rare in AML (Cheong et al., 2003, Liu et al., 2000). PML, a tumour suppressor gene highly expressed in HSC and chronic myeloid leukaemia (CML) LSC, is required for stem cell maintenance by repressing mTOR activity (Ito et al., 2008). PML is also involved in the t(15;17) chromosomal translocation of acute promyelocytic leukaemia (Bernardi and Pandolfi, 2007). Studies show that deletion of Pten or mTOR in mice results in stem cell hyper-proliferation and exhaustion which rapidly evolves acute myeloid leukaemia (Yilmaz et al., 2006, Blagosklonny, 2008). Up-regulated TSC-mTOR activity is also observed in association with a higher level of intracellular ROS, which plays a critical role for regulating HSC maintenance. Targeted mutations of TSC1 lead to increased biogenesis of mitochondrion and ROS accumulation (Jang and Sharkis, 2007). Stem cells are highly sensitive to ROS which cause functional defects. However, these defects can be effectively restored by inhibiting ROS activity *in vivo* (Chen et al., 2008). Thus, the TSC-mTOR pathway plays an important role in regulating dormancy and LT-HSC function.

Inhibition of oxidative stress by the protein kinase ataxia telangiectasia-mutated (ATM) is indispensable for maintenance of HSC self-renewal and

dormancy (Ito et al., 2004). ATM-deficient mice exhibit increased ROS, less dormancy in HSCs and progressive bone marrow failure (Ito et al., 2004). Reversal treatment with antioxidants can recover ATM functional defects. ATM has also shown striking multi-tasking ability in regulating cell cycle checkpoints upon its activation by DSB damage, telomere erosion, and oxidative stress (Shiloh, 2003). This protein kinase regulates G1/S checkpoint by phosphorylating Ser15 on p53, the key regulator of G1/S transition, which is subsequently activated as a transcription factor (Ashcroft et al., 1999, Dumaz and Meek, 1999). ATM phosphorylates and activates checkpoint kinase 2 (CHK2), which phosphorylates and activates p53 on Ser20 (McGowan, 2002). Interestingly, it also contributes the degradation of p53 by directly phosphorylating the p53 inhibitor, mouse double minute 2 homolog (Mdm2), on Ser395 (Maya et al., 2001).

The mammalian FoxO family of transcription factors (FoxO1, FoxO3, FoxO4, and FoxO6) plays an important role in mediating the PI3K/Akt pathway (Greer and Brunet, 2005). Activated by oxidative stress (Brunet et al., 2004), FoxOs control G0/G1, G1/S, and G2/M checkpoint transitions by directly activating or repressing target genes, including Rb/p130, p21, p27, Cyclin G2 (G0/G1 arrest) (Martinez-Gac et al., 2004), Cyclin D (G1/S arrest) (Kops et al., 2002), Cyclin B and polo-like kinase (G2/M arrest) (Alvarez et al., 2001, Tothova et al., 2007). Studies indicated that HSCs from FoxO3-deficient mice had elevated levels of ROS, and increased number of cycling compartment, defective long-term self-renewal ability, resulting in apoptosis and a limitation in repopulating abilities (Tothova et al., 2007). Driven out of dormancy, these HSCs

accumulate in S/G2/M, or G1 phase of the cell cycle, resulting in stem cell pool exhaustion (Tothova and Gilliland, 2007, Miyamoto et al., 2007). Nonetheless, HSC defects caused by FoxO-deficiency can be reversed by antioxidant rescue (Tothova et al., 2007).

A functional connection was identified between FOXO3 and the transcriptional activation of the tumour suppressor gene Liver Kinase B1 (LKB1), which is known for blocking cell cycle progression through direct phosphorylation of different AMP-activated protein kinase (AMPK) family members (Tiainen et al., 1999, Shaw et al., 2004b, Shaw et al., 2004a, Hawley et al., 2003, Cheng et al., 2009). AMPK is found to protect the dormant LSCs from metabolic stress which is characterised by high AMP and glucocorticoid concentrations and may disrupt leukaemogenesis (Saito et al., 2015a, Lagadinou et al., 2013, Wang et al., 2014). In the event of metabolic stress, AMPK switches on glycolysis, an ATP-producing pathway and sustains energy homeostasis by inhibiting ATP-consuming processes (Hardie, 2007). Deletion of AMPK leads to LSC depletion in the hypoxic BM environment by attenuating glucose metabolism and in return suppresses AML (Saito et al., 2015a). In contrast, inhibition of AMPK does not affect normal HSC function (Nakada et al., 2010), providing a therapeutic window in AML treatment.

1.2.7.ii The BM Microenvironment and the Extrinsic Factors Regulating Dormancy in AML

Like HSCs, LSCs reside in specialised areas in the BM, namely osteoblastic (endosteal) and vascular niches (Perry and Li, 2007). The osteoblastic niche, consisting of abundant bone-forming osteoblasts with the proximal stromal cells and generally hypoxic, plays an important role in sustaining long-term functions and dormancy of stem cells (Arai et al., 2005, Perry and Li, 2007), through release of soluble factors such as stroma-derived factor-1 (SDF-1/CXCR12), angiopoietin-1 (Ang-1), osteopontin, and cell contact interaction factors such as SCF and N-cadherin (Zhang et al., 2003). The vascular niche, on the other hand, is thought to provide support for the growth and differentiation of fast-cycling, short-term stem cells (Kopp et al., 2005). The niche systems work in an inter-cooperative fashion. In response to BM damage, a special vascularised niche is formed by the endosteum to support expansion of HSCs (Xie et al., 2009). Both osteoblastic and vascular niches are essential for LSC survival, proliferation and differentiation (Arai et al., 2004, Naveiras and Daley, 2006, Nilsson et al., 2005, Zhang et al., 2003).

Normal haematopoiesis needs homeostatic interactions between the BM niche and the HSCs, which is pivotal for the maintenance of HSC self-renewal. SDF-1, which is highly expressed in the BM HSC niches, is a key molecule governing HSC maintenance. In leukaemia patients, SDF-1 levels are elevated and normal haematopoiesis is often suppressed (Mirshahi et al., 2009). It is suggested that the signaling mechanisms of BM niches for normal HSC homing may be ‘hijacked’ by LSCs (Konopleva and Jordan, 2011, Lane et al.,

2009) and a tumour-like microenvironment in the BM is created by leukaemic cells (Colmone et al., 2008). LSCs escape chemotherapy by taking refuge in the protection of the niche, which provides nurturing cytokines to LSCs. Cell surface chemokine receptor CXCR4 is a predictor of increased relapse risk in AML (Rombouts et al., 2004). SDF-1 binding to CXCR4 plays a key role in governing HSC function and localisation (Tavor and Petit, 2010). AML cells retain their responsiveness to SDF-1. It is suggested that SDF-1/CXCR4 interactions contribute to the chemoresistance of leukaemic cells to chemotherapy-induced apoptosis. Studies using *in vitro* murine stromal cell models, mimicking the physiological BM microenvironment, have demonstrated that inhibition of CXCR4 diminished the protective effects of stromal cells on chemotherapy induced AML apoptosis (Zeng et al., 2006, Zeng et al., 2009). CXCR4 signalling can be activated by FLT3 gene mutation and AML/stroma coculture significantly reduced the effects of FLT3 inhibitors in cells carrying FLT3 mutations (Zeng et al., 2006, Zeng et al., 2009). It has also been reported that under niche-like conditions with fribronectin (FN) and a cocktail of cytokines, *in vitro* toxicity of FLT3 inhibitors such as AG1296 is significantly reduced in FLT3-mutated AML cells, particularly in the dormant subpopulation (Mony et al., 2008). The CXCR4 inhibitor AMD3100/Plerixafor has been used in priming chemotherapy for the relapsed and refractory AML patients, sensitising leukaemic cells to chemotherapy and improving the CR rate (Shen et al., 2015, Uy et al., 2012). SDF-1 activates adhesion molecules VLA-4 and CD44, which are expressed on leukaemic cells, and regulate cell growth and chemoresistance (Tavor and Petit, 2010). VLA-4 is considered a crucial molecule maintaining BM MRD (Matsunaga et al., 2003). Interaction

between VLA-4 and stromal cells upregulates the PI3K/Akt pathways and increases the level of anti-apoptotic molecule B-cell lymphoma-2 (Bcl-2). Increased binding of VLA-4 with soluble vascular cell adhesion molecule 1 (VCAM-1) has been associated with longer OS in AML patients (Becker et al., 2009). CD44 supports leukaemic cell proliferation, differentiation arrest, and BM retention (Charrad et al., 1999). Higher expression of the isoform CD44-6v on AML cells is correlated with shorter OS (Legras et al., 1998). Wnt/β-catenin signalling plays a key role in the BM niches in maintenance and self-renewal of dormant LSCs (Wang et al., 2010). Selectively inhibiting this pathway may impair cell dormancy. Anti-Wnt ligand monoclonal antibodies have shown promising effects in suppressing tumour growth *in vivo* (Takahashi-Yanaga and Kahn, 2010).

Hypoxia is present in the endosteal region of the BM niche, which is thought to contribute to stem cell dormancy, self-renewal, and chemoresistance in AML (Nilsson et al., 2001, Ishikawa et al., 2007). The key molecular response to hypoxia is the stabilisation of hypoxia-inducible factors (HIFs), a transcription factor family consisting of the O2-labile α subunits (HIF-1α, 2α and 3α) and the constitutively expressed β subunit (HIF1β), that activates over 100 target genes involved in the processes of angiogenesis, metabolism, and proliferation (Semenza, 2012, Semenza and Wang, 1992). Deletion of HIF1α in HSCs leads to a loss of self-renewal during serial transplantation (Takubo et al., 2010). HIF1α might also be activated by other signals in the BM, such as thrombopoietin (TPO) and SCF, which in return stabilise HIF1α in the absence of hypoxia (Nombela-Arrieta et al., 2013). Under normoxia, HIF1α plays an

important role in the maintenance of AML LSC proliferation and self-renewal by repressing a negative feedback loop in the Notch pathway (Wang et al., 2011, Rouault-Pierre et al., 2013). In addition, SCF-induced HIF1α is transcriptionally active, and the accumulation of HIF1α is dependent on the PI3K/Akt/mTOR growth pathway (Pedersen et al., 2008). Interestingly, studies found that LSCs require higher levels of HIF1α for their maintenance compared with normal HSCs, which makes them more sensitive to HIF inhibition (Zhang et al., 2012). Therefore, a therapeutic window that destroys LSCs but spares normal cells may exist.

Several HIF1α target genes are also involved in dormancy regulation. The hairy and enhancer of split-1 (HES1), a transcription factor that is activated via Notch signalling, is required for cell dormancy to be reversible. It prevents both premature senescence and inappropriate differentiation in dormant fibroblasts (Sang et al., 2008) and also protects tumour cells from differentiation (Sang et al., 2010). More recently, it was reported that HES1 suppresses AML development through FLT3 gene repression (Kato et al., 2015). The HIF1 inhibitor, the transcriptional co-activator CBP/p300-interacting-transactivator-with-an ED-rich-tail 2 (CITED2), competes with HIF1 for binding with CBP/p300 and thereby to displace HIF1 and downregulate its transcriptional activity (Bhattacharya et al., 1999, Freedman et al., 2003). It is suggested that CITED2 essentially regulates HSC dormancy through HIF1-dependent and HIF1-independent pathways (Du et al., 2012). Studies found that additional deletion of HIF1α in CITED2-knockout BM partially restored impaired HSC dormancy and reconstitution capacity caused

by CITED2 deficiency (at the transcriptional level, deletion of HIF-1α restored expression of p57 and Hes1 to normal levels) (Du et al., 2012). The fact that CITED2 expression is essential for HSC maintenance suggests that it could play a critical role in LSC maintenance. AML cells were found to have an elevated CITED2 expression (Andersson et al., 2007). Also, $CD34^+$ cells from a subset of AML patients displayed higher CITED2 expression compared with normal $CD34^+$ HSCs (Korthuis et al., 2015). In addition, knockdown of CITED2 in AML $CD34^+$ cells led to a loss of long-term expansion *in vitro* and *in vivo* (Korthuis et al., 2015).

The transcription factor gene GATA binding protein 2 (GATA2) is shown to increase dormancy (G_0 residency) of human and murine HSCs (Tipping et al., 2009). And transcriptional profiling has shown high expression of GATA-2 in dormant haematopoietic cells (Ezoe et al., 2002, Venezia et al., 2004). The interaction between GATA2 and Notch1 is essential for the onset of definitive haematopoiesis found in murine studies (Robert-Moreno et al., 2005). Also, GATA2 acts as a molecular entry point into the transcriptional programme regulating dormancy in human HSCs and progenitor cells, indicated by its activity inhibiting cell cycle *in vitro* and *in vivo* (Tipping et al., 2009).

1.3 AML Chemotherapy

1.3.1 The Traditional AML Chemotherapy

The mainstay of traditional chemotherapy is generally divided into two phases: remission induction and post-remission consolidation therapy. The aim of

induction treatment in AML is to achieve CR. The front-line regimen is based on a combination of the nucleoside analogue Ara-C (also called cytosine arabinoside or Cytarabine) and an anthracycline, such as daunorubicin (Herzig et al., 1985, Champlin and Gale, 1987, Ravandi et al., 2004). Ara-C is an antimetabolic agent which interferes with the synthesis of DNA and therefore mainly kills cells in the S-phase (Ravandi et al., 2004). Daunorubicin stabilises the complexes between DNA and the nuclear enzyme topoisomerase II, after the DNA chain has been broken for replication and thereby preventing the formation of DNA helices and causing the accumulation of double-stranded breaks (Come et al., 1999). This induction therapy is followed by postremission chemotherapy, which often includes multiple cycles of Ara-C, in patients who achieve remission to eradicate the residual leukaemia cells and to prevent relapse (Champlin and Gale, 1987, Jordan and Guzman, 2004a).

The limitation of the conventional regimen is that the drugs mainly target the rapidly proliferating progenitor cells in the cell cycle, but not the more primitive and mainly dormant LSCs, which may also possess natural mechanisms of survival such as drug efflux pumps (Jordan and Guzman, 2004a). Also, patients who have relapsed once are at high risk of relapse in the future, which leads to a reduced chance of achieving second remission and increases chemo-resistance. This clinical observation suggests that for a stem cell disease like AML (Blair and Sutherland, 2000, Bonnet and Dick, 1997, Lapidot et al., 1994), the old regimens do not effectively target the source of the disease.

In leukaemia research, a current major focus on LSCs is helping to improve our understanding of these primitive malignant cells, and is a promising way to the development of novel therapeutic strategies to target and eliminate the leukaemic stem and progenitor cells and to improve patient survival.

1.3.2 Targeted Therapy

Contemporary research on leukaemia therapy has focused on eliminating LSCs and developing anti-LSC strategies. Also, it is important that normal HSCs remain unharmed while novel drugs targeting the specific properties of LSCs. Cell surface markers may be exploited for antibody-based therapies. Ideally, the target antigen would be exclusively expressed on LSCs in abundance, so as to maximise the targeted therapeutic effects and minimise side effects (ten Cate et al., 2010). The search for such a 'perfect' antigen is still on-going. CD123 (Jordan et al., 2000), CD25 (Saito et al., 2010), CD32 (Saito et al., 2010), CD47 (Majeti et al., 2009), CD44 (Jin et al., 2006), CD96 (Hosen et al., 2007), CLL-1 (van Rhenen et al., 2007b), and CD33 (Taussig et al., 2005) have all been demonstrated to be differentially expressed on AML LSC compared with normal HSC.

Therapeutic monoclonal antibodies work by a number of different mechanisms to target leukaemia cells (Horne et al., 2015), either indirectly by inducing cell lysis, phagocytosis, and eventually cell death, or directly by targeting cells through conjugation to a cytotoxin, a drug, radionuclides or other cytotoxic substances to induce cytotoxicity (Figure 1.4).

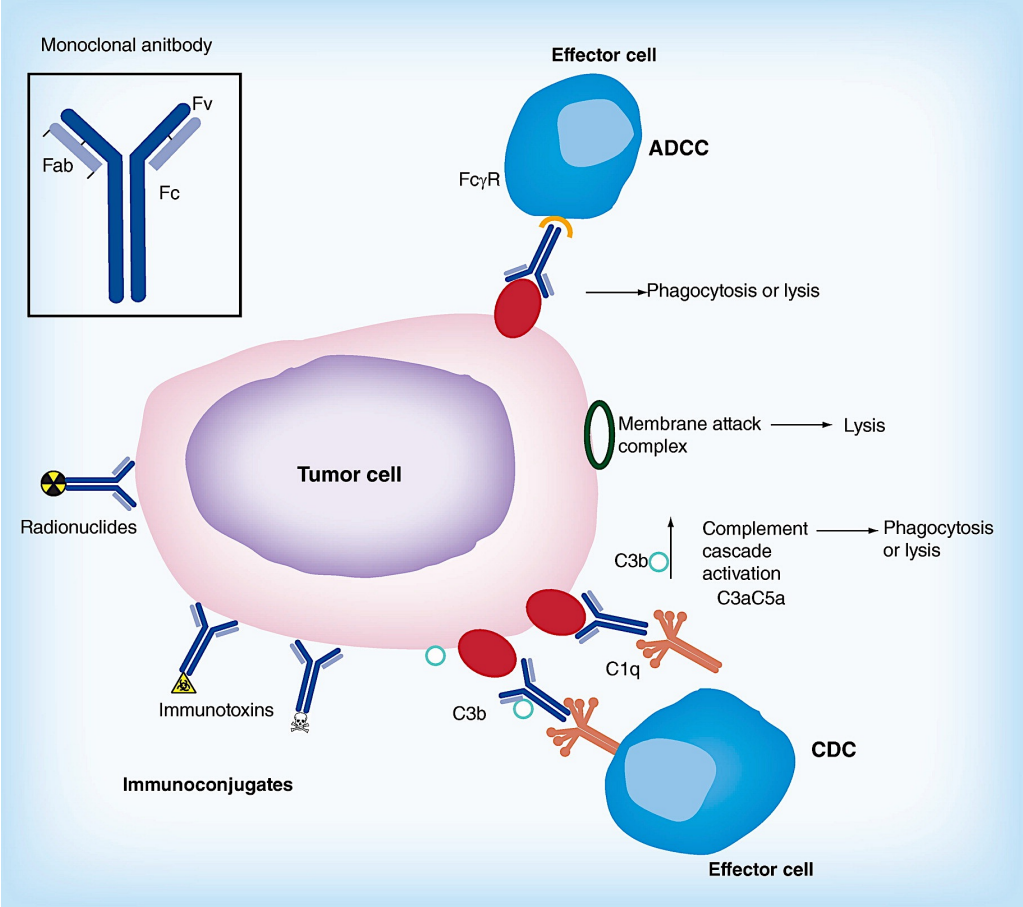


Figure 1.4. Mechanisms of action of monoclonal antibody-mediated therapy to induce cell death. An immune process, namely complement dependent cytotoxicity, occurs following binding of an antibody to an antigen, where the 'complement cascade' is activated. This leads to the formation of the terminal membrane attack complex in the cell membrane leading to lysis and death. Antibody-dependent cellular cytotoxicity occurs when the Fc receptor of effector cells binds to the Fc portion of an antibody that has bound to a target cell. Following binding the effector cell can release cytokines for example that cause lysis of the target cell. Therapeutic monoclonal antibodies can also directly target cells by being conjugated to radionuclides, toxins, drugs and other substances that cause cells to die. *ADCC: Antibody-dependent cellular cytotoxicity; CDC: Complement-dependent cytotoxicity.* From Horne, G. A., et al. (2015). *Pharm Pat Anal.* 4(3): 187-205. (Horne et al., 2015)

Antibody-dependent cellular cytotoxicity (ADCC) occurs after binding of the Fc portion of an antibody to the Fc receptor (FcR) on the effector cell surface. ADCC is typically mediated by activation of NK cells, but neutrophils,

macrophages and eosinophils also participate in ADCC. Upon binding to the FcR, the NK cells release cytokines (such as IFN-γ), which then send signals to other immune mediators and release cytotoxic factors to initiate apoptosis in the target cell (Horne et al., 2015). Monoclonal antibodies to CD123 (CSL362) have shown great effectiveness in enhancing ADCC through NK cells *in vitro* and *in vivo* (Busfield et al., 2014).

A good example for the antibody-cytotoxin conjugation is Mylotarg, a humanised monoclonal murine antibody (hP67.6) targeting CD33 antigen, with the antibiotic calicheamicin γ1 derivative attached through a bi-functional linker (van Der Velden et al., 2001, Larson et al., 2005, Stasi et al., 2008). Calicheamicin is a highly potent anti-tumour antibiotic that induces DSBs at specific sequences (Stasi et al., 2008). CD33 is expressed on more than 90% of AML myeloid blasts but is not found on HSCs (van Der Velden et al., 2001, Hauswirth et al., 2007), making CD33 a good target for specific drug delivery.

A study recently elucidated a novel therapeutic strategy by targeting stem cell-specific lipid metabolic pathways in normal and malignant haematopoiesis in order to eradicate LSCs while sparing HSCs. It was found that sphingolipid enzymes were differentially regulated throughout the haematopoietic hierarchy in particular. Myriocin, which is involved in the first step of sphingolipid synthesis, decreased AML cell viability and altered differentiation *in vitro* and reduced leukaemia burden *in vivo* following serial transplantation into NOD/SCID mice transgenic for human cytokines. In contrast, myriocin or its

derivative FTY720 treatment did not disrupt engraftment in mice bearing normal HSC grafts (Xie et al., 2014).

Disruption of adhesive interactions of residual cells after chemotherapy may be a favourable novel therapy for MRD. It is unclear whether targeting the SDF-1/CXCR4 interaction could effectively eradicate LSCs in the BM after standard chemotherapy (Tavor and Petit, 2010). CXCR4 inhibitors are considered more effective in patients in remission as part of maintenance therapy, in order to eliminate the residual LSCs (Tavor and Petit, 2010, Konopleva and Jordan, 2011).

Alternative strategies include disrupting the predominant dormancy and non-proliferative properties of LSCs, mediated by molecular checkpoints in the cell cycle machinery. CKIs are potential candidates participating in the sequential activation and inactivation of cyclin-dependent kinases, which are central to cell cycle progression (Cheng et al., 2000, Sherr, 1994). Furthermore, malignant cells are considered to be 'addicted' to the expression of survival molecules (Koumenis and Giaccia, 1997). It is possible that dormant cells are sensitive to transcriptional CKIs that target survival molecules, which are over-expressed in the LSC compartment.

As LSCs are regulated by numerous molecules, a complex network of signalling pathways, and the external microenvironment, which control the processes of dormancy, self-renewal, proliferation and differentiation, multi-

targeting agents or combined-targeted therapy may be the most optimal therapeutic approach in eradicating LSCs and preventing disease relapse.

1.3.3 Targeting the Mitochondrial Apoptotic and Survival Pathways By The BH3 Mimetics ABT-737 and ABT-199

Eliciting apoptosis initiated in the mitochondrion, a process primarily regulated by the BCL-2 protein family survival molecules (Figure 1.5), is another attractive approach to AML therapy (Strasser et al., 2011). Anti-apoptotic members of the BCL-2 family, including BCL-2, BCL-xL, BCL-w, Bcl-2-related protein A1 (BFL-1 or A1) and Myeloid Cell Leukaemia-1 (MCL-1), are considered as 'guardians' of mitochondrial integrity by opposing pro-apoptotic BCL-2 family proteins, such as the activator BH3-only 'death ligands', BIM and BH3 Interacting Domain protein (BID), and the multi-domain pro-apoptotic BCL-2-Associated X protein (BAX) and BCL-2-antagonist/Killer (BAK) (Strasser et al., 2011). Imbalanced expression of pro- and anti-apoptotic BCL-2 family proteins renders malignant cells resistant to chemotherapy (Campana et al., 1993). Overexpression of anti-apoptotic BCL-2 family proteins to evade apoptosis is a hallmark of many cancers including leukaemias (Choudhary et al., 2015, Hanahan and Weinberg, 2000). Strategies to restore apoptosis by antagonising anti-apoptotic BCL-2 members have led to the development of BH3 mimetics which have shown a robust pre-clinical response with reduced toxicity (Juin et al., 2013, Letai, 2008).

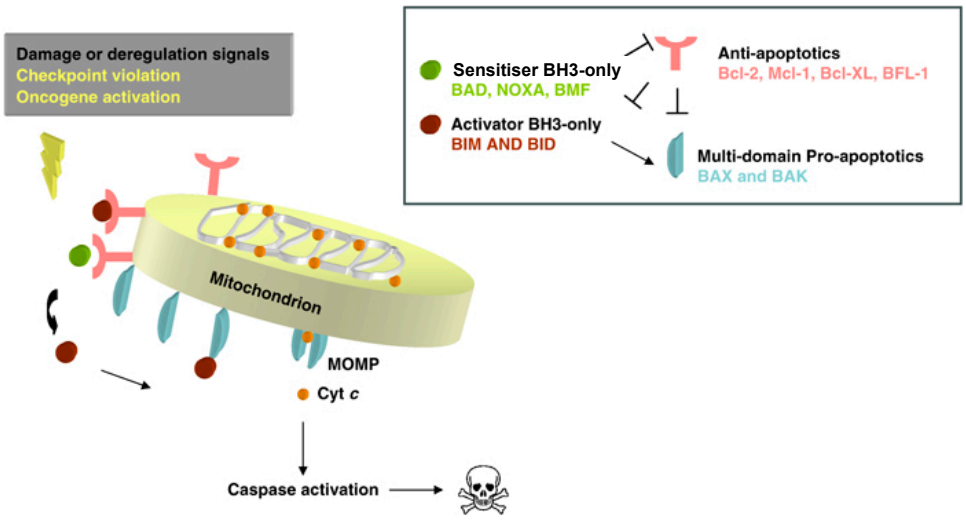


Figure 1.5 The mitochondrial intrinsic apoptotic pathway. Cellular stress activates both sensitiser BH3-only proteins (which inhibit the anti-apoptotic BCL-2 proteins) and activator BH3-only proteins (which cause the activation leading to oligomerisation and insertion of BAX and BAK into the mitochondrial membrane). Mitochondrial outer membrane permeabilisation (MOMP) triggers the release of Cytochrome C, which forms a complex with caspase-9, apoptotic protease activating factor 1 (APAF-1) and dATP/dADP leading to downstream apoptotic events. Adapted from Ni Chonghaile, T. and A. Letai (2009). *Oncogene* 27 Suppl 1: S149-157. (Ni Chonghaile and Letai, 2009)

ABT-737 (Figure 1.6-A, oral form ABT-263 or navitoclax) is a small molecule inhibitor that binds to the BH3 domain of anti-apoptotic BCL-2 proteins, including BCL-2, BCL-xL, and BCL-w, releasing BH3-only proteins and causing mitochondrial outer membrane permeabilisation through BAX/BAK activation (Oltersdorf et al., 2005, van Delft et al., 2006) (Figure 1.6-C). Moreover, ABT-737 is found to significantly potentiate conventional and novel chemotherapeutic drugs, indicating a promising future for novel drug combinations by CDK inhibitors and BCL-2 inhibitors to induce simultaneous targeting of MCL-1 and BCL-2 (Chen et al., 2007a, Kuroda et al., 2008, Kang and Reynolds, 2009, Chen et al., 2013, Abdul-Aziz et al., 2014). However,

owing to the essential role of BCL-xL in platelet homeostasis (Mason et al., 2007), the use of ABT-737/navitoclax was associated with transient thrombocytopenia in clinical trials (Roberts et al., 2012, Wilson et al., 2010). This has led to the re-engineering of ABT-737 into ABT-199 (venetoclax/GDC-0199/RG7601; Figure 1.6-B), an orally bioavailable BH3 mimetic which has high affinity for BCL-2 and lower affinity for BCL-xL, and has exhibited promising *in vitro* and *in vivo* anti-cancer activities, observed in advanced haematological malignancies and breast cancer (Abdul-Aziz et al., 2014, Burrows et al., 2014, Choudhary et al., 2015, Davids and Letai, 2013, Pan et al., 2014, Souers et al., 2013, Vaillant et al., 2013, Vandenberg and Cory, 2013).

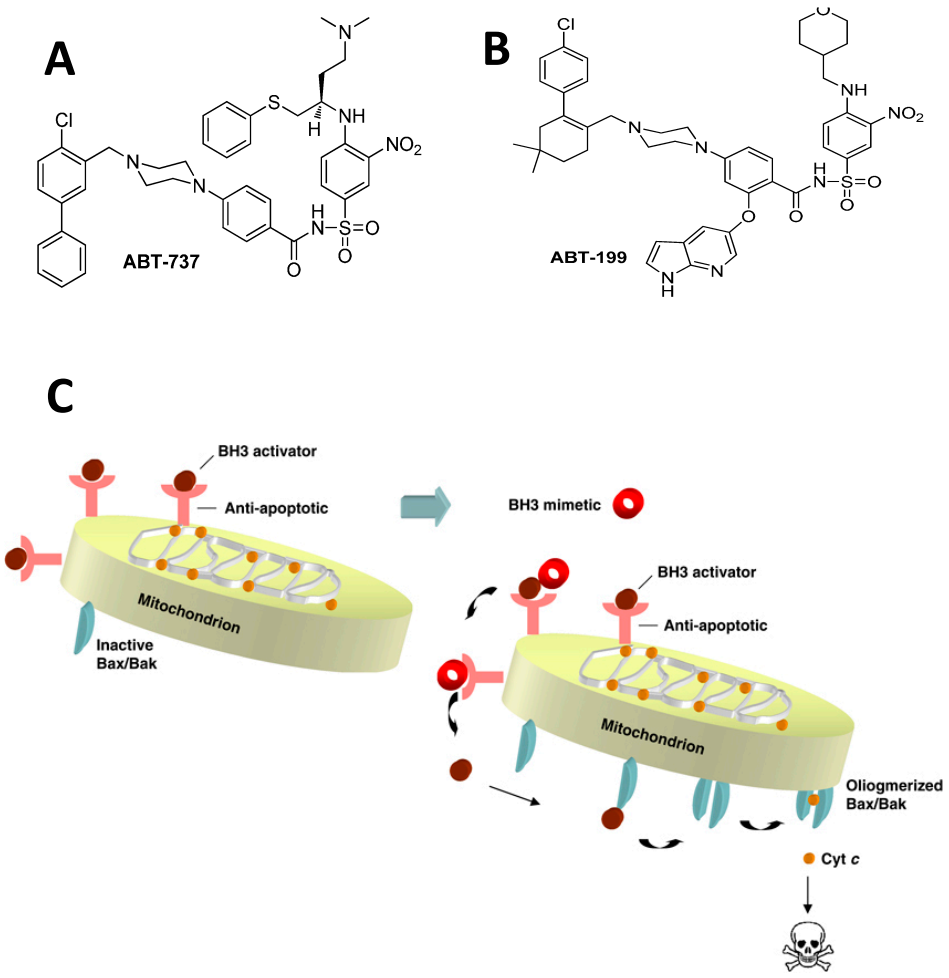
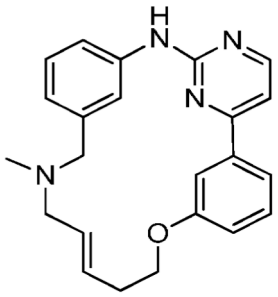


Figure 1.6 The BH3-mimetics ABT-737 and ABT-199. The chemical structure of ABT-737 (A) and ABT-199 (B), from Żołnowska B., et al. *Molecules* (2015), 20 (10): 19101-19129. (Żołnowska et al., 2015). (C) BH3 mimetic-induced apoptosis. BCL-2 family survival molecules buffer the death signals activated by deregulated growth of cancer cells. Upon binding to the hydrophobic groove of the anti-apoptotic BCL-2 members and displacing bound activator BH3-only proteins, BH3 mimetics (e.g. ABT-737 and ABT-199) induce apoptosis enable the activation of BAX/BAK and initiate the downstream apoptosis. Adapted from Ni Chonghaile, T. and A. Letai. *Oncogene* (2009), 27 Suppl 1: S149-157. (Ni Chonghaile and Letai, 2009)

1.3.4 The Multikinase Inhibitor TG02

TG02 (SG1317) is a pyrimidine-based synthetic macrocycle (Figure 1.7) with a wide spectrum of kinase targets including CDKs, FLT3, and Janus kinase 2 (JAK2) (Goh et al., 2012). It mainly inhibits signaling pathways downstream of

CDKs 1, 2, 3, 5 and 9 (William et al., 2011, Goh et al., 2012), which have important roles in cell cycle control (CDK1, 2 and 3), initiation of transcription (CDK9), and neuronal development (CDK5). Aberrations in these CDK-pathways have been observed in various tumours, including those of the breast, colon, liver and brain (Shapiro, 2006). Simultaneous inhibition of CDK1, 2, and 9 has shown better efficacy in killing malignant cells in lung cancer and osteosarcoma, compared to targeting single CDKs (Cai et al., 2006). Additionally, TG02 has its secondary, less sensitive targets including FLT3 and JAK2 (Goh et al., 2012). Mutations of JAK2 are found to sensitise haematopoietic cells to growth factors such as erythropoietin (EPO) and TPO in leukaemias (Kralovics et al., 2005). Constitutively activated FLT-3 is found to confer long-term proliferation and to activate Janus kinase 2 (JAK2) (Tse et al., 2000) and the direct FLT3 target, signal transducer and activator of transcription-5 (STAT5) (Choudhary et al., 2007), both of which play important roles in transducing cytokine-mediated signals via the JAK-STAT pathway (Yamaoka et al., 2004).



TG02

Figure 1.7. The chemical structure of TG02 (SB1317). Adapted from Mariaule G., et al. *Molecules* (2014), 19 (9): 14366-14382. (Mariaule and Belmont, 2014).

The combined inhibition of CDKs and JAK2/FLT3 by TG02 showed benefit in cell lines as well as primary cells, when compared to reference inhibitors that block only one of the main target kinases (Goh et al., 2012). *In vitro,* TG02 could induce at least an IC_{90} (the concentration that induced 90% inhibition of cell growth), with a narrow range of IC_{50} (the concentration that induced 50% reduction in cell number) in cell lines (Pallis et al., 2012), suggesting its ability in bypassing the protective mechanisms seen in responses to conventional anthracycline/nucleoside analolgue-based therapy, such as cell cycle arrest, DNA damage repair and the ability to maintain in dormancy (Raza et al., 1987, Hope et al., 2004). *In vivo,* TG02 has excellent pharmacological and pharmaceutical properties, with the flexibility of oral administration. A study showed that TG02 induced tumour regression after oral dosing in a murine model of mutant-FLT3 leukaemia (MV4-11) and prolonged survival in a disseminated AML model with JAK2 mutations and wild-type FLT3 (Goh et al., 2012). The fact that TG02 is active in various leukaemia models gives a rationale for the current clinical trials in advanced leukaemias. Furthermore, studies using xenotransplanted NOD/SCID mice with primary AML cells suggested the ability of TG02 mobilising LSCs out of BM niche, into the periphery. Also, the addition of TG02 may sensitise chemoresistant cells to Ara-C (Salman et al., 2014).

Moreover, TG02 is equally or more active against $CD34^{+}CD38^{-}$ leukaemia cells than against bulk AML blasts (Pallis et al., 2012). The serine 2 phosphorylation of RP2 by CDK9, the main TG02 target, predominates in later stages of transcription, during elongation and termination (Cho et al., 2001).

Although fatal to all cells, inhibition of RP2 provides a crucial therapeutic window by selectively affecting short-lived anti-apoptotic molecules such as MCL-1 and X-linked inhibitor of apoptosis protein (XIAP) (Koumenis and Giaccia, 1997, Lam et al., 2001), observed in AML cell lines and patient cells (Goh et al., 2012, Pallis et al., 2012, Burrows et al., 2014).

1.3.5 The Future Outlook of AML Therapy

The treatment of AML is entering a new era. With our improved understanding of the steps that a normal cell transforms into AML, and the new discoveries that lay the groundwork to detect and target the disease-initiating cells, we will be able to potentially stop the disease at a very early stage of development when it may be more amendable to treatment. The future sees us treating AML in induction and consolidation, but with more directed targeted therapy which will hopefully improve upon remission and OS rates.

1.4 Project Objective

This study explores new approaches *in vitro* to identify, characterise and target dormant AML cells which are thought to cause relapse. Firstly we planned to optimise *in vitro* cell culture systems for maintaining dormancy, especially in the presence of BM stroma cell support. Next, we aimed at distinguishing leukaemic entities from their normal counterpart by using special cell dyes to track cell division and cell surface markers to analyse LAPs. Moreover, we sought to investigate the efficacy of novel small molecule therapeutic agents TG02 and BH3 mimetrics ABT-737 and ABT-199 in targeting dormant

leukaemia cells. Also, we planned to identify dormant cells by cell markers that label cells with low/high RNA content, which would aid us to further characterise dormancy on the molecular level.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

Recombinant cytokines were purchased from R&D Systems (Abingdon, UK); antibodies were from BD Biosciences (Cowley, UK) and reagents were from Sigma-Aldrich (Poole, UK), unless otherwise stated below. Chemotherapeutic agent Cytarabine (Ara-C) was purchased from Sigma-Aldrich. TG02 (SB1317) was kindly provided by Tragara Pharmaceuticals (Carlsbad, CA, USA). ABT-737 was purchased from Sequoia Research Products (Pangbourne, UK) and ABT-199 was purchased from Active Biochemicals (Hong Kong). For all assays TG02, ABT-737 and ABT-199 were reconstituted in Dimethyl sulfoxide (DMSO) to a 10 mM stock solution, and then aliquoted and stored at -20°C. Working solutions were diluted from thawed stock solutions, using culture medium to final concentrations: TG02 at 25, 50, 100 nM, ABT-737 at 30, 100, 300 nM, and ABT-199 at 30, 100, 300 nM, respectively. Ara-C was reconstituted in RPMI-1640 (200 μM), and diluted fresh in culture medium when used at the final concentrations at 50, 100, 200 nM, 500 nM and 1 μM. Mitogens Phytohaemagglutinin (PHA) and Concanavalin A (ConA) were used to stimulate human T lymphocytes from dormancy to cycling states. The PHA (1mg/mL) stock solution was kindly gifted by Dr Lee Machado from Scancell Ltd (Nottingham, UK) and was diluted to final concentration (5 μg/mL) using culture medium. ConA was reconstituted in phosphate-buffered saline (PBS) to a stock solution (40 mg/mL) and diluted fresh in culture medium to a final concentration of 5 μg/mL. 5-Fluorouracil (5-FU) was reconstituted in DMSO to a stock solution (100 mM) and was diluted fresh in culture medium to a final concentration of 300 μM (40 μg/mL).

2.2 Stromal Cells and Cell Culture

2.2.1 The Murine MS-5 Stromal Cells and Cell Culture

The murine MS-5 stromal cell line was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany). MS-5 cells, routinely used for long-term support of primitive haematopoietic progenitors (Berardi et al., 1997, Gan et al., 1999, Konopleva et al., 2002a), were maintained at a density of 5 x 10^4/mL in α-minimum essential medium (α-MEM; Gibco, Paisley, UK), supplemented with 10% heat-inactivated foetal calf serum (FCS; First Link UK), 1% penicillin/streptomycin and 2mmol/L L-glutamine.

2.2.2 Isolation and Cell Culture of The Human Bone Marrow Mesenchymal Stem Cells (BM-MSCs)

The human adult BM-MSCs were obtained from healthy donors undergoing bone marrow harvest (BMH) for allogeneic BM transplantation (we have ethics in place to use the leftover BM from donors). Mononuclear cells were isolated by the following density gradient/centrifugation procedure. Samples were diluted 1:2 with RPMI-1640, and then layered onto an equal volume of HISTOPAQUE®-1077 drop by drop at room temperature. The mixture was subjected to centrifugation at 600g for 20 minutes, during which an opaque interface, i.e. a mononuclear layer, was formed between the plasma and the HISTOPAQUE®. Mononuclear cells were carefully 'hoovered' up by a sterile

pastette into a clean tube containing 10 mL RPMI-1640, followed by a centrifugation at 600g for 10 minutes. The cell pellet was incubated in cold 0.89% ammonium chloride for 10 minutes on ice to deplete contaminating red blood cells. Cells were washed with RPMI-1640 for 10 minutes at 400g, followed by a further centrifugation at 200g for 10 minutes.

The cell pellet was resuspended in growth medium containing α-MEM, 20% heat-inactivated FCS, 1% L-glutamine and Penicillin-streptomycin, with the initial plating density at 1 x 10^6 cells/cm^2. MSCs cultures were washed with PBS after 2-3 days to remove non-adherent cells. The remaining adherent cells were maintained with medium changes every 3-4 days until reaching 70-80% confluence. Following trypsinisation (0.25% trypsin with 0.1% EDTA), cells were used for experiments during the third or fourth passages and were sub-cultured for 48 hours at 2 x 10^4 cells/cm^2 in 96-well flat-bottom plates, before addition of AML cells. MSCs were labelled with anti-CD73-phycoerythrin (PE; #550257), anti-CD90-fluorescein isothiocyanate (FITC; #561969), anti-CD34-allophycocyanin (APC; #3458013;BD Pharmingen, Oxford, UK) and anti-CD45-Allophycocyanin-Cyanine 7 (APC.Cy7; #348795; BD Biosciences, Cowley, UK) antibodies to check for positivity of CD90, CD73 and the lack of expression of CD34 and CD45. Fluorescence of test antibodies was compared with matched isotype controls, i.e. mouse IgG1κ FITC (BD Biosciences, Cowley, UK), mouse IgG1 κ PE and mouse γ1 APC (BD Pharmingen, Oxford, UK).

2.3 Leukaemia Cell Lines and Cell Culture

The FLT3-ITD mutated MOLM-13 human acute monocytic leukaemia cell line was purchased from DSMZ (Braunschweig, Germany). The KG-1a primitive hematopoietic cell line was from EACC - the European Collection of Animal Cell Cultures (Salisbury, UK). MOLM-13 cells were maintained in RPMI-1640 with 10% FCS, 2mmol/L L-glutamine, and 1% penicillin-streptomycin. KG-1a cells were maintained as above, with 20% FCS. Cells were kept at 37ºC in 5% CO_2 in a humidified incubator and had no more than 20 passages. All experiments were performed with cells in log phase. Continuous testing using a panel of monoclonal antibodies and DNA genetic fingerprinting analysis with short tandem repeat (STR) was performed to validate the cell lines.

2.4 AML Patient Samples

Fresh or cryopreserved BM or PB samples of patients presenting with *de novo* or relapsed AML were collected at diagnosis at the Nottingham University Hospitals after written consent. The use of these samples was approved by the Ethics Committee of the Nottingham University Hospitals NHS Trust (Reference 06/Q2403/16). The patient demographics are summarised in the Appendix 1 to this thesis.

2.5 Preparation of AML Patient Cells

2.5.1 Isolation of Mononuclear Cells From AML Patient Samples

Patient PB or BM samples were received in BD Vacutainer® tubes (BD Biosciences, UK) containing preservative-free heparin or EDTA. Mononuclear cells were isolated using the same density gradient/centrifugation method described in Section 2.2.2. The cell pellet was resuspended in fresh RPMI-1640.

2.5.2 Cryopreservation of Primary AML Cells

Mononuclear cells were counted and resuspended in cold RPMI-1640 medium at 1 x 10^8/mL for primary cells. 2ml Cryovials (Nalgene®, Thermo Fisher Scientific, Loughborough, UK) were labelled with patient/sample code, cell concentration, date of cryopreservation, and were kept ice cold before the addition of 700 μL freezing solution (1 volume of DMSO, 2 volumes of FCS, and 4 volumes of RPMI-1640 medium) and 300 μL cells. The cryovials were then placed in a 'Mr Frosty' Cryogenic Freezing Container (Nalgene®, Thermo Fisher Scientific) and stored at -80°C for 2 to 24 hours before being transferred to the liquid nitrogen tank for long-term storage (up to 10 years). The laboratory logbook and database were updated accordingly with the sample code, sample date, cell number, vial number and cryovial location.

2.5.3 Thawing and Resting Patient Cells

Cryopreserved samples were removed from liquid nitrogen, placed into a water bath (37°C) for about 2 minutes and then transferred dropwise to a tube containing cold Thawing Solution, containing RPMI-1640 with 10% FCS and 1% heparin (Wockhardt UK Ltd, Wrexham, Wales). Cells were then centrifuged at 250g for 5 minutes and resuspended in Rest Medium, containing RPMI-1640 with 20% FCS and 1% L-glutamine, for 90 minutes in an incubator (37°C, 5% CO_2). Rested samples were then subjected to viability analysis using Trypan Blue, and only samples with ≥ 85% viability were used (described in section 2.9.1).

2.5.4 CD34 Enrichment

Mononuclear cells from BM or PB were magnetically labelled with the CD34 MicroBeads (Miltenyi Biotec Ltd, Bisley, UK), then passed through a MACS® column according to Manufacturer's instructions. $CD34^+$ labelled cells were retained in the column whereas $CD34^-$ cells ran through the column. Positively selected cells were eluted and collected after the column was removed from the magnet.

2.5.5 PKH26 Labelling of CD34 Enriched AML Blasts

The PKH26 Red Fluorescent General Cell Linker Kit was used to track cell division throughout the 12-day cultures. The hydrophobic dye molecules have

been shown to be stably incorporated into lipid regions of the cell membranes by phase-partitioning, and then faithfully divided into daughter cells during karyokinesis, resulting in a 50% dilution of the fluorescence intensity in cells without contaminating neighbouring cells (Horan and Slezak, 1989). $CD34^+$ blasts were suspended in the labelling solution 'Diluent C' to a final concentration of 6 μM PKH26 per 10^6 cells. Cells were primed for 3 minutes by incubation at room temperature (20 – 25 ˚C). An equal volume of heat-inactivated FCS was added to the cell mixture for 1 minute before the dye was washed out by rinsing cells four times in RPMI-1640 supplemented with 10% FCS before cell culture.

2.6 72-hour AML Cell Culture For Chemosensitivity Assays

To assess 72-hour AML primary cell chemosensitivity to agents TG02, ABT-737, and ABT-199, patient samples were cultured in 96-well, flat-bottom plates in triplicate at 1 x 10^6 cells/mL in suspension medium consisting of RPMI-1640 with 10% FCS, 2 mmol/L L-glutamine, supplemented with 20 ng/mL Interleukin-3 (IL-3), 20ng/mL stem cell factor (SCF), 20 ng/mL Interleukin-6 (IL-6), 25 ng/mL granulocyte colony-stimulating factor (G-CSF; Amgen, Thousand Oaks, USA) and 0.07 μL/mL β-mercaptoethanol (βME), or in Gartner medium consisting of alpha-minimum essential medium (α-MEM) supplemented with 12.5% FCS, 12.5% heat-inactivated horse serum (Stem Cell Technologies), 1% penicillin/streptomycin, 2 mmol/L L-Glutamine, 57.2 μM βME and 1 μM hydrocortisone (Gartner and Kaplan, 1980) with additional 20

ng/mL IL-3, human recombinant Thrombopoietin (TPO) and G-CSF on MS-5 feeder layers. The feeder layers were cultured as described in Section 2.2. The co-culture system was as reported before (van Gosliga et al., 2007).

For assessment of AML cell line (MOLM-13) chemosensitivity to Ara-C, TG02, ABT-737 and ABT-199, stromal cells (MS-5 and MSC) were seeded as feeder layers at 2 x 10^4 cells/mL in flat-bottom 96-well plates in triplicate 24 hours before addition of MOLM-13s at 3 x 10^5 cells/mL in RPMI-1640 supplemented with 10% FCS, 2 mmol/L L-glutamine and 1% penicillin/streptomycin.

Cells were incubated with drugs for an additional 72 hours, followed by collection of suspension cells. Remaining cultures were washed by PBS and were incubated with 0.25% Trypsin/EDTA solution at 37°C for 2 minutes before collection.

2.7 12-day Primary Cell Culture

2.7.1 Expansion Culture of AML Cells In Serum-Free Medium

PKH26-labelled $CD34^+$ AML primary cells (methods are described in section 2.5.5) were suspended in the AML blast expansion medium, StemSpan® Serum-Free Expansion Medium (SFEM), supplemented with 1% pre-mixed recombinant human (rh) cytokine cocktail StemSpan® CC100 (StemCell Technologies, Vancouver, Canada) containing 100 ng/mL of FLT3 Ligand, 100 ng/mL of SCF, 20 ng/mL of IL-3 and 20 ng/mL of IL-6. Cells were plated

at 2.5 x 10^5 cells/mL and cultured at 37˚C in 5% CO_2 for 12 days. Cells were checked daily under the microscope and diluted every 2 to 3 days to a density of 3 x 10^5 cells/mL.

2.7.2 12-day AML-stroma Co-culture

For the 12-day 'primary-cell-on-stroma' co-culture studies, cell culture plates were pre-coated with gelatin before media and cells were introduced. Briefly, 0.1% gelatin solution was diluted from a 2% stock solution (Sigma) in sterile, distilled water and was added to the plates to cover each well. Following an incubation period of 1 hour at 37°C (5% CO_2), excess gelatin was removed and the plates were placed in a laminar flow hood to dry, for at least 15 minutes. MS-5 cells were sub-cultured at 2 x 10^4/mL for 24 to 48 hours to form a monolayer before addition of PKH26-labelled $CD34^+$-enriched AML cells, which were seeded at 3 x 10^5/mL on the MS-5 feeder layer in Gartner Medium. Only freshly prepared medium was used for initiating and maintaining the cultures. Cells were kept in culture for 12 days and demi-populated on Day7. Drugs (Ara-C, TG02, ABT-737 and ABT-199) were added to cells on Day8 of the cell culture.

2.8 Isolating The Human T Cells And Cell Culture

2.8.1 Isolation of Normal T Cells

Mononuclear cells were separated from healthy donors' PB (we have ethics in place to use the leftover PB from donors), using the same density gradient/centrifugation method described in section 2.2.2, and were magnetically labelled with CD34 MicroBeads (Miltenyi Biotec Ltd, Bisley, UK), then passed through a MACS® column which was in a magnetic field of a MACS separator. $CD34^+$ labelled cells were retained in the column whereas $CD34^-$ cells ran through the column and were collected after the column was removed from the magnet. The enriched $CD34^-$ cells were then labelled with the pan T cell Isolation Kit (Miltenyi Biotec Ltd, Bisley, UK) containing biotinylated antibodies and anti-Biotin MicroBeads, before passing through the MACS® column. Non-T cells were retained in the column whereas T cells passed through the column and were collected as enriched cell fraction.

2.8.2 T Cell Mobilisation by Mitogens

Purified T cells were resuspended at 2 x 10^6 cells/mL in fresh medium (RPMI-1640 with 10% FCS, 200mM L-glutamine and 1% penicillin/streptomycin). Cells were cultured with or without phytohaemagglutinin (PHA) (5 μg/mL) or concanavalin A (ConA) for 72 hours (37˚C, 5% CO_2) before cells were subjected to further analysis.

2.9 Cell Counting and Cell Viability Analysis

2.9.1 Trypan Blue

The trypan blue test was used to quickly determine the number of viable cells in a cell suspension, based on the principle that living cells have intact cell membranes that can exclude trypan blue, where those with damaged cell membranes uptake the dye and subsequently become visually blue (Strober, 2001). Briefly, 10 μL of cell suspension were mixed with an equal volume of 0.4% trypan blue. A drop of this mixture was then transferred to a haemocytometer and was observed under a binocular microscope. Cells with a clear cytoplasm were counted as viable whereas those with blue staining were nonviable. The percentage of viable cells in a cell suspension was calculated as follows:

$$\% \text{ viable cells} = \frac{\text{Total number of viable cells (per mL)}}{\text{Total number of cells (per mL)}} \text{ x } 100\%$$

2.9.2 7AAD/FSC Viability Assay by Flow Cytometry

2.9.2.i. Preparation of fixed-stained cells (FSC)

To provide an internal standard for counting cells on the flow cytometer, PB mononuclear cells were isolated by a standard density gradient/centrifugation technique, from healthy donors after the peripheral stem cell harvest (SCH) procedure. Cells were suspended in 200 μL PBSAA buffer (PBS with 1% FCS

and 0.1% sodium azide), incubated with anti-CD45-FITC monoclonal mouse-anti-human antibody (BD Biosciences) for 20 minutes in the dark, washed twice in PBSAA and resuspended in 2mL 2% paraformaldehyde solution (VWR International, UK). The final cell concentration was determined by trypan blue using a haemocytometer (Pallis et al., 1999). FSC were kept at 4°C and were checked against deterioration before use.

2.9.2.ii. FSC/7AAD Cell Viability Assay by Flow Cytometry

The dye 7-amino-actinomycin D (7AAD) was used as an indicator of cell viability (Schmid et al., 1994, Philpott et al., 1996). An internal standard method incorporating fixed-stained cells and 7AAD that allows us quickly and accurately enumerate cell populations by flow cytometry had been developed in the department (Pallis et al., 1999). 100 μL AML cells were seeded at a density of 2 x 10^5/mL (cell lines) or 1 x 10^6/mL (primary cells) in triplicates on a 96-well plate. Following short-term cell culture (within 72 hours), 80 μL cells were harvested and were mixed with an equal volume of the 7AAD solution (25 μg/mL). The cell mixture was then incubated for 20 minutes in the dark at room temperature before addition of the FSC. The FSC alone were counted with a haemocytometer by trypan blue exclusion. Analysis for cell viability was carried out using a FACS Canto II™ flow cytometer (Becton Dickinson) and data were further processed by the FACSDiva® software (Becton Dickinson). Figure 2.1 demonstrates the FSC/7AAD flow cytometric method for viable AML cells, and the calculation was done as follows:

$$AML\ count\ (cells/ml) = \frac{P4}{P3} \times \frac{FSC\ Vol\ (ml)}{AML\ Vol\ (ml)} \times FSC\ count\ (cells/ml)$$

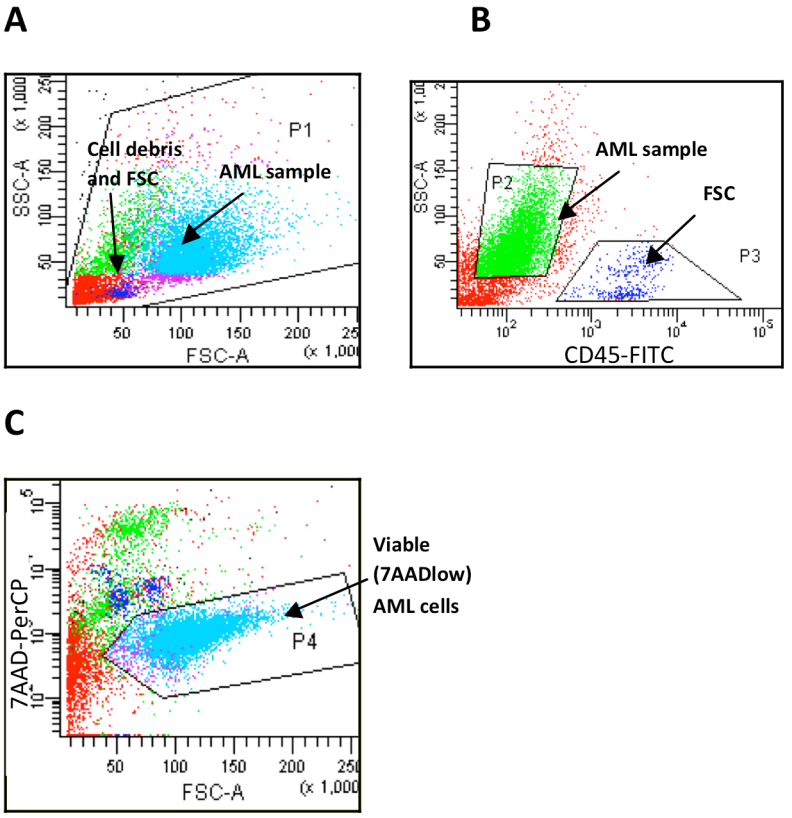


Figure 2.1. The FSC/7AAD flow cytometric cell viability assay. (A) FSC/SSC plot. (B) Derived from P1, the fixed CD45 pre-labelled normal mononuclear cells (FSC), P3; and the unlabelled AML sample cells, P2. (C) P4 is set around 7AADlow/FSChigh events (viable cells) derived from P2.

2.9.3 Fluorosphere Beads Enumeration by Flow Cytometry

The FITC-conjugated Dako CytoCount™ control beads (Dako UK Ltd, Ely, UK) were used as a reference population for the absolute count of sub-populations of interest (e.g. the dormant and cycling subsets), which require careful fluorescence compensation settings for multicolour flow cytometric analyses. Samples were stained with a combination of relevant fluorochrome-conjugated antibodies, e.g. anti-CD45- APC.Cy™7, anti-CD34-APC, and anti-LAP-FITC, followed by addition of a carefully measured volume (100µL beads/10^6 cells) of CytoCount™ beads to the samples using reverse pipetting technique. Samples were analysed by flow cytometry and cells of interest were counted using an established gating strategy (Gratama et al., 1999). The number of CytoCount™ beads was determined by setting a region around the beads in a dot plot where the beads were clearly separated from the stained cells (Figure 2.2). Concentration of the beads was stated on the vial by the manufacturer (1053/µL in current batch). Dilution Factor (DF) was determined by the volume ratio of 10^6 cells per sample (v/v). The absolute cell count was calculated using the following equation:

$$\textit{Cells of interest (cells/µL)} = \frac{\textit{Events of cells of interest}}{\textit{Events of Beads}} \; x \;\; \textit{Beads concentration x DF}$$

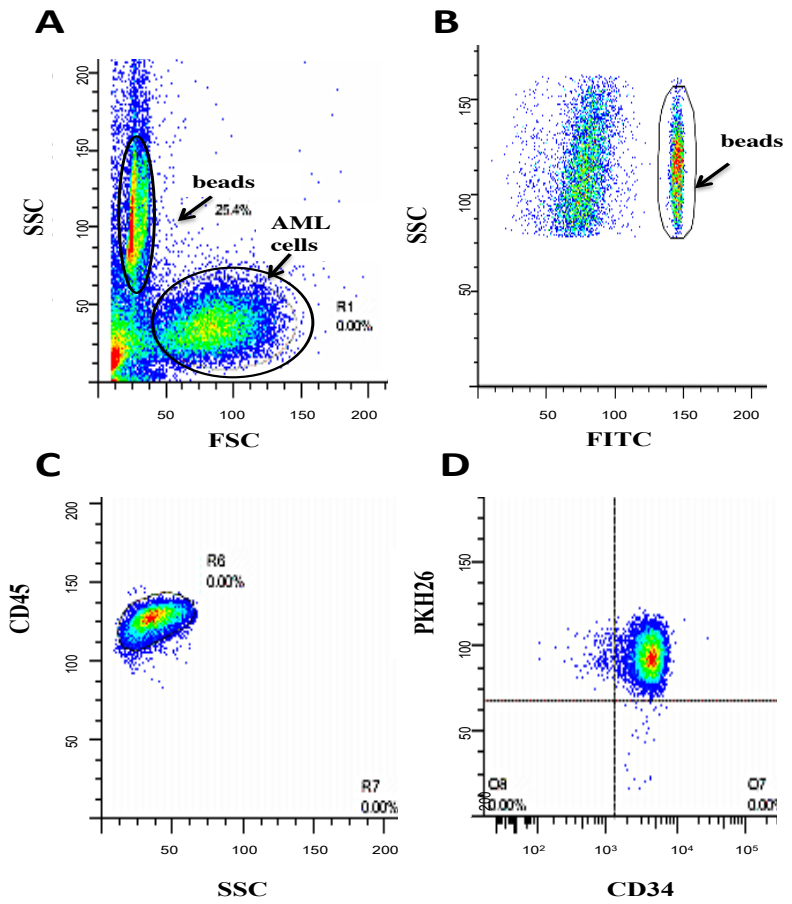


Figure 2.2. Determination of AML cells by CytoCount™ beads. Representative plots from a patient sample (AML-4) are shown. $CD34^+$ cells were enriched upon thawing, followed by labelling with PKH26 dye. Cells were stained with relevant fluorochrome-conjugated antibodies anti-CD45-APC.Cy™7, anti-CD34-APC, and anti-CD7-FITC. $1x10^6$ events were collected for analysis. (A) Forward scatter vs. side scatter showing an AML sample and beads. (B) A region was set around the beads in a dot plot (CD7-FITC/SSC) where the beads are clearly separated from the stained cells. (C) CD45-APC.Cy™7/SSC dot plot for CD45 expression. (D) CD34-APC/PKH26-PE dot plot showing PKH26 fluorescence intensity ($CD34^+/PKH26^{high}$) and the horizontal line shows isotype control**.**

2.10 Flow Cytometric Analysis of AML Phenotypes

Cells were suspended in 100 µL PBSAA at 2 x 10^6 cells/mL per tube. To minimise non-specific mAb binding of immunoglobulin, 2.5 µL normal mouse serum (NMS) was added to the cells, followed by incubation for 5 minutes in

the dark at room temperature. 2.5 μL of antibodies or isotype controls or PBS blank controls were added to the corresponding tubes (Table 2.1). Cells were incubated for 15 minutes in the dark at room temperature, followed by three washes with PBSAA. Cells were then resuspended in 300 μL PBSAA and were subjected to phenotypic analysis on a FACS Canto II cytometer (BD Biosciences). A minimum of 10,000 events was collected for each sample and the FACSDiva® software (BD Biosciences) was used for acquisition and analysis.

The IgG1-FITC (#345815), IgG1-PE (#444749), IgG1-PerCP (#345817) and IgM-FITC (#555583) isotype control antibodies, and anti-CD117-PE (#555714; IgG1κ), anti-CD34-APC (#345804; IgG1κ), anti-CD19-FITC (#345776, IgG1κ), anti-HLADR-FITC (#347400; IgG2a), anti-CD15-FITC (#555401; IgM), and anti-CD33-PE (#345799; IgG1κ) antibodies were purchased from BD Pharmingen (Oxford, UK). The IgG2a-FITC isotype control (#X0933), anti-CD2-FITC (#F0767; IgG1κ), and anti-CD13-FITC (#F0831; IgG1κ) antibodies were purchased from Dako (Ely, UK). The anti-CD7-FITC antibody (#A07755; IgG1κ) was purchased from Beckman Coulter (High Wycombe, UK). The anti-CD45-APC.Cy^{TM}7 antibody (#348795; IgG1κ) was purchased from BD Biosciences.

Table 2.1 Panel of markers for LAP analysis.

Tube number	FITC	PE	PerCP	APC.Cy7
1	PBS	PBS	PBS	CD45
2	IgG1	IgG1	IgG1	CD45
3	IgG2a	IgG1	IgG1	CD45
4	IgM	IgG1	IgG1	CD45
5	CD2	CD117	CD34	CD45
6	CD7	CD117	CD34	CD45
7	CD15	CD117	CD34	CD45
8	CD19	CD117	CD34	CD45
9	HLADR	CD117	CD34	CD45
10	CD13	CD33	CD34	CD45
11	CD13	CD117	CD34	CD45

2.11 Fluorescence-activated Cell Sorting (FACS)

Cell sorting was performed on a MoFlo™ XDP high-speed cell sorter (Beckman Coulter, High Wycombe, UK) to enrich and to purify cell populations of interest.

To study the clonogenicity of dormant cells, on Day12 of culture, cells were harvested and labelled with anti-CD45-APC.Cy™7 (#348795; BD Biosciences), anti-CD34-APC (clone BIRMA-K3; # C7238; Dako, Ely, UK), and additional anti-LAP antibodies, where appropriate. 7-AAD at 5 μg/mL was used to exclude non-viable cells. The photomultiplier (PMT) settings on the sorter were determined using the PKH26-unstained control and isotype antibodies (APC.Cy™7, IgG1κ, #557873; CD34-APC, IgG1κ, #345818; and FITC, IgG1κ, #345815). The plots in Figure 2.3 demonstrate the detailed sequential gating strategy for flow cytometric analysis on Day12. Scatter

(FSC/SSC) plots were combined with 7-AAD plots to distinguish live cells from dead cells and debris. The CD45 plots were set up to exclude residual stromal cells, and to distinguish myeloid cells ($CD45^{+/dim}$) from normal lymphoid and monocytic cells, which had higher CD45 expression regularly as the FSC/SSC characteristics of these populations were often overlapping (Lacombe et al., 1997). The LAP, CD34 and PKH26 gates were used to further distinguish the dormant ($PKH26^{high}$) and cycled ($PKH26^{low}$) AML blasts for cell sorting analysis. Dormant leukaemia cells were identified as a $CD45^{+}CD34^{+}PKH26^{high}LAP^{+}$ population and the cycling compartments were $7\text{-}AAD_{low}/CD45^{+}/CD34^{+/-}/LAP^{+}/PKH26^{high}$ (Figure 2.3).

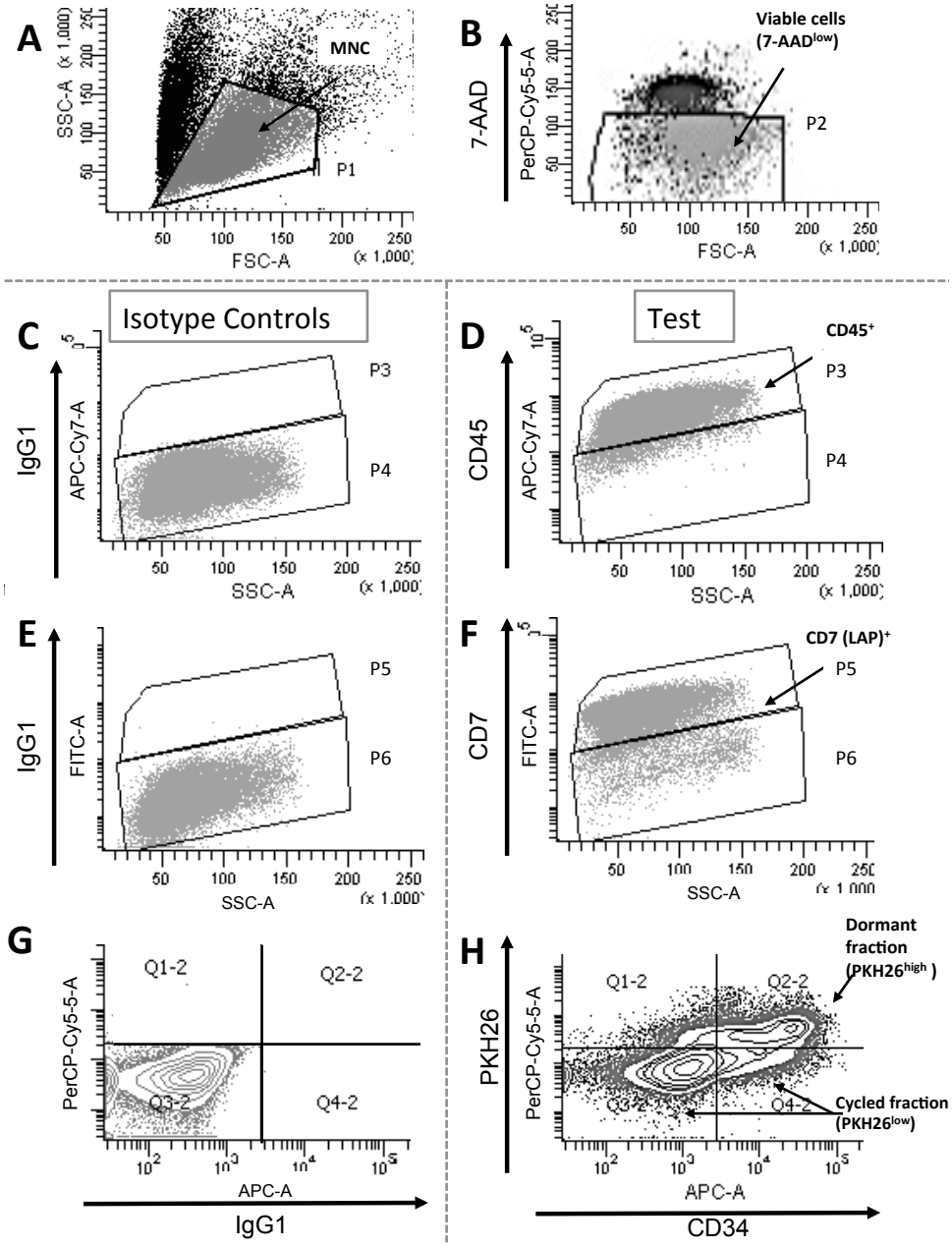


Figure 2.3. Flow cytometric gating strategy for the identification and sorting of dormant LAP$^+$ AML cells. Representative plots from Sample #4 are shown. On Day12 of the cell culture, FSC/SSC (A) and 7-AAD (B) were used to distinguish viable cells (P1+P2). (D) CD45 gate (P3) was set up to identify myeloid blasts (P3). (F) LAP gate (P5) was used to identify leukaemic cells. (H) A quadrant plot was used to distinguish the CD34 (horizontal axis) and PKH26 expressions (vertical axis) in cells. Isotype controls for CD45 (C), LAP (E) and CD34 (G; horizontal axis) were used to set up FACS gates and cut-off points. The sorter baseline for PKH26 was set up by PKH26-unlabelled cells that were maintained in culture for 12 days (G; vertical axis). The cell sorting criteria were: 7-AAD$_{low}$/CD45$^+$/CD34$^+$/LAP$^+$/PKH26^{high} gates ($P_1/P_2/P_3/P_5/Q_{2\text{-}2}$) for dormant cells; and 7-AAD$_{low}$/CD45$^+$/CD34$^{+/-}$/LAP$^+$/PKH26^{high} gates ($P_1/P_2/P_3/P_5/Q_{3\text{-}2}$ and $P_1/P_2/P_3/P_5/Q_{4\text{-}2}$) for cycling cells.

For molecular characterisation of dormancy studies, 1μg/mL Pyronin-Y (PY) solution was used to label cellular RNA. At least 2 x 10^5 cells/mL per tube were washed in PBSAA and were resuspended in 100 μL PBSAA, followed by addition of 5 μL of anti-CD34-APC (clone 581; #560940; BD Pharmingen), and anti-CD45-APC.Cy^{TM}7 antibodies (BD Biosciences) in each reaction tube. The cells were then incubated at 4°C for 30 minutes, and washed once in 2 mL of PBSAA. The washed cells were resuspended in 0.5 mL of Hanks buffered saline solution (HBSS) with 2% FCS. 5 μL of a 0.1 mg/mL PY stock solution was added to the cell suspension to achieve a final concentration of 1 μg/mL. Cells were then incubated on ice for a further 30 minutes and were washed once in 2 mL of HBSS (with 2% FCS) and were resuspended in 0.5 mL of HBSS (2% FCS) before FACS analysis. Dormant cells were identified as the $CD45^{dim}CD34^{+}PY^{low}$ population (10% at the start of the PY histogram), in contrast to the $CD45^{dim}CD34^{+}PY^{peak}$ population (10% at the start of the PY peak), which had a higher RNA content indicating a substantial cycling status.

All cell pellets were either processed for RNA extraction, or were stored in RNA-later® solution (Ambion, Thermo Fisher Scientific, Loughborough, UK) at -20°C before being processed for RNA extraction.

2.12 Colony-forming Unit (CFU) Assays

Freshly isolated or FACS sorted mononuclear cells were washed, resuspended in methylcellulose-based medium Methocult^{TM} H4034 Optimum (Stem Cell Technologies, Grenoble, France), or Methocult^{TM} H4534 (Stem Cell

Technologies, Grenoble, France), supplemented with 3 U/mL rhEPO and 50 ng/mL G-CSF, or in Methocult^{TM} H4100 (Stem Cell Technologies, Grenoble, France), diluted in Iscoves modified Dulbecco medium (IMDM) to a Methylcellulose concentration of 0.9%, supplemented with 10% FCS, 1% L-glutamine and Penicillin-Streptomycin, 1% HEPES buffer, 100 U/μL Interleukin-3 (IL-3), 100 U/mL GM-CSF (Novartis, Camberley, UK), and 25ng/ml G-CSF (Pallis et al., 1998), with or without the addition of 25 nM TG02, 100 nM ABT-737, 200 nM Ara-C. Cells were plated onto 96-well flat-bottom plates at 2 x 10^4 cells/100 μL in triplicates and were kept at 37 ˚C, with 5% CO_2 in a humidified incubator for 14 days. Colonies of > 50 cells were counted.

2.13 Investigating Dormancy Markers

2.13.1 Pyronin Y (PY) Assay

Thawed and rested primary cells were subjected to PY (1 μg/mL) and multi-colour cell surface labelling. For each tube, 2 x 10^5 cells were washed in PBSAA and then resuspended in 100 μL PBSAA, followed by incubation with 2.5 μL NMS in the dark for 5 minutes at room temperature. Cell surface antibodies, including 2.5 μL anti-CD45-Alexa Fluor®488 (BioLegend, # 304019; Cambridge, UK) and 2.5 μL anti-CD34-APC (clone 581; #560940; BD Pharmingen) were added to the cells followed by incubation on ice for 30 minutes. Cells were then washed once in 3 mL PBSAA, and resuspended in 0.5 ml of HBSS (Hanks Buffered Saline Solution) containing 2% FCS. 5 μL of PY stock solution (0.1 mg/mL) was added to the cell suspension. This mixture was

incubated on ice for 30 minutes in darkness. Cells were then washed once again with 2 mL of HBSS with 2% FCS and the cell pellet was resuspended in 0.5 mL of HBSS with 2% FCS, before flow cytometric analysis. For cell sorting studies, cells were kept on ice during the transit to the Flow Cytometry Facility Core Services, prior to FACS analysis.

2.13.2 Ki-67 and CD71 Combined Staining Method

Proliferation-associated cell analysis was based on a previously described method with some modifications (Jordan et al., 1996), using anti-Ki-67-FITC monoclonal antibody (#556026; BD Pharmingen, Oxford, UK). Cells were harvested, washed and resuspended in 100 µL PBS with HFH buffer (20mM hepes and 10% FCS in Hanks balanced salt solution). Cell surface labelling was by 5 µL of anti-CD71-PE (BD Pharmingen; # 551143), 2.5 µL of anti-CD34-PerCP (#345802), anti-CD38-APC (#555462) and anti-CD45-APC.Cy^{TM}7 (#348795) antibodies (BD Biosciences, Cowley, UK) or isotype controls (Table 2.2). Cells were then incubated at 4°C for 20 minutes, washed once in 2mL HFH, and then resuspended in 0.5 µL HFH. Cells were fixed at 2 x 10^6 cells/mL in PBS plus 4% paraformaldehyde for 30 minutes on ice. Subsequently, samples were permealised by 1 mL PBS plus 0.1% Triton X-100 and were incubated overnight at 4°C. Afterward, samples were washed and resuspended in 100 µL of PBSAA and labelled with 5 µL of anti-Ki-67-FITC and incubated for 15 minutes in the dark at room temperature. Cells were washed twice more with PBSAA and finally suspended in 100 µL PBSAA

before flow cytometric analysis.

Table 2.2. Antibodies for the Ki-67/CD71 assay.

Tube #	FITC	PE	PerCP	APC	APC.Cy™7
1	IgG1	IgG2a	IgG1	IgG1	CD45
2	IgG1	IgG2a	CD34	CD38	CD45
3	Ki-67	IgG2a	CD34	CD38	CD45
4	Ki-67	CD71	CD34	CD38	CD45

2.13.3 CD71 Staining In Unfixed Cells

For each tube, 2 x 10^5 cells were washed in PBSAA and then resuspended in 100 μL HFH. 2.5 μL of NMS was added and cells were incubated in the dark for 5 minutes at room temperature, 5 μL of anti- CD71-PE or Mouse IgG2a-PE isotype control (BD Pharmingen, Oxford, UK) was added to the according tube. Cells were then incubated at 4°C for 20 minutes followed by two washes with HFH buffer. Cell pellet was resuspended in 0.5 mL of HFH and cells were subjected to flow cytometric analysis.

2.13.4 The Aldehyde Dehydrogenase (ALDH) Assay

As demonstrated in Figure 2.4, cells were processed according to manufacturer's instructions (attached in Appendix 2). Briefly, cells were counted and resuspended at 1 x 10^6 cells/mL in 1mL Aldefluor™ Assay Buffer provided in the kit. 5 μL of the activated Aldefluor™ reagent was added. 500 μL cell/reagent mixture was transferred to a negative control tube containing DEAB (to prevent ALDH activity). Cells were incubated for 30 minutes at

37˚C, and then resuspended in 0.5 mL of Aldefluor™ Assay Buffer and kept cold (0 - 4 °C). Cell surface labelling was carried out by adding 2.5 µL of anti-CD34-PerCP, anti-CD38-APC and anti-CD45-APC.Cy™7 (#348795) antibodies (BD Biosciences, Cowley, UK) or isotype controls. Cells were then incubated at 4°C for 20 minutes, and washed twice with PBSAA before being resuspended in 100 µL PBSAA for flow cytometric analysis.

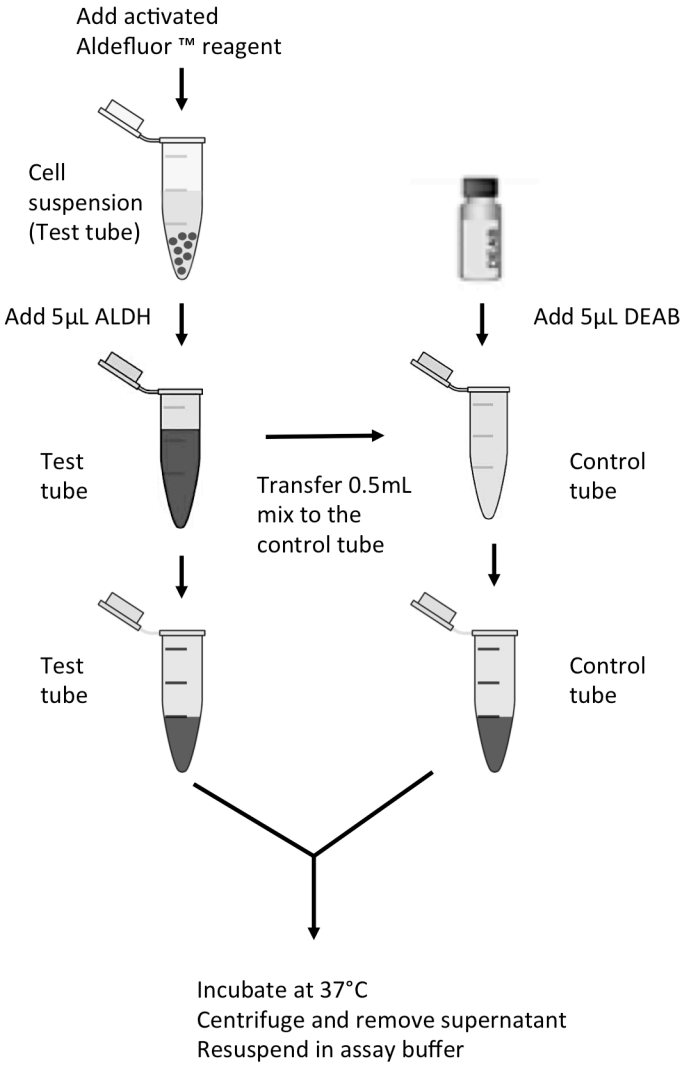


Figure 2.4. The ALDH assay staining procedure, using the Aldefluor™ kit. (Adapted from the manufacturer's Direction for Use, by Stem Cell Technologies; www.stemcell.com)

2.14 Molecular Biology Techniques

2.14.1 RNA Extraction with the Qiagen Mini Blood Kit and cDNA Synthesis

For T cell samples, total RNA was isolated from the cell lysates by QIAamp RNA Blood Mini Kit (Qiagen, Manchester, UK) *as per* the manufacturer's protocol. An additional DNase digestion step was added to the standard procedure using the Qiagen RNase-free DNase set.

A volume (1 μg) of RNA was added to each micro-centrifuge tube and was topped up to 13 μL with RNase-free water and then heated in a thermo cycler at 65°C for 10 minutes. The mixture was quickly chilled on ice for 5 minutes and pulse spun, followed by the addition of 12 μL 'Master Mix' consisting of 10.75 μL reverse transcription (RT) mix [5x First Strand Buffer (Invitrogen, Paisley, UK), 25mM dNTPs (Invitrogen, 0.1M DTT (Invitrogen), 3 μg/μL Random primers (Invitrogen), DEPC treated water], 0.5 μL RNasin (Promega, Southampton, UK), and 0.75 μL Moloney murine leukaemia virus (MMLV) reverse transcriptase (Invitrogen). The reaction tubes were then placed in a thermal cycler (PTC-100, Bio-Rad, CA, USA) programmed to run for 60 minutes at 37°C, 10 minutes at 95°C and then 4°C to cool. All reaction tubes were then pulse spun and the cDNA was stored at -20°C if not used immediately. The RNA concentration was determined by the optical absorbance at 260/280 nm using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Hemel Hempstead, UK).

2.14.2 RNA Amplification by the Eberwine Method

In this study, the Eberwine RNA amplification method (Phillips and Eberwine, 1996, Morris et al., 2011) was used to attain abundant mRNA from small cell samples (i.e. the dormant/cycling cell compartments derived from FACS sorting) while preserving the relationships among transcripts. The procedure is outlined in Figure 2.5 and is described in detail below.

2.14.2.i. First Round First Strand cDNA Synthesis

The following description is for every 5 µL of cell collection. The reaction can be scaled up for larger collection volumes (e.g. 2x for 10 µL collection volume, etc). 'Mastermixes' were created by multiplying the reagents by the number of collection tubes, plus 10-20% to account for pipetting error. A reagent list specifying the manufacturers is attached in Appendix 3.

To each tube, A volume (1x) of Mastermix #1, containing 1.2 µL dNTP's (at 2.5 mM each), 2.4 µL 5x first strand buffer, 0.3 µL T7-oligo(dT) primer (100 ng/µL), and 1.2 µL DTT (100mM), was added on ice, to create mRNA/cDNA hybrids. The volume was brought up to 10.25 µL with nuclease-free water, followed by a gentle mix by pipetting, and a brief spin in a microfuge. The mixture was then incubated for 5 minutes at 70˚C to denature any secondary structure of the mRNA, and was then immediately placed on ice for at least 5 minutes. A volume of Mastermix #2, containing 0.3 µL RNasin (40 U/µL),

0.45 µL Superscript III (200 U/µL), and 1 µL nuclease-free water, was added to each tube, attaining a total volume of 12 µL. The reaction was once again gently mixed by pipetting and briefly spun in the microfuge, followed by incubation at 42˚C for 1 hour to synthesis cDNA, and then at 70˚C for 15 minutes to inactivate the Superscript.

2.14.2.ii. First Round Second Strand cDNA Synthesis

Next, RNA primers were created from the mRNA portion of the mRNA/DNA hybrids to aid in synthesis of double stranded cDNA. A volume of Mastermix #3, containing 5.6 µL 5x second strand buffer, 0.75 µL dNTP mix (2.5mM each), 0.25 µL DNA ligase (10 U/µL), 1 µL DNA polymerase I (10 U/ µL), 0.25 µL Rnase H (2U/ µL) and 8 µL nuclease-free water, was added to each reaction tube on ice. The contents were mixed gently but thoroughly by pipetting and spun briefly, followed by a 2-hour incubation period at 16˚C. Afterward, 1 µL T4 DNA polymerase (5 U/ µL) was added, and the reaction was again thoroughly mixed and briefly spun and was then incubated for 10 more minutes at 16˚C. The reaction was cleaned up using the Qiagen MinElute kit as per the manufacturer's instructions with slight modifications (i.e. 2 washes with 500 µL wash buffer instead of once with 750 µL) and was eluted in nuclease-free water. The reaction was concentrated to 4 µL by ethanol precipitation. To each tube, 1 µL glycogen (3 mg/mL), 1/10 volume of 3M sodium acetate, 2.5 volumes of cold 100% ethanol, and 40 µL nuclease-free water were added and the reaction was precipitated at -80˚C for 30 minutes (or overnight), followed by 20 minutes centrifugation at 4˚C. The pellet was

washed with 800 µL 70% ethanol (diluted from 100% ethanol in nuclease-free water). Care should be taken to fully dislodge the pellet either by pipetting or vortexing, to remove any excess salt. The cDNA pellet was then resuspend in 4 µL nuclease-free water, and was used immediately to synthesise aRNA (or it could be stored at -20 or -80˚C for later usage).

2.14.2.iii. First Round in vitro transcription (IVT)

The synthesis of aRNA from the T7 promoter incorporated into the double stranded cDNA was performed using the Ambion MEGAscript T7 kit as per the manufacturer's instructions except for an adaptation to scale for a 10 µL reaction, instead of 20 µL. The procedure was performed at room temperature but the NTPs and the enzyme mix were kept on ice when not in use. The resuspended double stranded cDNA was transferred to a thin walled PCR tube, followed by the addition of 4 µL NTP mix (18.75mM each), 1 µL 10x reaction buffer, and 1 µL 10x enzyme mix. The reaction was incubated for 14 hours at 37˚C in a water bath, and was then cleaned up by ethanol precipitation using the Ambion MEGAclear kit, as per manufacturer's instructions with some modifications (two washes with 500 µL wash buffer instead of one wash with 750 µL buffer). To elute, 50 µL of nuclease-free water was added to the centre of the column. The reaction was then incubated at 70˚C for 10 minutes, and spun at 10,000g for 1 minute. The elution step was repeated in a fresh collection tube, and the eluates were combined and concentrated to 4 µL by ethanol precipitation. Ammonium acetate was used in place of sodium acetate to prevent co-precipitation of free nucleotides with the nucleic acid. To each reaction, 2 µL glycogen (5 mg/mL), 1/10 volumes of 5M ammonium acetate,

and 2.5 volumes of cold ethanol were added and the mixture was precipitated at -80˚C for at least 30 minutes (or overnight). Afterward, the reaction was centrifuged for 20 minutes at 4˚C. The pellet was washed with 800 µL 70% ethanol, and was fully dislodged to remove all of the excess salt. Then, the aRNA pellet was centrifuged for another 20 minutes at 4˚C, and was resuspended in 4 µL nuclease-free water.

2.14.2.iv. Second Round First Strand cDNA Synthesis

To every 4 µL aRNA, 1 µL random primers (0.05 mg/mL) was added (on ice). The reaction was heated at 70˚C for 10 minutes, and was then immediately placed on ice for at least 5 min. A volume of mastermix #4, containing 1 µL DTT (100mM), 0.5 µL dNTP's (2.5 mM each), 0.5 µL RNasin (40 U/ µL), and 1 µL Superscript III (200 U/ µL) was added. The mixture was rested at room temperature for 10 minutes, allowing extension of the short random primers before the reverse transcription reaction began. The reaction tube was incubated at 42˚C for 30 minutes to synthesise cDNA, 95˚C for 5 minutes to denature the RNA in DNA/RNA hybrids, and then chilled on ice for 5 minutes. The cDNA products were used to synthesise a second strand cDNA (or could be stored at -20 or -80˚C for later use).

2.14.2.v. Second Round Second Strand cDNA Synthesis

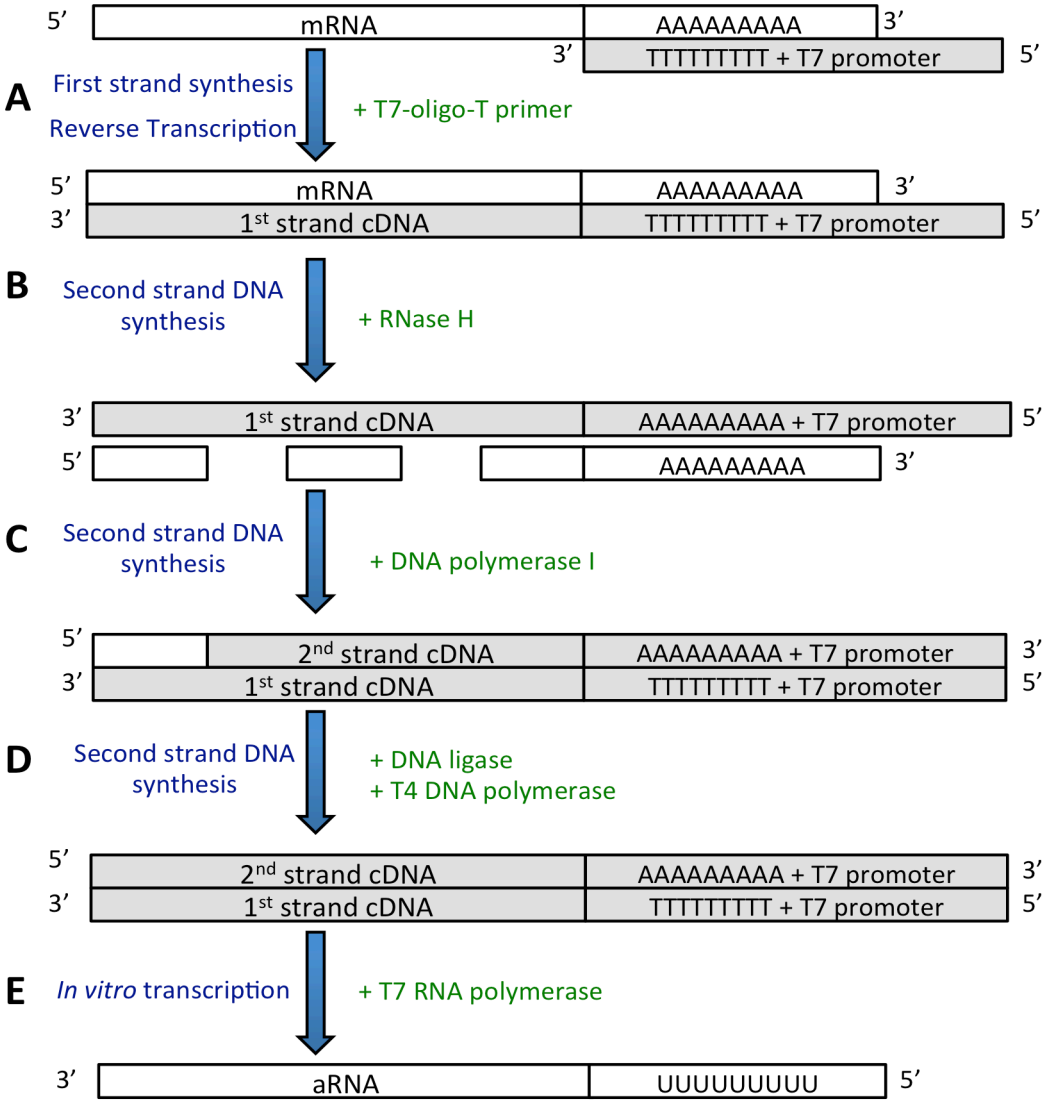
2 μL T7-oligo (dT) primer (10ng/μL) was added to each cDNA reaction tube placed on ice, followed by incubation at 70˚C for 5 minutes and another 5 minutes cooling on ice. Then, 15 μL 5x second strand buffer, 1.5 μL dNTP mix (2.5 mM each), 2 μL DNA polymerase I (10 U/ μL), and 43.5 μL nuclease-free water were added, followed by 2 hours incubation at 16˚C. 2 μL T4 DNA polymerase (5 U/ μL) was then added, followed by a further incubation at 16˚C, for 10 minutes. The reaction was cleaned up by the Qiagen MinElute kit, as described previously in Section 2.14.2.ii. The double stranded cDNA was concentrated to 4 μL by ethanol precipitation as previously (Section 2.14.2.ii).

2.14.2.vi. Second Round IVT and the Subsequent Round of Amplification

The IVT procedure is as described in Section 2.14.2.iii. The RNA amplification procedure can be repeated for a number of times. However, as each subsequent round of amplification results in shorter RNA products (Morris et al., 2011), the number of rounds should be limited. We have performed 3 rounds of amplification in this study.

Eberwine amplification process

First round amplification



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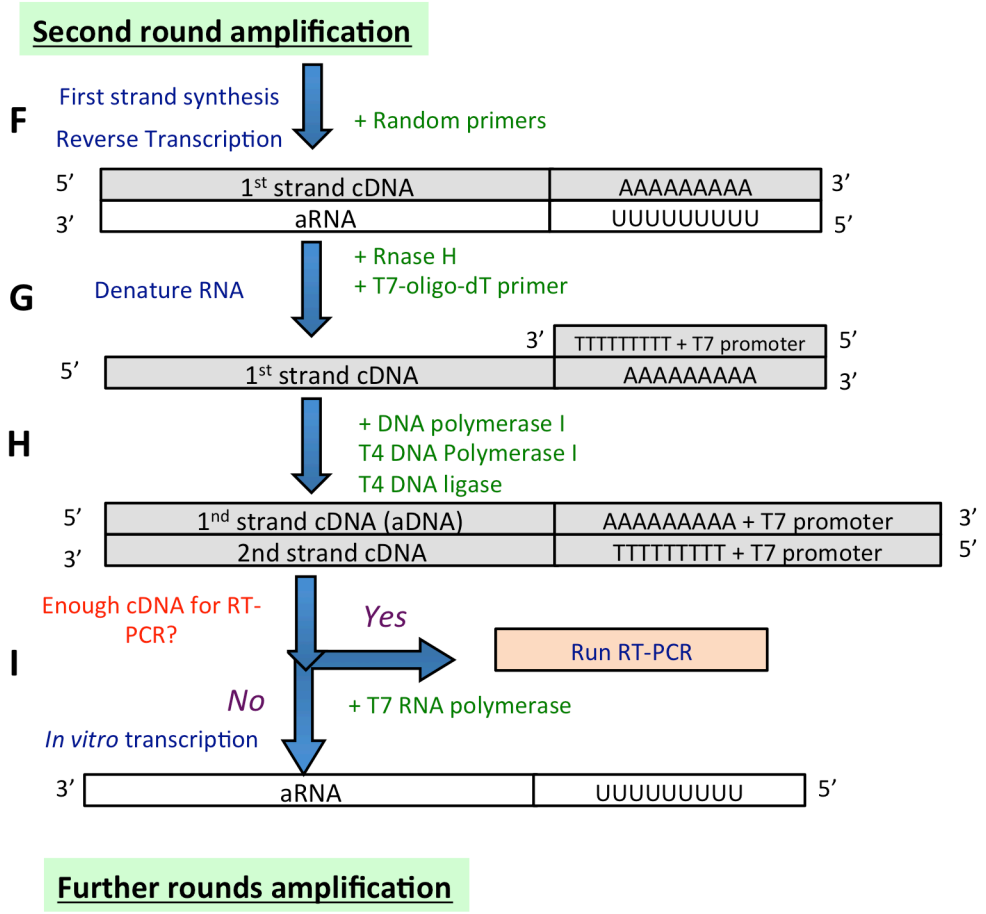

Figure 2.5 Schematic diagram of the Eberwine amplification procedure in this study. (A) First strand cDNA synthesis was primed by an oligo-dT primer containing a T7 polymerase binding site. Reverse transcriptase synthesises a cDNA strand from this primer using the mRNA as template. (B) – (C) The mRNA strand from the mRNA/cDNA hybrid was digested by RNase H treatment, leaving small fragments of mRNA which are used by DNA polymerase I to prime second strand cDNA synthesis. (D) Any incomplete replacement strands were ligated by DNA ligase with T4 polymerase filling in the gaps. (E) An *in vitro* transcription (IVT) step using T7 RNA polymerase then produced multiple copies of antisense-RNA (aRNA). (F) The amplified RNA was used as a template for a second and further rounds of amplification by priming the first strand cDNA (antisense-DNA, aDNA) synthesis with random primers. (G) RNase H was used again to digest the aRNA strand, and to leave small fragments of RNA to prime second strand cDNA (sense-DNA). (I) The amplified sense-DNA could be used for qRT-PCR or continue with a further IVT step to achieve more rounds of amplification.

2.14.3 Real-time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Real time PCR was performed on the thermal cycler 7500 Fast Real Time PCR System with 7500 Fast Sequence Detection and was analysed by the 7500 software v2.0.1 (Applied Biosystems, Paisley, UK), using a mastermix with SYBR Green (Applied Biosystems). The PCR mastermix consisted of 1 μL of sample cDNA, 5 μL SYBR Green mastermix (Applied Biosystems), 1pmol of the specified primer for the relevant gene (see Table 2.3), and was made up to 10 μL by molecular grade water. The thermal cycler conditions were comprised of an activation step (incubation at 50°C for 2 minutes), an enzyme activation step (95°C for 10 minutes), followed by 40 cycles of denaturation (95°C for 15 seconds) and anneal/extension steps (60°C for 1 minute). The products were then heated from 60°C to 95°C for 20 minutes during which a melting curve analysis was performed. This process was to determine the specificity of the products, indicated by the presence of a single melting peak, and also to confirm the absence of products generated by primer-dimer association. No template controls (NTCs) were included in each plate and all reactions were run in triplicate. To enable the levels of transcripts to be quantified, cDNA from the KG-1a cell line was used to construct a standard curve with doubling dilutions, giving concentrations between 0.78125 and 50 ng. And the Ct or "threshold cycle" values, i.e. the number of cycles to detect a real signal from the sample at the level above background fluorescence, were obtained from each RT-PCR run.

Table 2.3 Primers used in RT-PCR.

Gene	Primer Sequences (5' to 3')	Product code	Manufacturer	References
HIF1a	(Forward) ACT GCC ACC ACT GAT GAA TCA AAA ACA G (Reverse) TTC CAT TTT TCG CTT CCT CTG AGC ATT C	----------	Invitrogen	(Lukashev et al., 2001)
FPGS	'QuantiTect'® primer assay set	QT00052150	Qiagen	----------
TRAP1	'QuantiTect'® primer assay set	QT00007861	Qiagen	----------
DECR1	'QuantiTect'® primer assay set	QT00076986	Qiagen	----------
PPIB	'QuantiTect'® primer assay set	QT00067186	Qiagen	----------
STK11/LKB1	'QuantiTect'® primer assay set	QT01008980	Qiagen	----------
FOXO3	'QuantiTect'® primer assay set	QT00031941	Qiagen	----------
GATA2	'QuantiTect'® primer assay set	QT00045381	Qiagen	----------
CITED2	'QuantiTect'® primer assay set	QT00014364	Qiagen	----------
HES1	'QuantiTect'® primer assay set	QT00039648	Qiagen	----------

The quantitative data were generated from the standard curves. The relative expression levels of gene transcripts were calculated as the ratio between the level of candidate genes and the level of the housekeeping gene.

2.15 Statistical Analysis

Data were analysed using SPSS 16.0 (Chicago, IL, USA). Results are shown as mean ± standard deviation (SD) of values obtained in independent experiments ($n \geq 3$). Chemosensitivity experiments were analysed using Student paired *t* test. Confidence intervals (CI) and co-efficient of variability (Cv) were calculated to investigate dormancy marker reproducibility. And the Spearman's rank correlation coefficient (rho) was calculated to indicate the correlation

between each dormancy marker. Wilcoxon signed rank test was used to compare dormant marker expressions in bulk and LAP^+ populations. *P* values of ≤ 0.05 were considered to represent significance.

CHAPTER 3.

TARGETING BM NICHE-MEDIATED CHEMORESISTANCE IN AML

3.1. Background

Although front-line therapy (typically including Ara-C and an anthracycline compound, such as daunorubicin) is usually effective at remission induction, most AML patients suffer from relapse and chemoresistance. An altered haematopoietic microenvironment and impaired apoptosis are two contributors (among other factors such as drug influx and efflux, and up-regulated DNA repair mechanisms, etc.) to the complex, simultaneous processes that lead to chemoresistance (Gottesman, 2002). Leukaemic cells disrupt and hijack normal haematopoietic BM niches, and create a tumour microenvironment with dominant signals that favour cell proliferation and growth (Sipkins et al., 2005, Colmone et al., 2008). Up-regulation of the pro-survival BCL-2 and MCL-1 pathways is often observed in leukaemic cells growing in contact with BM-derived stromal cells (Konopleva and Jordan, 2011). Like other cancerous cells, leukaemia cells are prone to be addicted to producing survival molecules, making specific targeting of the mitochondrial pathways of apoptosis an important way of eradicating the disease (Adams and Cory, 2007). Novel agents that selectively target BCL-2 family pro-survival molecules would provide an advanced approach to AML therapy. We selected two categories of novel agents: (1) the multi-kinase inhibitor TG02 and (2) the BCL-2 BH3 mimetics ABT-737 and ABT-199. TG02 selectively inhibits the cell cycle and transcriptional cyclin-dependant kinases 1, 2, 3, 5, and 9. Studies showed that TG02 exerts the greatest activity against CDK9 (Goh et al., 2012), a kinase which phosphorylates the C-terminal domain of RNA polymerase II (RP2) and activates transcription (Cho et al., 2001, Yik et al., 2003). As a result TG02

depletes short-lived proteins such as the BCL-2 family member MCL-1 (Pallis et al., 2012). Novel pan BCL-2 inhibitors ABT-737 and ABT-199, on the other hand, avidly bind the pro-survival proteins and induce Bax/Bak-dependent cell killing at very low doses (nM range) (van Delft et al., 2006, Pan et al., 2014). This study aimed to investigate whether these novel chemotherapeutic agents, which target the intrinsic apoptotic pathway, could efficiently target niche-mediated chemoresistance in AML.

3.2. Are the Stromal Cells MS-5 And MSCs Susceptible To the Drugs Used in the Co-culture Study?

BM stromal cells are the main source of SDF-1 and are known to support haematopoietic cell growth and survival *in vitro* (Aiuti et al., 1997, Majumdar et al., 2000, van Gosliga et al., 2007, Rozenveld-Geugien et al., 2007, Walenda et al., 2010), and to facilitate leukaemia chemoresistance (Andreeff and Konopleva, 2002, Zeng et al., 2009). Two types of BM stromal cells, the murine MS-5 and the human MSCs, were used as feeder cells in this project to provide an *in vitro* 'niche' environment for AML cells. MSCs were tested positivity for CD90, CD73 and negativity for CD45 and CD34 (Figure 3.1).

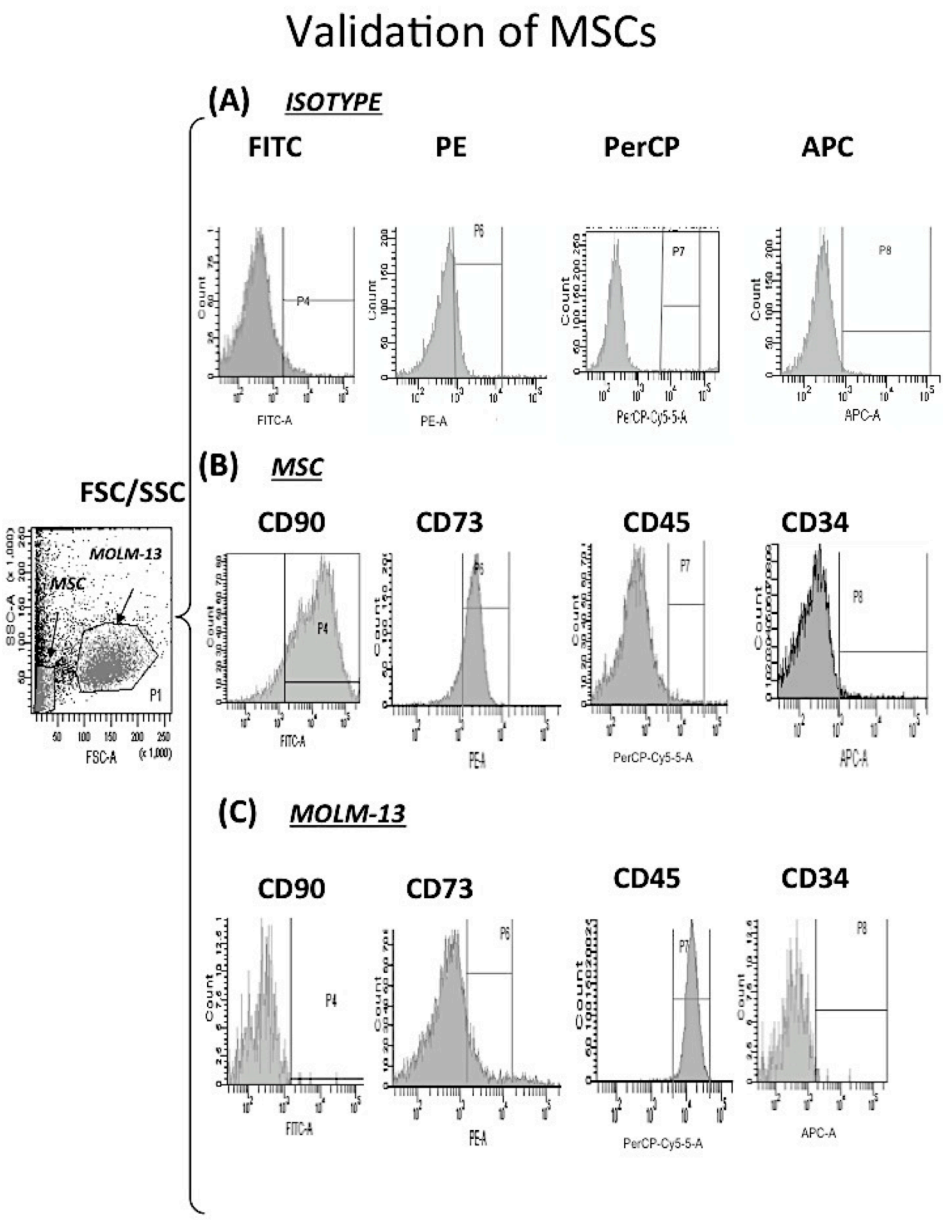

Figure 3.1. Distinguishing MSCs from MOLM-13 AML cells. MSCs were verified for surface antigen profiles with positivity for CD90 and CD73 and negativity for characteristic haematopoietic antigens CD45 and CD34, fluorescence expression of test antibodies was compared with according isotype control antibodies. (A) MSCs were labelled with mouse-anti-human isotype control antibodies. (B) MSCs were tested positivity for CD90, CD73 and negativity for CD45 and CD34. (C) The known CD34-negative AML cell line MOLM-13 (source: DMSZ) was tested negativity for CD73, CD34 and positivity for CD45.

In order to study chemosensitivity of AML cells to various therapeutic agents, e.g. Ara-C, TG02, ABT-737 and ABT-199, it was important to validate the suitability of the AML-stroma cell culture systems. We investigated whether these chemotherapeutic agents were toxic to the feeder cells. MS-5 and MSCs were seeded at a density of 5 x 10^4/ml and were maintained at 37°C in a 5% CO_2 humidified atmosphere for 24 hours to form a monolayer, before drugs (Ara-C at doses up to 1000 nM, TG02 at doses up to 100 nM, ABT-737 and ABT-199 at doses up to 300 nM, respectively) were added to the culture. The range of drug concentrations given to the stromal cells alone was inclusive of the doses given to AML cells cultured on and off stroma in the impending chemosensitivity study (detailed in Sections 3.3 and 3.4). Results represent the percentage of viable cells versus the untreated controls (Mean ± SD, N=3). No significant cell kill was observed in stromal cells after 72 hours of exposure to drugs (Figure 3.2). This implied that neither type of the stromal cells was susceptible to the drugs in this study, confirming the usage of the "AML-on-stroma" cell culture model in this study was valid.

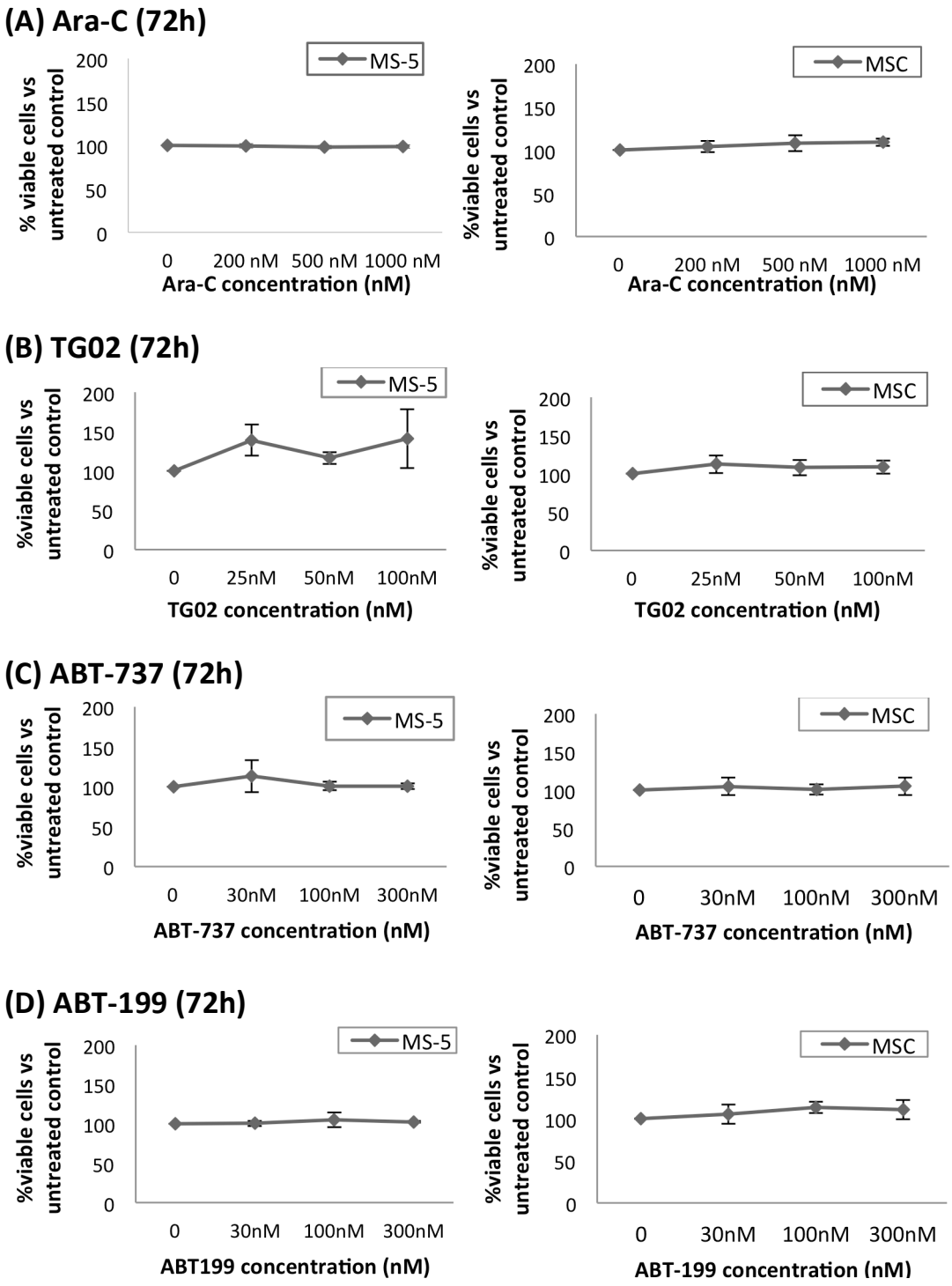

Figure 3.2 Stromal cells MS-5 and MSC were not susceptible to Ara-C, TG02, ABT-737 and ABT-199 after 72 hours exposure to drugs. MS-5 and MSC stromal cells were plated 24 hours prior to treatment with chemo-agents, in order to allow the cells attaching to the plastic surface. The following drugs: (A) Ara-C at 0, 200, 500, 100 nM; (B) TG02 at 0, 25, 50, 100 nM; (C) ABT-737 at 0, 30, 100, 300 nM; and (D) ABT-199 at 0, 30, 100, 300 nM were given. The stromal cells were exposed to the drugs for 72 hours before flow cytometric analysis using the 7-AAD/FSC methods. Results are shown as Mean ± SD, N=3.

3.3. MS-5 And MSC Stromal Cells Exhibited Similar Efficacy In Supporting AML MOLM-13 Cell Survival

The human BM MSCs represent an important cellular component of the BM microenvironment supporting haematopoiesis and are found to support expansion of HSCs *ex vivo* to simulate physiological stromal conditions (Majumdar et al., 2000, Liang et al., 2010, Morrison and Scadden, 2014). We did a comparison to see whether MS-5 and MSCs have different impacts on AML chemosensitivity. The human MOLM-13 cells were cultured with or without the presence MS-5 or MSCs for 72h, and were treated with various drugs: Ara-C (200 nM), TG02 (25 nM), ABT-737 (100 nM), and ABT-199 (100 nM). The drug concentrations were determined by preliminary work on AML cell lines (data not shown). Results represent cell kill (%) for drug-treated cells versus untreated controls, and are based on three independent experiments (Figure 3.3). MSC and MS-5 cells exhibited strikingly analogous patterns in terms of supporting AML cell survival, under Ara-C ($p = 0.344$), TG02 ($p=0.93$), ABT-737 ($p = 0.536$) and ABT-199 ($p = 0.188$) treatment. Ara-C had poorer toxicity to co-cultured AML cells than those in suspension culture over the 72-hour period, shown by only 3.5% cell kill in the presence of MS-5 ($p = 0.005$), and 5.9% cell reduction in the presence of MSCs ($p = 0.029$), compared to nearly 40% cell kill when MOLM-13 were cultured alone. TG02 presented a good capacity in targeting AML cells on stroma, achieving an additional 15% ($p = 0.041$) and 16% ($p = 0.050$) cell kill in MOLM-13 cells

co-cultured with MS-5 and MSCs, respectively, compared to those in suspension culture. The toxicity of the two BH3 inhibitors was not impeded by the presence of stromal cells. No reduced chemosensitivity to ABT-737 was observed, when MOLM-13 cells were co-cultured with either MS-5 ($p = 0.219$) or MSCs ($p = 0.101$), compared to those in suspension cultures. Likewise, no significant reduction in MOLM-13 sensitivity to ABT-199 was detected, either when cells were co-cultured with MS-5 ($p = 0.149$) or MSC ($p = 0.164$).

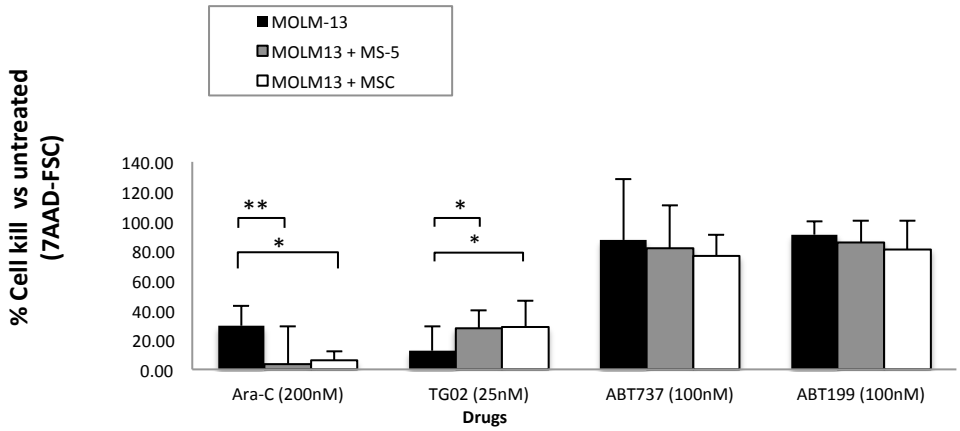

Figure 3.3 72-hour chemosensitivity (% cell kill vs. untreated) of the MOLM13 cell line to TG02, ABT-737 and ABT-199 on and off stroma. Cells were cultured with various drugs, i.e. Ara-C (200nM), TG02 (25nM), ABT-737 (100nM) and ABT-199 (100nM) on or off stromal cells (MS-5 and BM-MSCs) for 72 hours. Results (percentage cell kill vs. untreated conditions) are shown as Mean ± SD, representative of three independent experiments. P values were determined by paired T test to compare drug toxicity under different culture models. **$P < 0.01$, *$P \leq 0.05$.

3.4. Novel Agents Effectively Impaired AML Survival On Stroma in Short-term Culture

In this study, the toxicity of individual drugs to AML cells was evaluated by 72h chemosensitivity assays in 16 primary AML samples (#AML1, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 27, 28, and AML29; patient characteristics

are tabulated in Table 1, Appendix 1) under stromal conditions (with MS-5) or in suspension culture (i.e. without MS-5). Most samples had significantly higher survival rate when cultured on stroma than in suspension culture without drug exposure (p=0.013; Figure 3.4-A), suggesting that stromal cells provided good support for AML cell maintenance *in vitro*. The toxicity of Ara-C was impeded by the stroma niche, shown by the significantly higher viable counts of AML cells co-cultured with MS-5, than those cultured without MS-5 (p=0.042), Figure 3.4-B. To compare effects, and since Ara-C reduced the number of cells by no more than 40% of cells in suspension culture, IC_{40} (the concentration which induced 40% reduction in cell number) measurements were used to analyse the cytotoxicity of each drug to AML cells on stroma. The IC_{40} of these drugs in suspension culture were determined by the dose-response curves shown in Figure 3.4-B, with Ara-C at 200 nM, TG02 at 25 nM, and ABT-737 and ABT-199 both at 100nM. When this dosage was given to AML cells co-cultured with MS5, compared to those treated with the traditional drug Ara-C which had a mean cell kill of 14%, the mean cell kill was increased to 33% with TG02 ($p = 0.042$), 36% with ABT-737 ($p = 0.013$) and 36% with ABT-199 ($p = 0.02$), indicating a better efficacy of these novel agents in targeting AML cells maintained in stromal niche than Ara-C (Figure 3.4-C). Also, none of the novel drugs showed preference for proliferating cells, i.e. cells in suspension culture ($P_{TG02} = 0.452$; $P_{ABT\text{-}737} = 0.596$; $P_{ABT\text{-}199} = 0.423$). Ara-C, by contrast, had better efficacy in proliferating cells ($p = 0.006$; Figure 3.4-C).

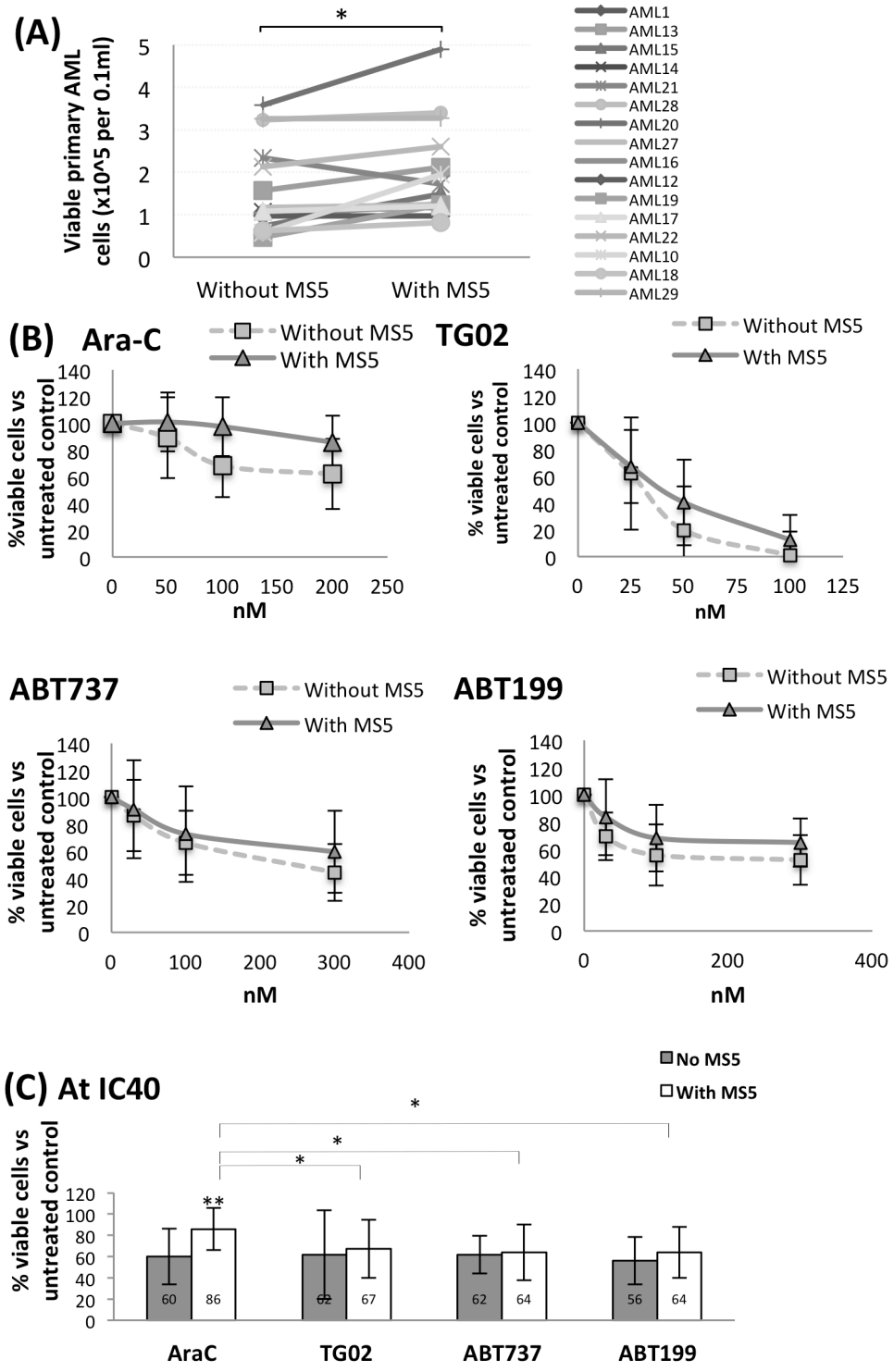

Figure 3.4 Novel agents TG02, ABT-737 and ABT-199 effectively impaired AML cell survival on stroma in short-term culture. Cells from 16 AML patient samples were cultured on or off MS-5 feeder cells for 72 hours. (A) Comparison of viability (%, vs untreated control) when the same AML samples were cultured with or without MS5. (B) Dose response for Ara-C, TG02, ABT-737 and ABT-199. (C) Comparison of viability (%, vs untreated control) after AML samples were treated with drugs at a dose equivalent of IC_{40} derived from suspension culture. ** $P < 0.01$, * $P \leq 0.05$.

3.5. Discussion

The BM niche-protected, dormant cells within a leukaemic clone present challenges to AML treatment, as the mainstay chemotherapeutic drugs are designed to target proliferating cells and therefore chemoresistance is often observed. Agents with novel mechanisms of action are needed. In this study, we focused our attention on to the mitochondrial apoptotic pathway in dormant leukaemia cells.

An *in vitro* AML-on-stroma, short-term chemosensitivity assay was set up, using two types of BM stromal cells, the murine stroma cell line MS-5 and the human BM-MSCs. The MS-5 cells have been a popular *in vitro* feeder model supporting the survival, growth and differentiation of primitive AML cells and cell lines in short-term and long-term cell cultures, as well as cell expansion in colony assays (Issaad et al., 1993, Mohle et al., 1998, Konopleva et al., 2002a, Zeng et al., 2009, Zeng et al., 2012). Also, MS-5s were found to facilitate leukaemia chemoresistance by up-regulating anti-apoptotic proteins BCL-2 and BCL-xL levels and thus prevent apoptosis of AML cells (Konopleva et al., 2002a). Another advantage of MS-5 is that being a cell line, they are easy to access and maintain. The use of human BM-MSCs provides a more physiological approach compared to MS-5, and *in vitro* studies suggested that MSCs support proliferation and self-renewal of primitive haematopoietic cells (Walenda et al., 2010). However, as MSCs have multilineage potential (Pittenger et al., 1999) and are derived directly from BM which may contain various cellular types, it was important to confirm their identity prior to being

used in the co-culture study. We tested a range of cell surface markers, including MSC-positive markers, e.g. CD73 (Pittenger et al., 1999), CD90 (Colter et al., 2000); and MSC-negative, HSC-positive markers CD45 and CD34 (Pittenger et al., 1999, Colter et al., 2000). An additional step was taken to see whether these stromal cells were susceptible to the drugs in our study, which would confound the results. 'Mock' treatments were given to the stromal cells MS-5 and MSC, using the same dosage of drugs (Ara-C, ABT-737, ABT-199 and TG02), that would be given to the AML cells cocultured with stroma in the impending chemosensitivity study. No substantial cell kill was observed in stromal cells after 72 hours of drug exposure. This implied that neither type of stromal cells was a target to the drugs and ensured us to continue with the coculture study. To see whether one stroma type was advantageous to the other in terms of supporting AML cell survival, a comparison was carried out and we showed that MS-5 and MSC stromal cells had similar efficacy in supporting MOLM13 survival (Figure 3.3). We also demonstrated that *in vitro* stroma support (MS-5) significantly improved AML patient cell survival (Figure 3.4-A).

The therapeutic relevance of BCL-2 in AML has been long established (Keith et al., 1995, Konopleva et al., 2000). And inhibition of BCL-2 family members with small-molecule BH3 mimetics has shown promises for haematologic and solid tumours (Oltersdorf et al., 2005, Yecies et al., 2010, Vandenberg and Cory, 2013, Vaillant et al., 2013, Pan et al., 2014). However, the notorious setback for ABT-737 and its oral bioavailable compound ABT-263 is the non-selective inhibition of several BCL-2 family members, such as BCL-2, BCL-w

and BCL-xL, whilst Mcl-1 and Bfl-1 are untargeted. As circulating platelet survival is dependent on BCL-xL, treatment with ABT-737 may lead to dose-dependent thrombocytopenia in patients. Nevertheless, emerging studies argue that Bcl-2 is the optimal target of ABT-737, preferred over Bcl-xL and Bcl-w, in a cellular context (Rooswinkel et al., 2012, Merino et al., 2012). The highly variable expression of BCL-2 molecules in AML cells has highlighted the importance of cellular BH3 profiling (Deng et al., 2007, Pierceall et al., 2013, Pan et al., 2014), which can help decide which AML cases are likely to respond to BCL-2-targeting BH3 mimetics. The BH3 mimetic ABT-199 has a safer profile for not targeting BCL-xL. It was found to induce rapid apoptosis of AML cell lines and primary myeloblasts at very low concentrations (nM) and inhibited AML progression in mice xenograft *in vivo* (Pan et al., 2014). We investigated the two BH3 mimetics in parallel, by comparing the *in vitro* cytotoxicity of primary AML cells (n = 16) to these agents in stromal niches, using the AML/MS-5 co-culture model. After 72 hours of drug exposure, no significant difference was observed between BCL-2 inhibitors ABT-737 and ABT-199 in inducing cell kill on stroma. When given the same dose, these two BH3 mimetics showed analogous patterns in cytotoxicity (Figure 3.4-B). The novel multi-kinase inhibitor TG02 has selectivity against cell cycle and transcriptional Cdks, as well as FLT3-ITD mutations. Previous studies indicated that TG02 inhibition of RP2 successfully induced apoptosis in primary AML cells at nanomolar concentrations, possibly through rapid depletion of MCL-1 but not BCL-2 or BCL-w (Pallis et al., 2012). And here we have shown that TG02 had improved cytotoxicity under BM stromal conditions compared to the traditional drug Ara-C (Figure 3.4-B). Emerging

data suggest that the cytotoxic actions of TG02 and BH3 mimetics are complementary in AML cells (Abdul-Aziz et al., 2014, Burrows et al., 2014). The mechanism-based combination may provide an improved regimen for targeting apoptosis and chemoresistance in AML.

Taken together, our current investigation provided evidence that the intrinsic apoptosis-inducing agents, the BH3 mimetics (ABT-737 and ABT-199) and TG02 could efficiently target BM niche-mediated chemoresistance, which is thought to be one of the main issues with traditional chemotherapy.

CHAPTER 4.

IDENTIFYING AND TARGETING DORMANT AML SUBSETS *IN VITRO*

4.1 Background

The interaction with the BM 'niche' is essential for LSCs and progenitors to maintain a predominant dormant (G_0) cell cycle profile (Li and Bhatia, 2011, Ishikawa et al., 2007, Guan et al., 2003). The capability of these cells to lie dormant for long periods of time before dividing helps to explain why most AML patients appear to respond to remission induction treatment but eventually relapse, as traditional chemotherapeutic drugs mainly target cycling cells with active DNA replication but spare the dormant subsets, which may thus represent an important leukaemia reservoir and require effective targeting. In this chapter, we aimed to develop and optimise *in vitro* cell culture systems to identify dormant leukaemia cells in AML, and to determine their sensitivitiy to TG02, ABT-737 and ABT-199, which we have previously shown to efficiently target BM niche-mediated chemoresistance (Figure 3.4, Chapter 3), in dormant AML cells. Moreover, cells tend to enter senescence under *in vitro* conditions, as reviewed by Hwang et al (Hwang et al., 2009). Therefore we wanted to find out whether cells that had not divided *in vitro* still kept their clonogenicity.

4.2 Identification of AML Cells *In Vitro*

To investigate therapeutic responses in dormant AML cells, a prerequisite is to distinguish leukaemic cells from normal populations. The usage of flow cytometry to detect cell surface leukaemia-associated phenotype markers is a good way of identifying leukaemic properties. In particular, some lineage markers such as CD2, CD5, CD7, CD11b, CD11c, CD15, CD19, CD22 and

CD56, are almost completely absent on normal $CD34^{+}CD38^{-}$ HSCs (van Rhenen et al., 2007a, Feller et al., 2013, Terwijn et al., 2013), thus may define the leukaemic nature of $CD34^{+}CD38^{-}$ cells, without needing to add a myeloid marker (van Rhenen et al., 2007a).

In this study, immunophenotyping assays were carried out on nineteen patient samples, including 16 *de novo* (samples #AML1-7, 23-25, 31, 33, 35, 36, 37, 39 and AML40), 2 relapsed (#AML2R and 4R), and 1 secondary AML (Sample #AML3; transformed from treatment-related chronic myelomonocytic leukaemia, tr-CMML), using multi-colour antibody combinations with multi-parameter flow cytometry (MPFC). As described in Section 2.10 (Chapter 2), a panel of antibodies was selected consisting of myeloid markers (CD13 and CD33), stem/progenitor markers (CD34, CD38 and CD117) with CD45, and aberrant markers (e.g. CD2, CD7, CD15 and CD19), based on a recent multi-centre consensus that proposed some of the most commonly accepted markers for LAP analysis (Feller et al., 2013). On the basis of this report, the aim was to identify the cross-lineage antigen expression of CD2/CD7/CD15/CD19 with either CD34 or CD117. Asynchronous antigen expression was also investigated, such as an absence of CD13 while presenting CD117 and CD34, an absence of the Class II human leukocyte antigen molecule HLA-DR with the expression of CD34, and also an absence of CD33 while presenting CD34. 10% was a standard cut-off point for LAP expression among all mononuclear cells tested (Feller et al., 2013, Terwijn et al., 2012, Terwijn et al., 2013, Zeijlemaker et al., 2013) (Figure 4.1).

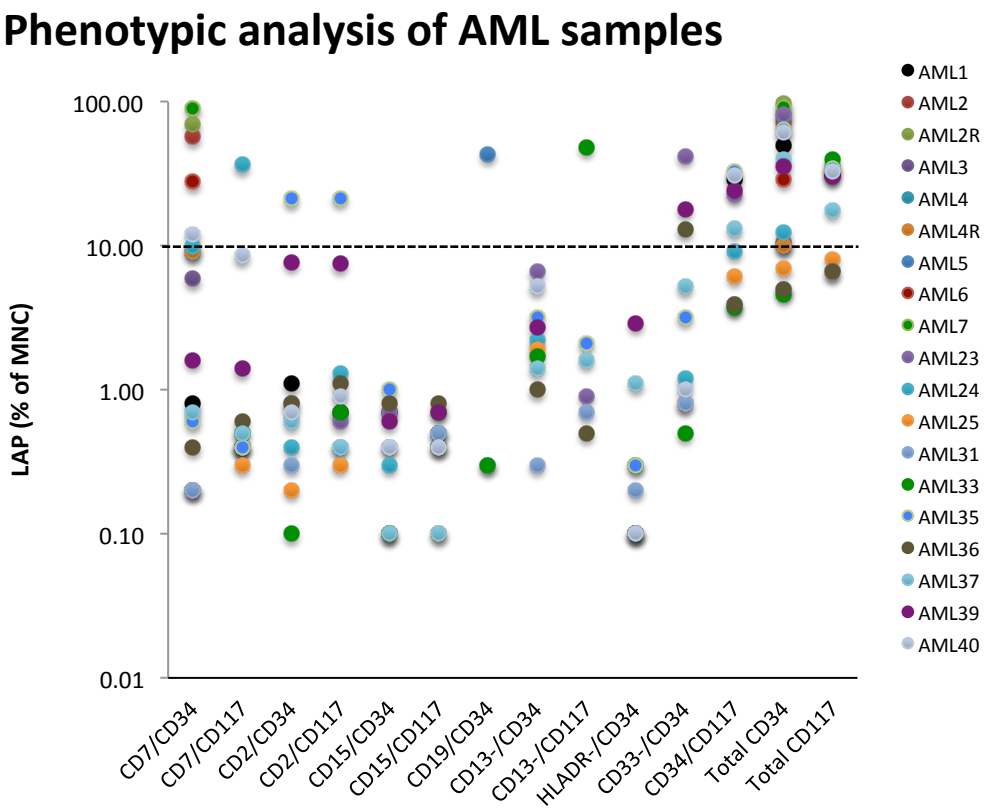

Figure 4.1. Expression of marker/marker combinations on MNC in AML samples. Different LAP immunophenotypes tested in patient MNC samples. All LAPs contained CD45. LAP expression is shown as % of MNC. 10% is a standard cut-off point for LAP expression among all mononuclear cells tested. Data are presented in Logarithmic scale.

Cross-lineage antigen expression was found in 11 tested AML samples. Nine samples, including 5 *de novo* (#AML2, 4, 6, 7, 24 and 40), 2 relapsed (#AML2R and 4R), and 1 secondary (#AML3) were identified as $CD7^{+}/CD34^{+}$ and/or $CD7^{+}/CD117^{+}$. One sample (#AML5) was $CD19^{+}/CD34^{+}$, and one sample (#AML35) presented $CD2^{+}/CD34^{+}$ and $CD117^{+}$ co-expression. Asynchronous expression of $CD33^{-}/CD34^{+}$ was found in 3 samples (#AML23, 39, and 36) (Figure 4.1; summary in Table 4.1). The immunophenotyping assays provided a practical step which helped to identify LAP^{+} samples containing at least 10% leukaemic cells. These samples were used in our long-

term cell culture studies to identify dormant leukaemic cells after 12 days and to specifically look at the therapeutic responses in this cell fraction.

Table 4.1. LAPs in AML patient samples.

AML sample	Aberrant surface markers (LAPs or overexpression)
AML 2	$CD34^+$, aberrant CD7
AML 2R	$CD34^+$, aberrant CD7
AML 3	$CD34^+$, aberrant CD7
AML 4	$CD34^+$, aberrant CD7
AML 4R	$CD34^+$, aberrant CD7
AML 5	$CD34^+$, aberrant CD19
AML 6	$CD34^+$, $CD117^+$, aberrant CD7
AML 7	$CD34^+$, $CD117^+$, aberrant CD7
AML 23	$CD34^+$ with $CD33^-$, $CD117^+$
AML 24	$CD34^+$, $CD117^+$, aberrant CD7
AML 35	$CD34^+$, $CD117^+$, aberrant CD2
AML 36	$CD34^+$ with CD33-, $CD117^+$
AML 39	$CD34^+$ with CD33-, $CD117^+$
AML 40	$CD34^+$, $CD117^+$, aberrant CD7

4.3 Identification of Dormant AML Cells *In Vitro*

Various *in vitro* culture systems were explored to identify dormant AML cells and to distinguish these undivided live cells from their cycling/divided counterparts after long-term (12-day) cell expansion. Initially, we chose the AML-stroma (murine MS-5 or human MSC) co-culture method, as stromal cells are known to support haematopoietic stem cell growth, proliferation, and long-term maintenance (Majumdar et al., 2000, van Gosliga et al., 2007, Rozenveld-Geugien et al., 2007, Walenda et al., 2010), as well as the facilitation of drug-resistance (Andreeff and Konopleva, 2002, Zeng et al., 2009) and anti-apoptotic mechanisms in AML cells (Garrido et al., 2001, Konopleva et al., 2002a). However, there were certain drawbacks to this

method. Firstly, this model required plating of stromal cells at least 24 hours before AML cells were added to the co-culture; secondly, there was a limited availability of human donor BM from which the MSC could be obtained. Considering the above limitations, it was sometimes not practical to study newly diagnosed, fresh AML samples which required immediate handling and placing into culture. We therefore ventured out to see if suspension culture systems with additional cytokines had comparable growth support capacity which the co-culture systems provide. We used a combination of the StemSpan^{TM} SFEM and the CC100 cytokine cocktail, which were shown to offer good support to the proliferation of human haematopoietic progenitors (Du et al., 2011, Goh et al., 2012). General methodology of the 12-day primary cell cultures is given in Chapter 2 and the essential steps are highlighted in Figure 4.2. On Day0, magnetically sorted $CD34^{+}$ cells were labelled with PKH26 dye and cultured with either stromal cells or seeded in the StemSpan SFEM at a density of 2.5 x 10^{5} per mL for 12 days. Cells in the co-culture were demi-populated, counted and phenotyped on Day7. Cells in the SFEM culture were demi-populated and counted every 2-4 days. After the initial 7 days, during which the majority of cells died off and the remaining cells established a growing pattern, a single dose of various drugs (Ara-C 200 nM, TG02 25 nM, ABT-737 and ABT-199 100 nM), determined by the chemosensitivity assays described in Chapter 3, was added to the culture on Day8. On Day12, cells were enumerated by CytoCount^{TM} beads (described in Chapter 2; Section 2.9.3), phenotyped and FACS-sorted into the dormant ($CD45^{dim}CD34^{+}LAP^{+}PKH26^{high}$) and cycling ($CD45^{dim}CD34^{+/-}LAP^{+}PKH26^{low}$) fractions for further analysis.

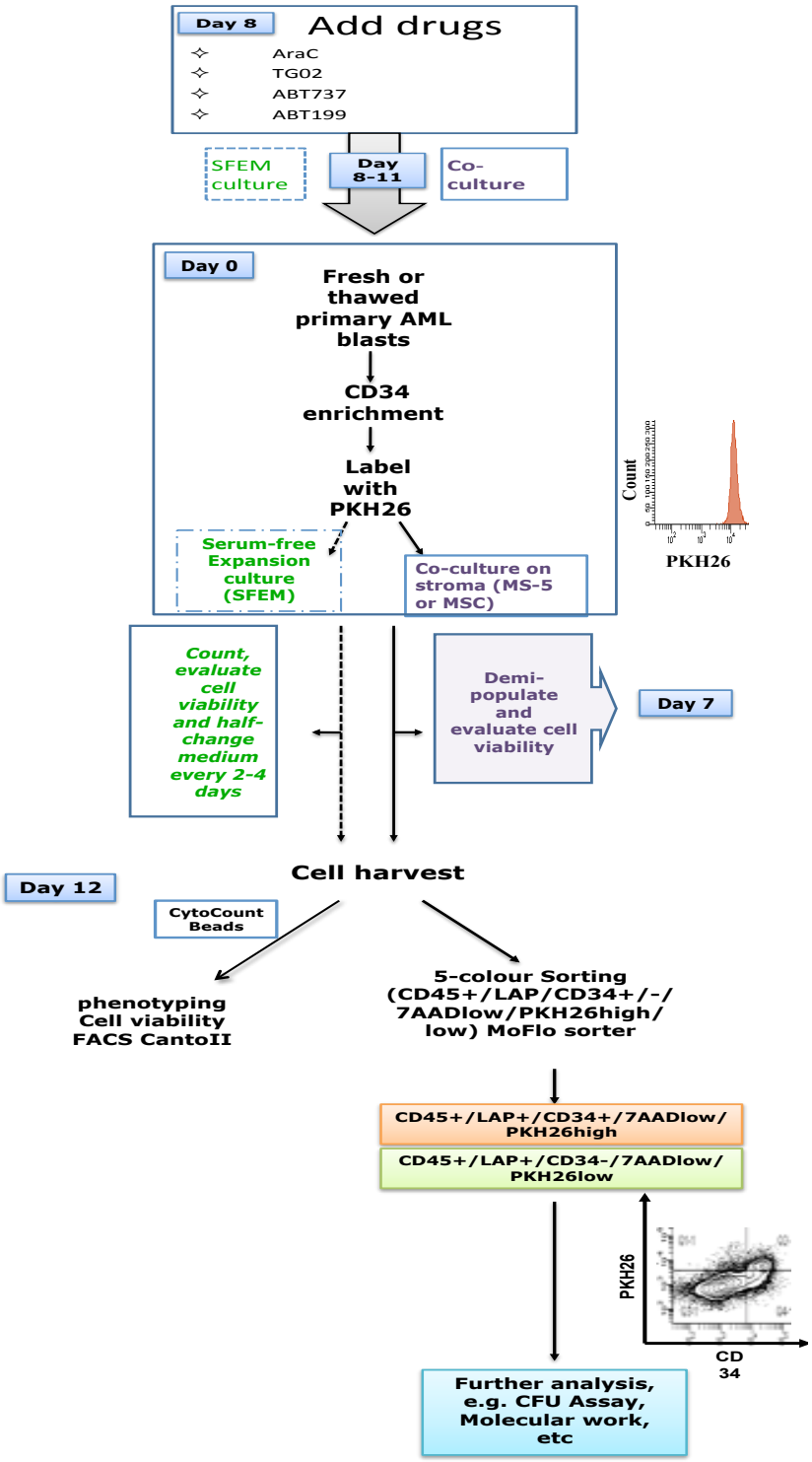

Figure 4.2. Schema of the 12-day cell culture systems. (A) On Day0, $CD34^+$ enriched cells were labelled with PKH26 and cultured with either stromal cells or in the SFEM culture at a density of 2.5 x 10^5 per mL for 12 days. Cells in co-culture were demi-populated, counted and phenotyped on Day7. Cells in SFEM culture were demi-populated and counted every 2-4 days. Drugs were added to the culture on Day8. On Day12, cells were phenotyped, counted and FACS sorted into the dormant and cycling fractions for further analysis.

Prior to using the cell dye PKH26, the toxicity was assessed to validate its use in the long-term cell culture study. Bulk and magnetically enriched $CD34^+$ cells from patient samples #AML1, 2 and 4R were labelled with PKH26 (10 μM), sham-treated with Diluent C (the diluent for PKH26), or unlabelled/untreated. After 14 days in the methylcellulose-based medium (Methocult^{TM} H4534), colonies were scored using the standard of over 50 cells per cluster under the microscope. No evident reduction of cell proliferation was observed in PKH26-labelled cells compared with untreated and sham-treated cells, indicating that PKH26 was not toxic to AML cells and it is a safe cell dye for long-term culture (Figure 4.3).

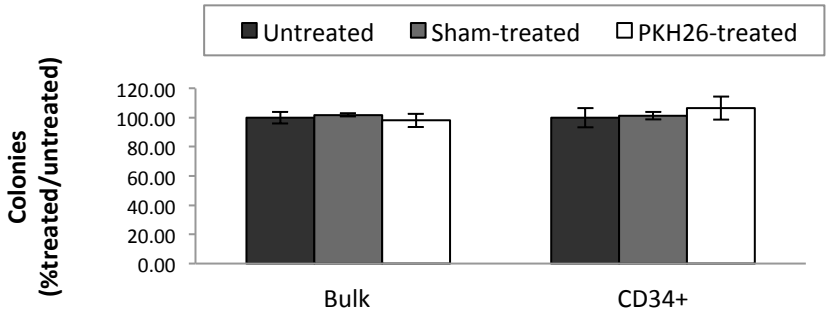

Figure 4.3. PKH26 is not toxic to AML cells. Bulk and magnetically sorted $CD34^+$ cells from samples #AML1, and 4R were labelled with PKH26 and then resuspended in methylcellulose-based medium, and cultured for 14 days at 37°C, 5% CO_2. No significant reduction of cell proliferation, as measured by colony production, was observed in PKH26-labelled cells.

Furthermore, we also investigated whether the cryopreservation and the subsequent thawing process would affect cell expansion capacity. Three thawed samples (#AML1, 2 and 4) and two freshly isolated AML samples (#AML8 and 9) from newly diagnosed patients were used. $CD34^+$ cells were seeded at 2.5 x 10^5 cells/mL on a 12-well plate, either co-cultured with MS-5 or in the StemSpan^{TM} SFEM for 12 days and cell expansion was determined by

the FSC/7AAD flow cytometric method (described in Chapter 2; Section 2.9.2). Under the same conditions (in terms of the procedure, vessels, incubation, etc.), cell expansion was greatly enhanced over 12 days when using fresh cells (Figure 4.4), up to 35.1 fold in co-culture and up to 41.4 fold when expanded in SFEM, in contrast to the expansion of frozen-thawed samples with up to 4.0 fold when expanded in co-culture and up to 1.5 fold in SFEM (Table 4.2).

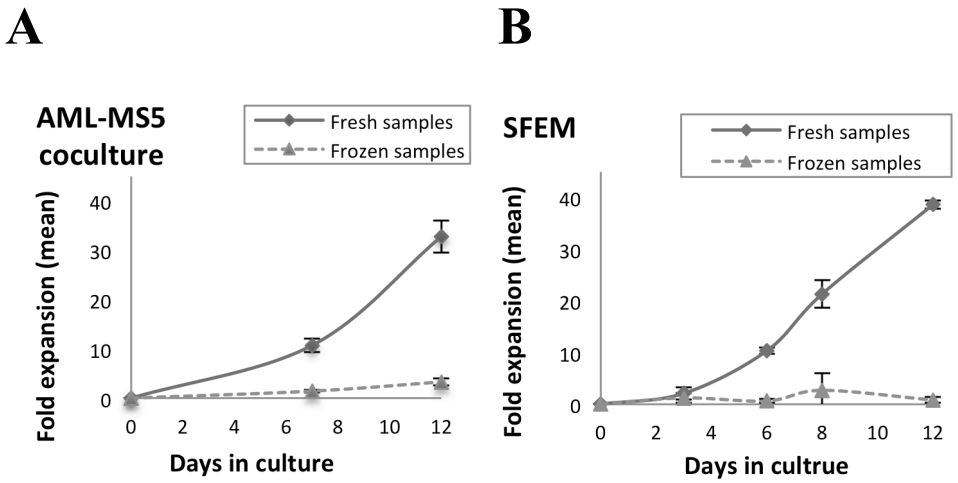

Figure 4.4. Freshly isolated CD34$^+$ cells achieved better expansion *in vitro* than frozen-thawed cells. Three frozen samples and two fresh samples were included. Cell count was determined by 7-AAD/FSC on a flow cytometer. (A) Fold expansion measured in the AML-MS-5 co-culture model on Day0, 7 and 12; (B) Fold expansion measured in the SFEM culture system on Day0, 3, 6, 8, and 12. Results are shown as Mean ± SD.

Table 4.2 Fold of CD34$^+$ cell expansion *in vitro*. Results are shown as mean ± SD. Three frozen-thawed samples, AML1, 2, 4 and two fresh samples, AML8, 9 were included. Expansion as compared to Day0.

Sample ID	Fold expansion in co-culture		Fold expansion in SFEM culture			
	Day7	Day12	Day3	Day6	Day8	Day12
AML1	1.08	3.27	0.7	0.3	1.3	0.5
AML2	1.64	2.55	0.7	0.5	0.3	0.5
AML4	1.55	4.04	2.3	1.1	6.5	1.5
AML8	11.67	35.12	2.5	6.1	18.2	36.3
AML9	9.74	30.5	1.7	14.7	24.4	41.1

To distinguish the dormant and the cycling AML subsets, cells were subjected to phenotypic analysis by a panel of markers including CD45, CD34, and LAPs, on Day0 (pre-culture), day7 (Mid-culture) and Day12 (post-culture). A small percentage (6.4% - 9.7% in the AML-MS-5 co-cultures; 5.7% - 8.5% in the SFEM cultures) of $CD45^{dim}CD34^{+}$ cells preserved Day0 PKH26 fluorescence intensity (Figure 4.5). In contrast, the majority of cells (76% - 83% in the AML-MS-5 co-cultures; 64.1% - 86% in the SFEM cultures) had lost their Day0 CD34 expression and PKH26 fluorescence, indicating a loss of 'stemness' and the occurrence of cell division.

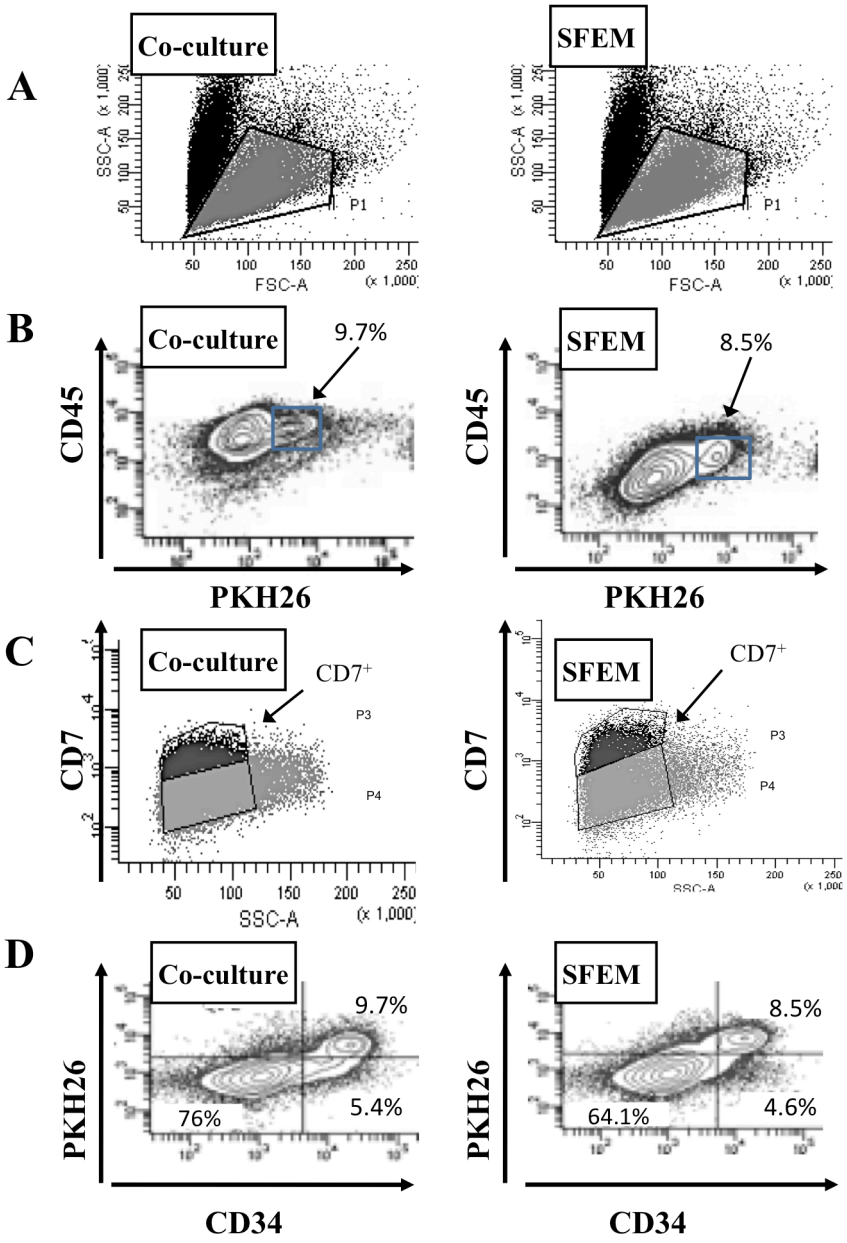

Figure 4.5. Identification of non-cycling AML subsets by PKH26 labelling and LAP expression *in vitro.* Primary AML cells were either co-cultured with MS-5 cells or maintained in the StemSpan^{TM} SFEM for 12 days. Representative plots from sample #AML2 are shown. On Day12, cells were labelled with anti-CD45-APC.Cy^{TM}7, anti-CD34-APC and anti-CD7-FITC monoclonal antibodies for phenotypic analysis. (A) FSC and SSC parameters are used to distinguish mononuclear cells from granulocytes, debris, etc. (B) A small sub-population of non-dividing (PKH26^{high}) cells were detected in CD45^{dim} myeloid blasts, in both culture systems. (C) LAP (CD7) positive cells were identified. (D) CD34/PKH26 plots are shown, gated on CD45^{dim} CD7^{+}cells. 1 x 10^{6} total events were collected for each analysis.

We observed that some LAP markers, such as CD7 and CD19, persisted in culture during the 12-day period. As is shown in Table 4.3, samples #AML2, AML4, AML6, and AML7 have aberrant CD7 expression at diagnosis whereas sample #AML5 is $CD19^{+}$. Our data indicate a high percentage of LAP expression in the magnetically enriched $CD34^{+}$ myeloid population on Day0 (54.7% - 98.4% in $CD45^{dim}CD34^{+}$ cells). The percentage of LAP expression in total cultured cells declined between Day0 and Day12 of the cell culture. However, expression of these aberrant markers persisted in most of the undivided cells ($CD45^{dim}CD34^{+}PKH^{26high}$), suggesting that the majority of dormant $CD34^{+}$ AML cells retained their aberrant phenotypes in cell culture. In these cases, we were able to exclude normal cells and to purify dormant leukaemic populations based on phenotypic expressions ($CD45^{dim}CD34^{+}LAP^{+}PKH26^{high}$) by FACS-sorting for further characterisation.

Table 4.3 The majority of non-cycling $CD34^{+}$ AML cells retained their aberrant surface markers (LAP) expression after 12-day culture

AML sample	Days in culture	LAP marker	LAP cells in total AML cells	LAP cells in the $CD45^{dim}CD34^{+}PKH26^{high}$ compartment (%)
AML 2	**Day 0**	CD7	85.5	79.1
	Day 7		68.1	77.4
	Day 12		13.8	77.2
AML 4	**Day 0**	CD7	84.6	89.1
	Day 7		73.6	83.4
	Day 12		12.5	83.9
AML 5	**Day 0**	CD19	43.1	54.7
	Day 7		38.5	44.8
	Day 12		37.3	41.3
AML 6	**Day 0**	CD7	61.3	85.1
	Day 7		55.4	84.3
	Day 12		53.9	80.4
AML 7	**Day 0**	CD7	86.4	98.4
	Day 7		73.1	87.2
	Day 12		41.4	86.9

4.4 Dormant Primary Leukaemia Cells Retained Clonogenic Capacity After 12 Days of Co-culture

Using flow cytometric cell sorting techniques to enrich for rare populations of AML cells after 12-day culture, we were able to prospectively study clonogenicity and to characterise the surface antigen phenotypes of both undivided (dormant) and proliferating (cycling) cells from AML samples. On Day12, dormant AML cells were identified as a $CD45^{dim}CD34^{+}LAP^{+}PKH26^{high}$ population, which retained PKH26 and the same fluorescence intensity of phenotypic markers as on Day0. In contrast, the cycling AML cells were identified as $CD45^{dim}CD34^{+/-}LAP^{+}PKH26^{low}$. Colony assays were performed with sorted cells plated in methylcellulose-based Methocult^{TM} H4034 Optimum medium for 14 days before scoring. Colony-growth rate was standardised as a calculated frequency of one repopulating progenitor in 1 x 10^4 sorted cells (Table 4.4).

Five AML samples, #AML2, 4, 5, 6, and 7 were FACS-sorted after 12 days co-cultured with MS-5 stromal cells. Overall, the sorted $CD45^{dim}CD34^{+}LAP^{+}PKH26^{high}$ (dormant) subpopulation retained significantly higher ($p = 0.048$) clonogenic capacity compared to the $CD45^{dim}CD34^{+/-}LAP^{+}PKH26^{low}$ cells that had already gone through the cell cycle (Table 4.4; Figure 4.6). This observation indicates that not only could the expression of some aberrant markers persist in the 12-day culture, but also that some LAP^{+}

leukaemia cells, that had not divided, maintained their primitiveness *in vitro* and were still clonogenic.

Table 4.4 Colony frequency in sorted dormant and cycled fractions from *de novo* AML samples after 12-day co-cultured with MS-5 stromal cells. The dormant subsets had a significantly higher colony-initiation potential than the cycling cells ($p = 0.048$).

AML sample	Subsets	Total sorted cells	Total colony count	Standard colony growth rate (per 10,000 cells)
AML 2	dormant	1471	11	75
	cycling	344	0	0
AML 4	dormant	717	17	237
	cycled	8510	2	2
AML 5	dormant	6530	31	47
	cycled	9830	3	3
AML 6	dormant	10390	55	53
	cycled	230750	3	0
AML 7	dormant	6306	54	86
	cycled	10757	0	0

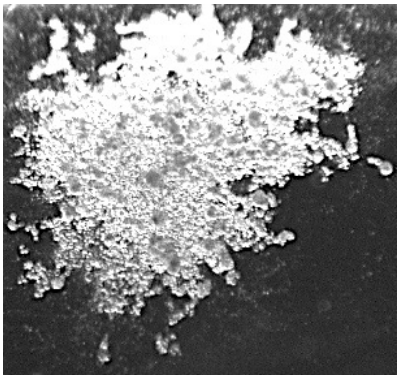

Figure 4.6. The clonogenic capacity of dormant AML cells. Representative photomicrograph of a single colony (x200), formed by $CD45^{dim}CD34^{+}LAP^{+}PKH26^{high}$ cells, plated in methylcellulose.

We had previously observed that both murine MS-5 and human MSC stromal cells provided good support for AML cell growth in short-term (72 hours) culture (Figure 3.3, Chapter 3). To see whether the clonogenicity of AML cells

change when co-cultured with different stromal types in our *in vitro* studies, we compared the colony-generating rate (colonies per 10^4 cells) of sorted dormant and cycled cells after 12-day co-culture with either MS-5 or MSC. Two samples, #AML2 and AML4, were co-cultured with either MS-5 or MSC simultaneously for 12 days using the same media (Gartner medium, ingredients are described in Chapter 2) under the same cell culture conditions. Cell sorting was performed on Day12. Similar to our previous observations in the AML-MS-5 co-culture, the sorted $CD45^{dim}CD34^{+}LAP^{+}PKH26^{high}$ (dormant) fraction retained clonogenicity compared to the $CD45^{dim}CD34^{+/-}LAP^{+}PKH26^{low}$ cells (Table 4.5). No evident differences regarding the standardised colony frequency were identified in either the dormant subsets or the cycled fraction between cells cultured on MS-5 and on MSC (Figure 4.7), suggesting that the stromal cell type used in the cell culture did not affect the capability of dormant cells to further proliferate.

Table 4.5. Colony frequency in sorted dormant and cycled fractions after 12-day AML+MSC co-culture.

AML sample	Subsets	Total sorted cells	Total colony count	Standard colony growth (per 10,000 cells)
AML 2	dormant	1070	4	37
	cycling	128	0	0
AML 4	dormant	352	15	426
	cycling	3359	1	3

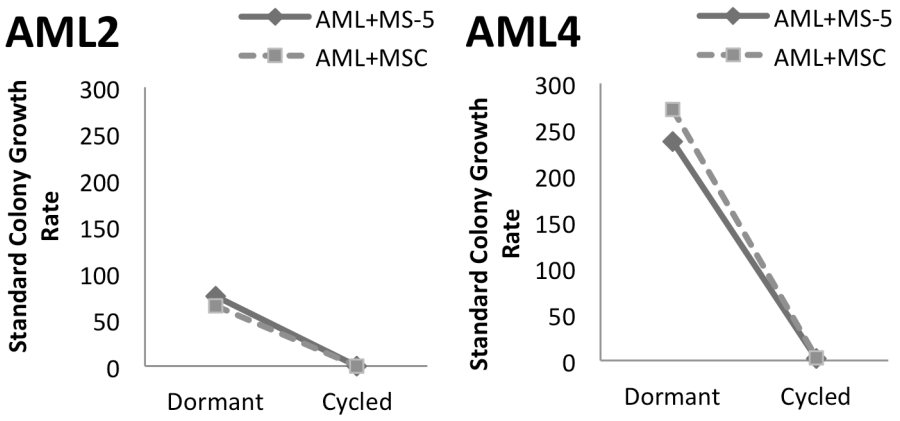

Figure 4.7. The clonogenicity of AML cells was unaffected by the type of supporting stromal cells in the co-culture system. *De novo* samples #AML2 and AML4 were cultured either on MS-5 or on MSC for 12 days and were sorted into the dormant and the cycled fraction. Data are standardised as colonies per 10^4 cells inoculated.

4.5 Dormant Primary Leukaemia Cells Are Effectively Targeted By The Novel Agents

Traditional chemotherapeutic agent Cytarabine (Ara-C) preferentially kills actively cycling cells but not dormant cells. We used our optimised *in vitro* models to investigate the efficacy of novel chemo-agents TG02 and pan-BCL2 inhibitors ABT-737 and ABT-199 in targeting non-cycling AML cells. Six LAP-positive samples (#AML2, 3, 4, 5, 6, and 7), were cultured on MS-5

stromal cells for 12 days. A single dose of Ara-C (200 nM), TG02 (25 nM), ABT-737 (100 nM) and ABT-199 (100 nM) was given at Day8. These chosen concentrations were based on the IC_{40} values determined by the preliminary 72-hour assays in primary AML samples (N=16, detailed in Chapter 3). Chemosensitivity was ascertained by 5-colour flow cytometric counting using a suspension of CytoCount^{TM} fluorospheres beads as a reference population (see Figure 2.2, Chapter 2). 200nM Ara-C reduced the cell number by 38% in the cycling fraction, but there was no reduction at all in the dormant fraction ($p = 0.009$, Figure 4.8). In contrast, exposure to TG02 and the BH3 mimetics markedly reduced the number of both dormant and cycling cells. Although there was some favouring of cycled cells with these agents (Table 4.6), we found that TG02, ABT-737 and ABT-199 were able to reduce the dormant cell fraction by 70%, 84% and 80%, respectively of their effects in proliferating counterparts.

Table 4.6. Percentage cell survival in six AML samples.

Drugs	Fractions	AML2	AML3	AML4	AML5	AML6	AML7
AraC	cycled	52	n/a*	62	51	48	97
	dormant	104	100	105	117	97	111
TG02	cycled	48	n/a*	41	17	42	59
	dormant	65	73	62	41	81	47
ABT-737	cycled	43	n/a*	31	8	37	39
	dormant	49	61	46	39	60	32
ABT-199	cycled	39	n/a*	29	30	32	7
	dormant	47	42	40	36	60	14

*Sample AML3 lacked a cycled subset after 12 days and was therefore excluded from the comparative graph.

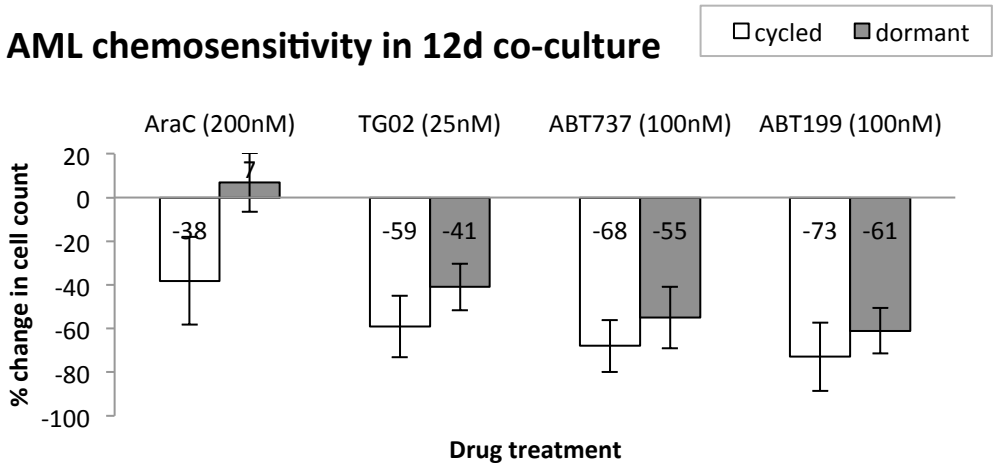

Figure 4.8. Percentage surviving cells compared with untreated controls in the dormant and cycled fractions of AML blasts after 12 days culture on MS-5 stromal cells. A single dose of each drug was given at Day8. The data are presented as Mean ± SD; n = 5.

4.6 TG02 and ABT-737 Inhibited Leukaemic Progenitor Function *in vitro*

To assess the inhibitory effects of TG02 and ABT737 on leukaemic progenitor function, colony assays were performed with freshly isolated or thawed AML samples, which were plated at 2 x 10^4 cells/100 µL in methylcellulose containing TG02, ABT737, as well as Ara-C. Figure 4.9 shows the effects of TG02 (25 nM), ABT737 (100 nM), and Ara-C (200 nM) on leukaemic colony formation in CFU assays using the optimised methylcellulose (Methocult™ H4100)-based medium. In untreated plates, #AML9 and AML10, which were freshly isolated, cultured and expanded, had relatively better colony growth capacities compared with cryopreserved sample #AML2 in the same culture conditions. Ara-C ablated over 99% colony formation in all 3 samples tested. Novel drugs TG02 and ABT-737 induced growth inhibition of primary AML blasts in all three samples tested.

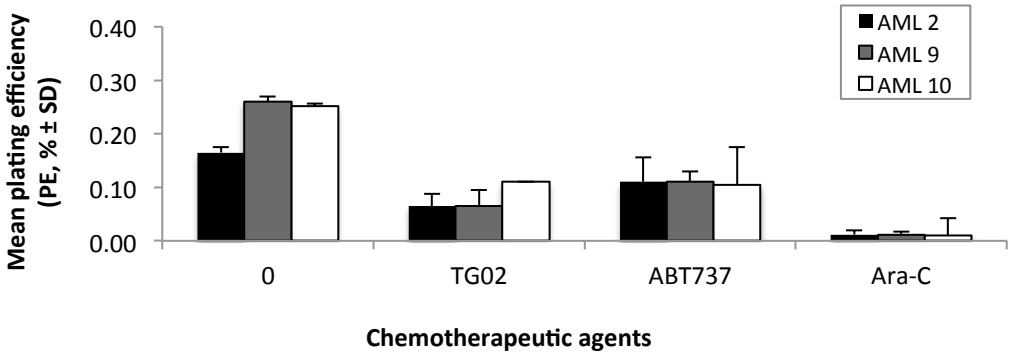

Figure 4.9. Inhibitory effect of chemo-agents on leukaemic colony formation in CFU assays. AML patient cells were plated in methylcellulose-based colony-forming medium for 14 days after exposure to TG02, ABT-737 and Ara-C. Values shown are mean plating efficiencies (± SD) for each samples.

4.7 Discussion

In Chapter 3, our short-term chemosensitivity assays indicated that in contrast to the standard chemotherapy drug AraC, novel agents TG02 and the two BH3 mimetics, ABT-737 and ABT-199 could efficiently target AML cells cultured on stroma. Also, ABT-737 and ABT-199 had no preference for proliferating cells. In this chapter, our work was largely focused on developing and optimising cell culture models to be able to distinguish the dormant leukaemic cells from normal haematopoietic cells and from proliferating cells *in vitro*. We designed and optimised a 12-day cell culture assay using the PKH26 cell linker dye to track cell division. The 12-day time span was to physiologically mimic clinical chemotherapy with Ara-C and an anthracycline (Seval and Ozcan, 2015), which thus gave an estimation of the length of time leukaemic cells would need to stay dormant to resist treatment. Previous studies demonstrated that the majority of *ex vivo* dormant AML cells rapidly re-enter the cell cycle *in vitro* (Guan et al., 2003), therefore analysis of freshly isolated or thawed cells with dormancy markers might overestimate the proportion of cells that would

remain dormant, whereas the 12-day model allowed us to study a clinically relevant subset of dormant cells. CD34-enriched primary AML cells from patients were cultured on MS-5 or MSCs for 12 days, using PKH26 to track cell division. Flow cytometric techniques were used and FSC_{low}/SSC_{low} restrictive gating was applied to exclude large and granular cells, including senescent cells (Hwang et al., 2009). On day12, we were able to detect a dormant ($CD34^{+}PKH26^{high}$) and in contrast a cycling ($CD34^{+}PKH26^{low}$) subset.

The expression of certain aberrant markers, including CD7 and CD19, persisted throughout the 12 days of culture. Two out of six presentation samples used in this study were from patients with complex cytogenetics and two were with normal cytogenetics. There were no good cytogenetic risk samples included in this investigation. The selection of $CD7^{+}$ aberrant phenotypes may have contributed to this bias. Previous studies have suggested the important prognostic value of CD7, which is associated with normal or adverse cytogenetics, and with multidrug resistance (Del Poeta et al., 1993, Kornblau et al., 1995, Venditti et al., 1998). Therefore, this is an interesting cohort to study, in favour of identifying therapies from which hard-to-treat and chemoresistant AML patients may benefit.

Our study showed that some dormant LAP^{+} leukaemia cells maintained their clonogenic potential in the 12-day culture, confirming that these cells had not become senescent in the *in vitro* conditions. Nonetheless, various factors in the cell sorting process could have had an impact on the quality of sorted cells, and contributed to the low yield (2-237 colonies per 10,000 sorted dormant cells;

Table 4.4). For example, cell hydrodynamic stress and damage may ensue during the cell sorting procedure; and whilst cells were kept on ice to reduce cell activities, the low temperature could also prevent cells from repairing sorting-induced damages.

It was observed that AML cells with high BCL-2 expression tend to have an impaired ability to enter the cell cycle (Konopleva et al., 2002b). A recent study indicates that dormant (ROS-low, Ki-67-negative) AML cells which overexpress BCL-2 are selectively targeted by ABT-737 (Lagadinou et al., 2013). Our data suggest that both ABT-199 and ABT-737 have efficacy against cells protected by the BM niche and dormancy. The efficacy of these agents in dormant cells cultured on MS-5 was over 80% of their efficacy against cycled cells. The multikinase inhibitor TG02 can rapidly deplete short-lived molecule MCL-1 through RP2 inhibition (Pallis et al., 2012). It selectively inhibits CDKs, FLT3 and JAK2 in myeloid malignancies both *in vivo* and *in vitro*, and was found to specifically induce G_0 cell cycle arrest (Goh et al., 2012). Here we have shown that TG02 had great efficacy against dormant AML patient cells under stromal niche conditions (Table 4.6 and Figure 4.8), consistent with our previous cell line work which suggested that TG02 may be a valuable drug in targeting dormant cells in AML (Pallis et al., 2013).

In summary, our work in this chapter shows that dormant AML cells are sensitive to TG02, and the BH3 mimetics ABT-199 and ABT-737, which thus have the potential to be useful to target MRD in AML patients.

CHAPTER 5

EXPLORING DORMANCY MARKERS

5.1 Background

As mentioned in Chapters 3 and 4, dormant leukaemic cells are considered a major culprit of chemoresistance and disease relapse in AML. Developing non-invasive ways to identify and isolate this population would be a prerequisite for characterising these dormant cells. In Chapter 4, we used long-term (12-day) cell culture models, a cell tracker (PKH26) and LAPs to distinguish dormant leukaemic cells from their cycling counterparts (Chapter 4; Sections 4.2 and 4.3), and have successfully separated these two subsets using FACS sorting techniques (Chapter 4; Section 4.4). Nonetheless, there are other possible ways, such as using cell markers, to identify the dormant population. For instance, dormant cells are known to have lower nucleic acid content than proliferating cells, and the RNA dye PY is a proven marker to identify and to characterise dormant cells in flow cytometry (Shapiro, 1981). PY-low cells are also found to have engraftment potential (Guan et al., 2003). However, PY has its own drawbacks such as its possible cytotoxicity to living cells, its dose-dependent labelling patterns (Darzynkiewicz et al., 1986, Darzynkiewicz et al., 1987), and more practically, its lack of negative control markers on flow cytometry. Emerging studies have suggested that other markers, such as the proliferation markers Ki-67 and CD71, can help to characterise dormant cells (Blair et al., 1998, Pallis et al., 2013, Dong et al., 2011, Holyoake et al., 1999, Holyoake et al., 2001, Jordan et al., 1996). More recently, it was suggested that the ALDHs, a group of enzymes that play a crucial role in oxidising retinol to retinoic acid during early cell differentiation, have increased activities in stem cell populations and thus provide a common marker to distinguish these primitive subsets from their more differentiated progenies (Pearce et al., 2005, Chute et

al., 2006, Schubert et al., 2011, Gerber et al., 2012). Furthermore, LAPs and other immuno-phenotypic markers would help to distinguish leukaemia cells from normal cells (discussed in Chapter 4). The aim of this study was to explore possible markers or marker combinations using flow cytometry to distinguish dormant cells from proliferating cells, and leukaemia cells from normal cells. Part of the work in this Chapter, where stated, was performed by Gary Swift (BMed Sci 2013), whom I co-supervised on his research project, including experimental design, research directions and daily bench-supervision.

5.2 PY As a Marker Used in Cell Sorting For Dormant Cells

One priority of this study was to develop a cell sorting strategy to separate living AML cells into the dormant and cycling compartments so we could study the two subsets in a comparative way and further characterise AML dormancy on a molecular level. This would require the cell staining and sorting procedures to be as non-invasive as possible, to preserve cell viability. The RNA dye PY can enter living cells with intact membranes (Shapiro, 1981, Darzynkiewicz et al., 1987), making it a non-invasive marker for sorting.

Cells needed to be labelled with PY and antibodies in our laboratory, before being transferred across to the Flow Cytometry Facility Core Services for sorting. The prolonged incubation on ice during transit and the delayed procedure (up to 60 minutes) before cell sorting had to be taken into account. It

was important to optimise the time course of the final staining steps, i.e. cell incubation with PY, and to determine the stability of stains over the delay.

PY (1 µg/mL) was used in combination with CD45 and CD34, to distinguish dormant and proliferating cells based on their RNA content. A comparison was conducted on PY incubation time (Figure 5.1). The staining remained stable at 15, 30 and 60 minutes and no significant reduction on cell viability was observed.

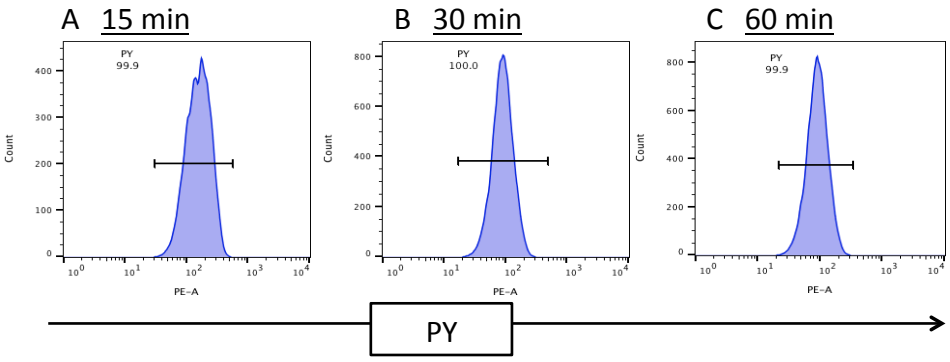

Figure 5.1. Time course comparison of PY staining on CD45$^+$CD34$^+$ cells at 15, 30 and 60 minutes. (A-C) Flow cytometry plots from Sample #AML1.

Having optimised the PY-based staining, nine AML samples underwent the staining and labelling process before cell sorting. All samples were successfully sorted into the CD45$^+$/CD34$^+$/PY^{low} (dormant; P4, Figure 5.2) and CD45$^+$/CD34$^+$/PY^{high} (cycling; P5, Figure 5.2) subsets and were subjected to further molecular analysis (detailed in Chapter 6).

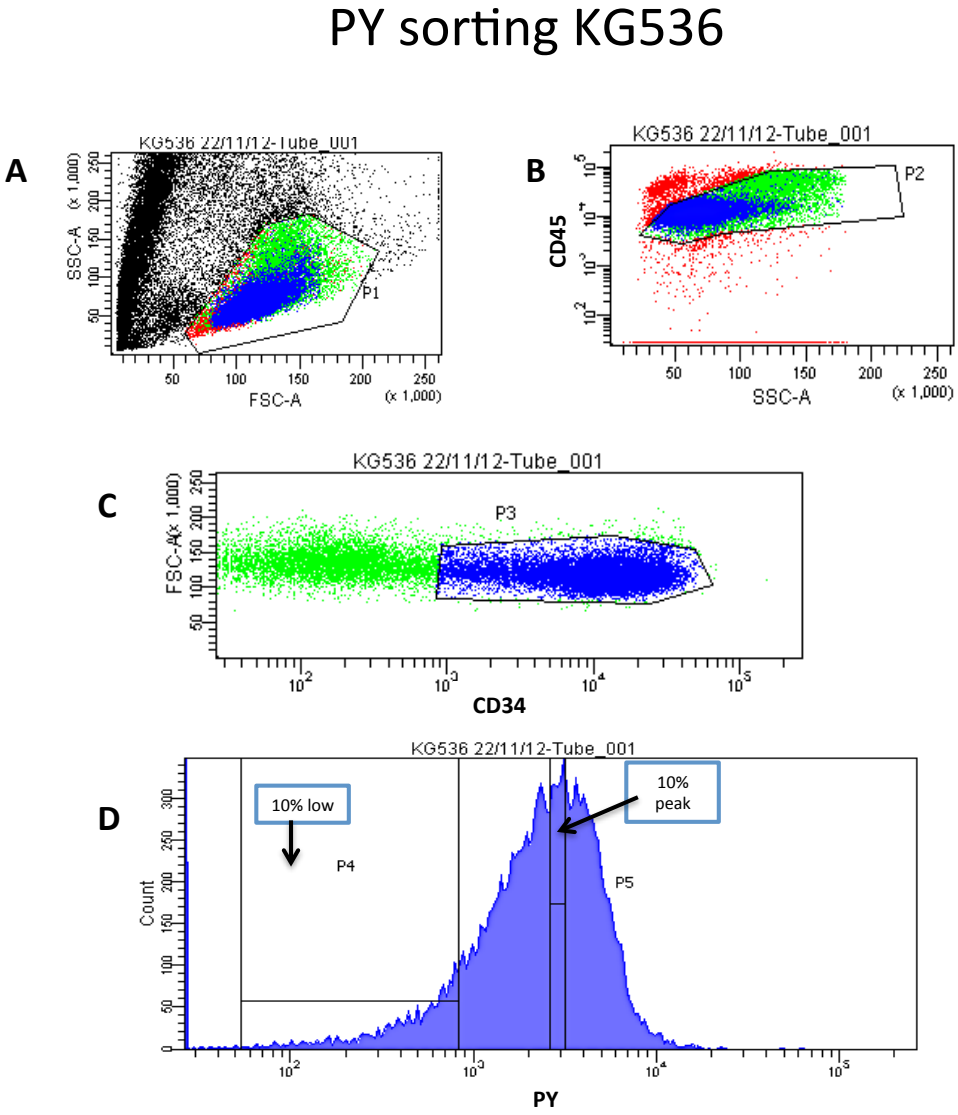

Figure 5.2 Cell sorting criteria according to cellular PY contents. Representative flow cytometric plots were obtained from sample AML6 (KG536). The majority of cells were selected for analysis based on forward vs. side scatter profile (A), CD45 (B) and CD34 status (C). Cells were then analysed for PY expression for FACS sorting of PY^{low} (10% at the start of the histogram, P4) and PY^{high} (10% cells on the peak of the histogram, with higher PY expression and the highest count indicating they are at cycling stages, P5) populations (D). The two subsets ($CD45^{+}CD34^{+}PY^{low}$ and $CD45^{+}CD34^{+}PY^{high}$) were subjected to further RNA work.

5.3 The Proliferating Markers Ki-67 and CD71

In addition to nucleic acid dyes, proliferation markers are potentially valuable in assessing the dormant state of cells. Two markers, Ki-67 and CD71 (also a maturation marker), stood out in our literature search. The nuclear antigen Ki-67 was found exclusively in proliferating cells (Jordan et al., 1996, Schluter et al., 1993) and is currently the gold standard marker for cell proliferation (du Manoir et al., 1991). The transferrin receptor CD71 has been reported absent in dormant lymphocytes and in cancer stem cells (Neckers and Cossman, 1983, Ohkuma et al., 2012). Both markers could be used to exclude, and thus help to identify dormant cells. Here we validated the two markers by using the T cell experimental model.

Human T lymphocytes, which remain in a dormant (G_0) state (Schmidtke and Hatfield, 1976, Habu and Raff, 1977, Pearce, 2010, Yang and Chi, 2012), are invaluable to study cell dormancy, as upon stimulation by mitogenic lectins such as ConA (Palacios, 1982) and PHA (Dasgupta et al., 1987), they can be directly activated and driven out of dormancy. Thus T cells serve to compare between the resting and proliferating cell cycle states. Freshly isolated T cells from healthy donors were cultured with or without PHA (5 μg/mL) for 72 hours before cells were subjected to the staining procedure. General staining methods are described in Chapter 2. As is shown in Figure 5.3-D, after 72h exposure to PHA, 45.5% of T cells remained dormant (Ki-67$^-$CD71$^-$) while the majority of T cells had been activated and started cycling (Ki-67 and CD71-

positive). The percentage of Ki-67^{+} and CD71^{+} cells are strikingly similar, at 55% and 56.2%, respectively (Figure 5.3-E and -F).

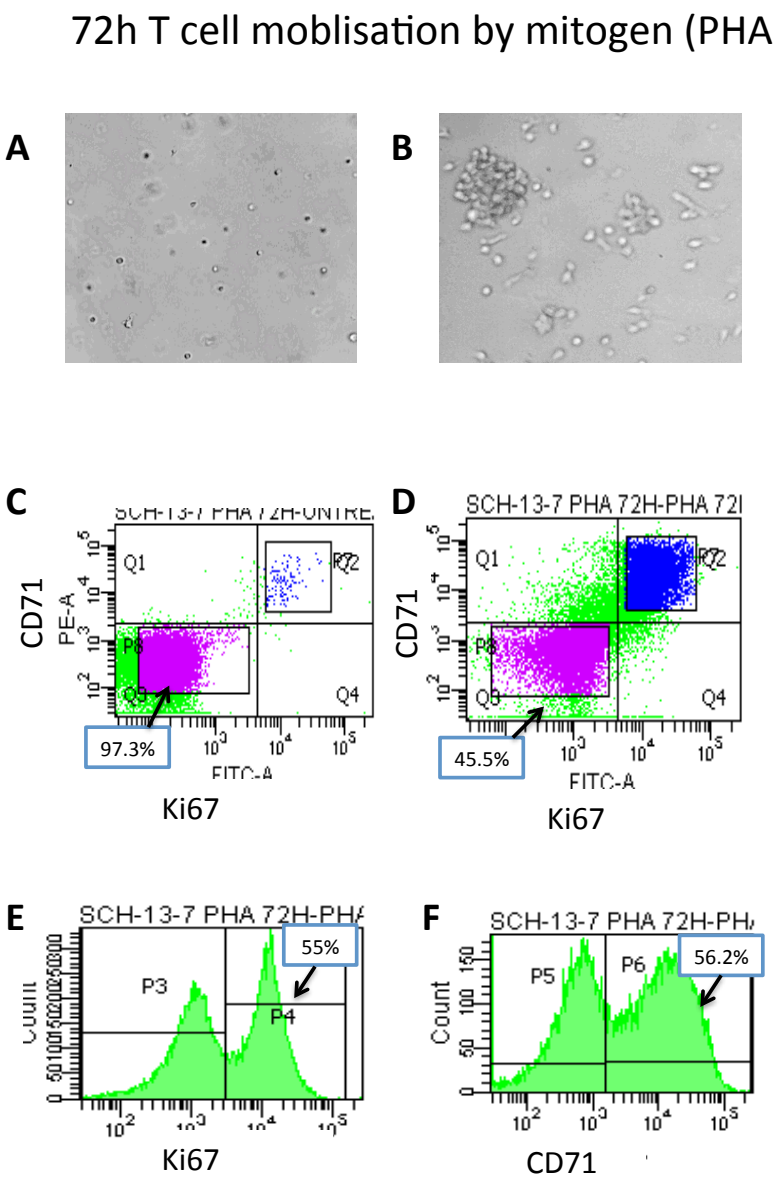

Figure 5.3. Ki-67/CD71 analysis of T cell mobilisation after 72h PHA treatment. Mitogen activation of T lymphocytes *in vitro*. Human T cells were isolated from healthy donor PB and were then cultured for 72 hours with PHA treatment (5 µg/mL). (A) Microscopic image showing untreated T cells in suspension culture for 72 hours. (B) Microscopic image showing T cells after 72 hours PHA exposure. (C) Ki-67/CD71 quadrant plots showing that almost all of the untreated T cells remained dormant (Ki-67^{-}CD71^{-}) after 72 hours *in vitro* culture. (D) Ki-67/CD71 quadrant plots showing the majority of cells proliferated. (E) Histograms showing the Ki-67^{+} population and (F) CD71^{+} population, respectively.

We then used Ki-67 and CD71 to analyse the content of dormant cells in AML samples. The anti-proliferation drug 5-Fluorouracil (5-FU) was used to enrich for leukaemic populations based on previous reports (Terpstra et al., 1996, Zhang et al., 2010). Five AML samples (#AML1, 2, 4, 28 and AML31) were incubated with or without 5-FU at 300 μM at 37°C for 24 hours before being subjected to cell staining and FACS analysis. Cell viability was assessed by the 7-AAD/FSC me thod (described in Chapter 2). Representative plots from sample #AML4 are shown in Figure 5.4. After 24 hours cell culture, 7% $CD34^{+}CD38^{-}$ cells remained dormant ($Ki\text{-}67^{-}CD71^{-}$) and with 5-FU enrichment, the dormant fraction within the $CD34^{+}CD38^{-}$ population has increased to 25%. As 5-FU is a potent chemotherapeutic drug targeting cycling cells, it was important to assess cell viability, at the time of plating (0h), 24h after drug-free cell culture, and 24h after 5-FU treatment (Figure 5.4-C). 79% primary cells were viable after 24h *in vitro* culture. And 5-FU treatment further reduced cell number by 35.4% (versus untreated cells at 24h).

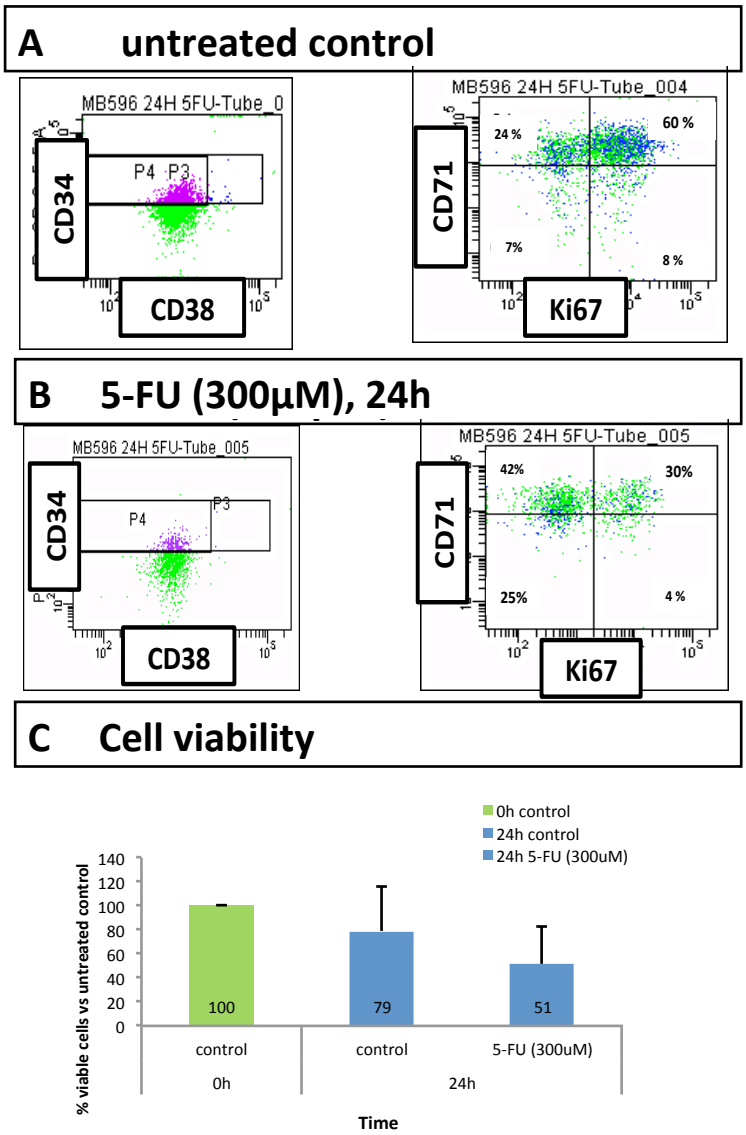

Figure 5.4. Ki-67/CD71 analysis of 5-FU enrichment for dormant AML cells. Cells from five AML samples were incubated with 5-FU (300 µM) for 24h prior to staining and FACS analysis. Representative plots from #AML 4 are shown. (A) untreated cells after 24h incubation at 37°C. 7% of the $CD34^{+}CD38^{-}$ population were $Ki\text{-}67^{-}CD71^{-}$ double negative (dormant). (B) 5-FU treated cells, with 25% $CD34^{+}CD38^{-}$ cells with negative Ki-67 and CD71 expression. (C) Cell viability analysis by 7-AAD/FSC. `

5.4 The Reproducibility of Dormancy Marker Staining Methods

The staining procedure of candidate markers PY, Ki-67 and CD71 consisted of complex and time-consuming steps. To see whether each of the staining methods was reproducible, and whether the marker expression results were reliable, a parallel investigation was carried out on each individual marker using the same AML samples. A comparison of CD71 expression with fixation- and non-fixation-based protocols was also performed. Measurement was taken for the percentage of positive populations for CD71 on unfixed cells and combined Ki-67 and CD71 after fixation. Each staining procedure was repeated on all four patient samples (AML1, AML31, AML33 and AML35), at least 3 times.

The mean values of coefficient of variation (Cv) and confidence intervals (CI) were derived from each set of patient samples by comparison of marker expression between samples (Figure 5.5). The C_V values suggested PY staining being the most reproducible procedure with the lowest mean Cv (15.4%), followed by Ki-67 after fixation (15.7%) and CD71 on unfixed cells (30.7%). In contrast, CD71 seemed the most variable marker after fixation, with a mean Cv of 95.1% and its CI ranged outside the CI values of all other markers (53.4% - 137%), suggesting that the epitope detected by CD71 antibody was destroyed during the fixation process. Hereafter, the CD71-fixation staining method was excluded from subsequent studies.

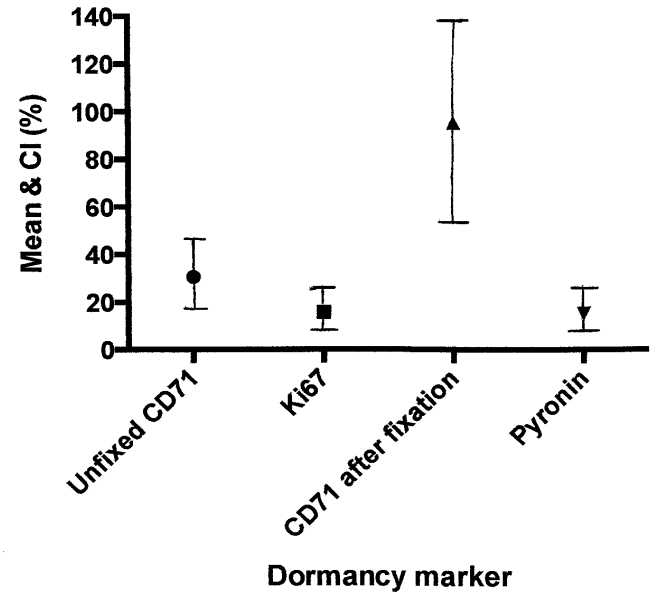

Figure 5.5. The comparison of marker reproducibility. The coefficient of variation (Cv) for each of the dormancy markers along with their upper and lower confidence intervals (CI). (Data by Gary Swift, BMedSci, 2013)

5.5 The Correlation Between PY, CD71 and Ki-67 Staining

Eight AML samples were used to investigate the correlation between the dormancy markers (PY, CD71 and Ki-67) and their staining/fixation procedure. Each of the markers was compared against the others individually, indicated by Spearman's rho. Among all these markers, a significant correlation was found between unfixed CD71 and PY staining (rho = 0.8095, p = 0.0218) (Figure 5.6-B). No strong correlation was found between CD71 (unfixed) and Ki-67, or PY and Ki-67.

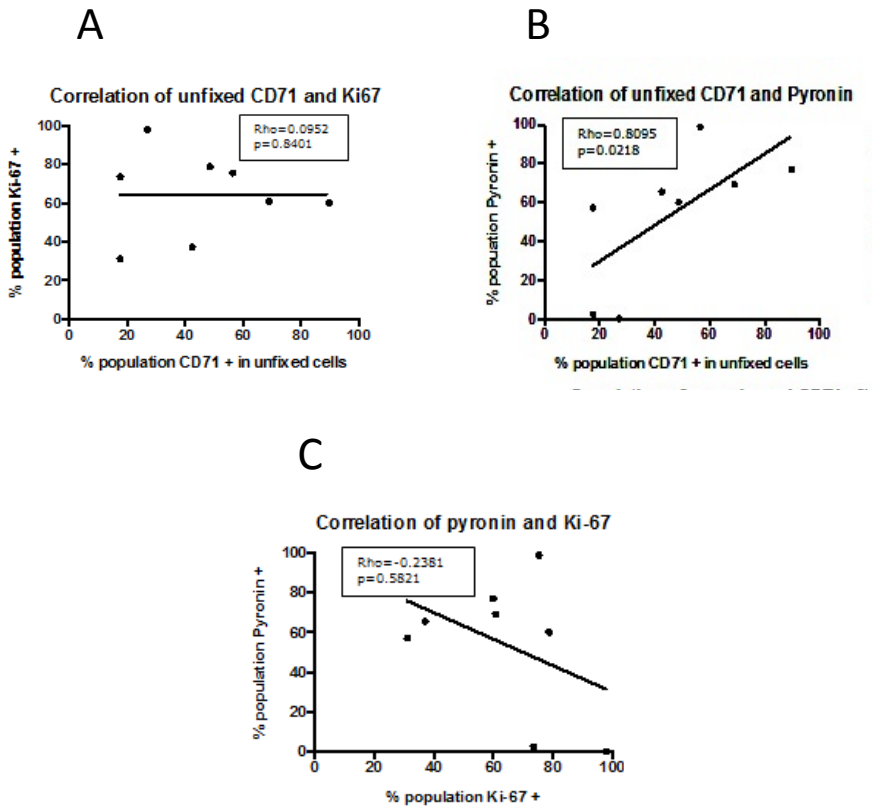

Figure 5.6. The correlation between dormancy markers. N=8. Each of the marker staining methods, i.e. PY, CD71 on unfixed cells, and Ki-67 on fixed cells, was compared to each other individually. The Spearman's correlation (rho) and p values are calculated. A significant correlation was observed between unfixed CD71 and PY staining (B). (Data by Gary Swift, BMedSci, 2013.)

5.6 Comparing Dormant Populations in Bulk Cells and LAP$^+$ Cells

As mentioned in Chapter 4, LAPs are unique markers which help to distinguish leukaemia cells from normal cells and we have determined LAP expressions in 14 AML samples (Chapter 4; Section 4.2; Table 4.1). For isolation of dormant leukaemia cells, it would be useful to identify a LAP-positive, dormant subpopulation. We therefore sought to establish whether such a subpopulation

exists. Five LAP^+ samples (AML#23, 35, 36, 39 and AML40) were included and were subjected to labelling with markers CD71 and Ki-67. As LAP^+ cells can be rare events within AML samples, to maintain data reliability, the acceptable coefficient of variation was set to 10%. Using a method described by Mony et al (Mony et al., 2008), data were only valid if there were over 100 events after subtraction of false positive from the isotype controls. In this particular study, a total of 10,000 events were processed through FACS from each tube. Thus, only those LAP^+, dormancy marker negative populations with a reading of over 100 events, i.e. above 1%, could be classified as significant. As is shown in Table 5.1, all 5 samples had a substantial number of dormant LAP^+ subsets when labelled with CD71 (in unfixed cells). 4 out of 5 samples were identified as having a subset of LAP^+, which was dormant when stained with Ki-67 (in fixed cells). It was not possible to identify marker positive/negative populations with PY due to its lack of negative controls.

Table 5.1 Percentage LAP^+ dormant cells in whole samples. (Gary Swift, BMedSci 2013)

Sample ID#	LAP type	LAP^+ in un-manipulated bulk (%)	$LAP^+/CD71^-$ in unfixed cells (%)	$LAP^+/Ki67^-$ in fixed cells (%)
AML23	CD33-/CD34+	41.9	25.86	21.92
AML39	CD33-/CD34+	18	13	8.86
AML36	CD33-/CD34+	12.9	8.87	11.7
AML35	CD2+/CD34+	21.1	8.18	0.961
AML40	CD7+/CD34+	12.1	1.86	1.69

A further analysis was performed to see if there was a significant difference in the percentage of dormant cells within the bulk cells and the LAP^+ populations. To allow comparison of PY expressions, the gate was set at 95% negative when looking at the bulk cells and the same gates were used to look at the

LAP$^+$ cells. The percentage of dormancy marker negative cells in bulk and in LAP$^+$ populations is shown in Figure 5.7. The level of marker expression was not significantly different in bulk cells compared to that in LAP$^+$ cells ($P_{\text{CD71 unfixed}} = 0.125$, $P_{\text{PY}} = 0.8125$, and $P_{\text{Ki-67}} = 0.71$).

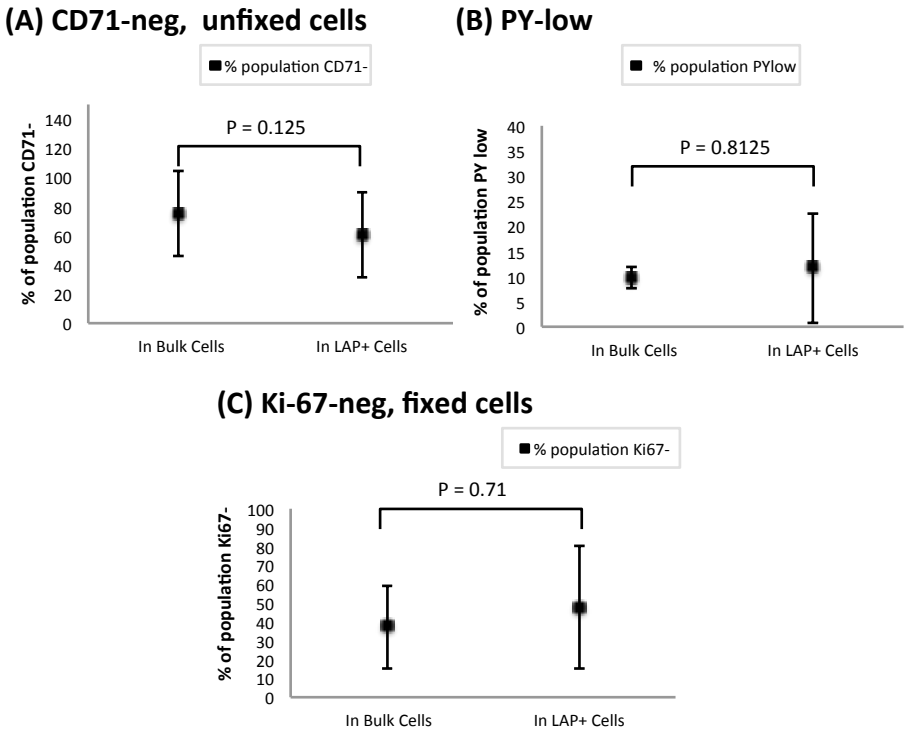

Figure 5.7. Comparison of % dormancy marker-negative cells in bulk and LAP$^+$ populations. The expression of dormancy markers (A) CD71, (B) PY, and (C) Ki-67 were assessed in 5 AML samples and the percentage of marker-negative cells was compared between bulk and LAP$^+$ populations. P values and confidence intervals were calculated by the Wilcoxon test (Data by Gary Swift, BMedSci 2013).

5.7 The Stem Cell Functional Marker ALDH

In addition to markers that indicate cellular RNA content and proliferation status which may help to identify dormant cells, there may be other markers available to help distinguishing the primitive, non-cycling stem cells from

more differentiated cells. It is known that cancer stem cells have increased metabolic activity through ALDHs (Eyler and Rich, 2008, Bao et al., 2006), and AML LSCs might be enriched in the $ALDH^+$ subsets which are associated with adverse prognosis (Pearce et al., 2005, Cheung et al., 2007, Ran et al., 2009, Ran et al., 2012). Here, we intended to see whether ALDH could be a suitable dormancy marker for AML cells.

Firstly, we tested ALDH activity in normal PB obtained from healthy donors after stem cell harvest. Cells were incubated in expansion culture for 72h before being harvest and subjected to cell labelling with ALDH/DEAB (the negative control for ALDH, included in the Aldefluor kit), combined with CD45, CD34 and CD38 labelling. As is shown in Figure 5.8-E2, 48.9% of cultured $CD34^+CD38^-$ cells showed active ALDH activity (ALDHbr).

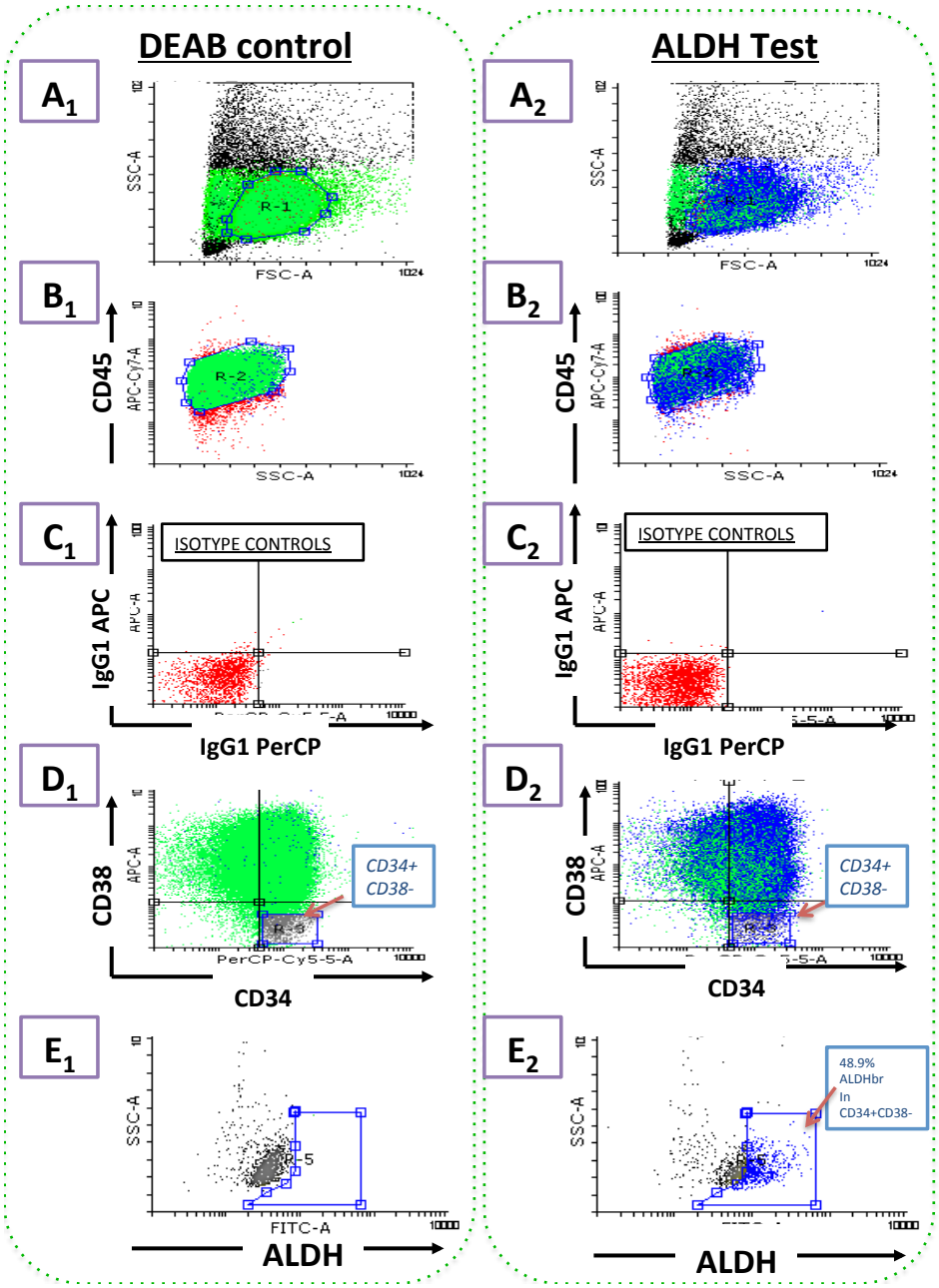

Figure 5.8. ALDH activity in healthy donor's PB. Peripheral blood obtained from stem cell harvest was enriched for $CD34^+$ by magnetic miniMACS cell sorting. Cells were in expansion culture for 72 hours and were subjected to Aldefluor staining, combined with CD45, CD34 and CD38 antibody labelling, followed by flow cytometric analysis. (A_1-E_1) DEAB negative control to set the ALDH gate; and (A_2-E2) ALDH staining. (C1 and C2) IgG1-PerCP and IgG1-APC isotype controls to set the CD34/CD38 quadrant gates, for DEAB and ALDH staining, respectively. DEAB: diethylaminobenzaldehyde.

Next, we used Aldefluor in patient samples #AML1 and AML4, which were

treated with 5-FU (300 µM, 24h) to enrich for dormant cells. ALDH expression in 5-FU-treated $CD34^{+}CD38^{-}$ cells was 17.45% lower in sample AML1 (Figure 5.9-A), but 1.05% higher in sample AML4 (Figure 5.9-C) compared to untreated cells. There was no evidence that ALDH could indicate dormancy enrichment by 5-FU, and therefore it is unlikely to be a promising dormancy marker. Further investigation of ALDH was discontinued.

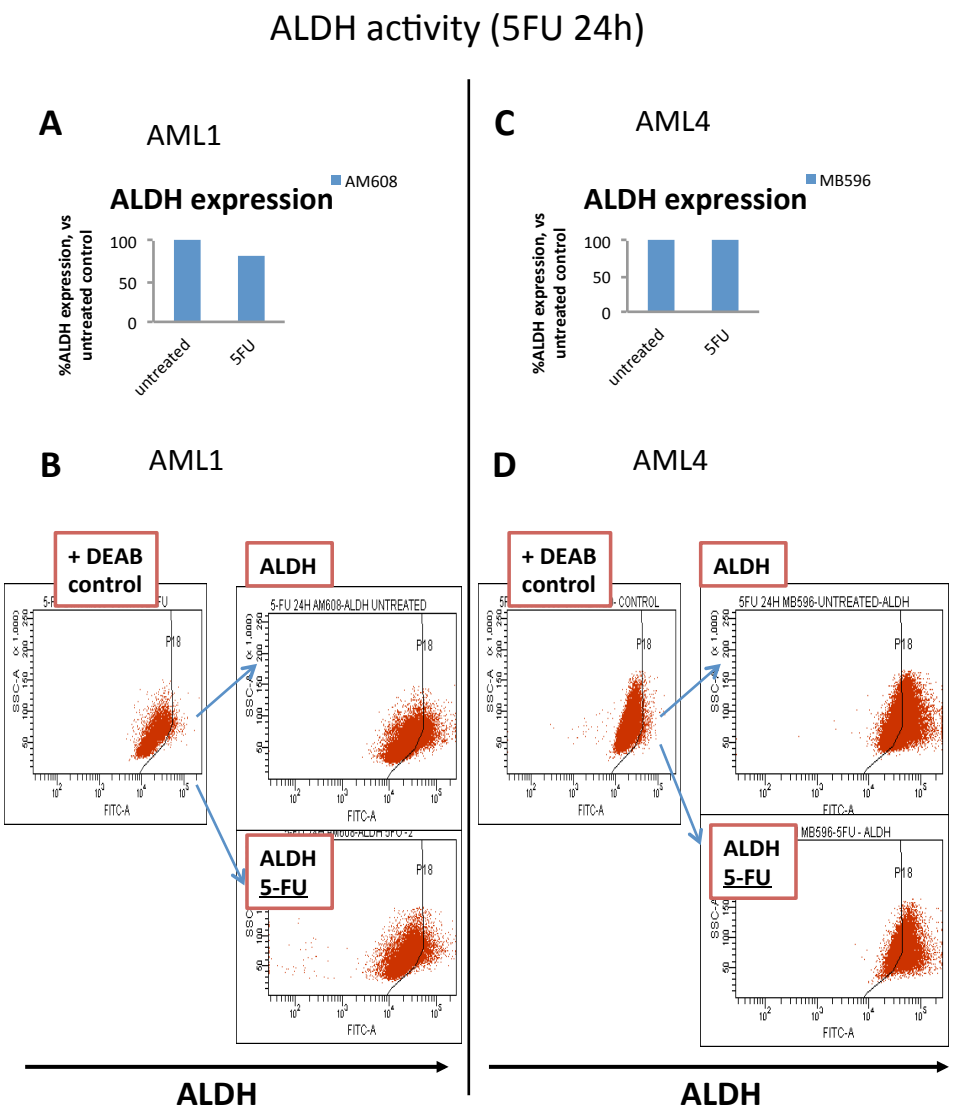

Figure 5.9. ALDH activity in 5-FU-treated $CD34^{+}CD38^{-}$ AML cells. Relative ALDH expression (%, 5-FU-treated cells vs untreated control) for samples AML1 (A) and AML4 (C) was shown in column charts. (B) and (D) Dot plots from samples AML1, and AML4, respectively, showing the shift of ALDH expression in gate P18, from negative DEAB control to $ALDH^{+}$ (with no 5-FU treatment), then to $ALDH^{+}$ (5-FU treatment).

5.8 Discussion

Dormancy is one of the main factors that could explain cancer relapse and drug resistance. This study explored different ways of using various markers to identify dormant cells in primary AML samples, using flow cytometric techniques.

Ever since the 1980s, a popular approach to distinguish between G_0 and cycling cells is by DNA and RNA staining (Shapiro, 1981, Darzynkiewicz and Traganos, 1990). Such an approach helps to identify cells beginning to transcribe and accumulate RNA from G_0 to G_1. PY is an intercalating cationic dye that shows specificity towards RNA (Darzynkiewicz et al., 1986) and has been successfully used for two-parameter cell cycle analysis (Cowden and Curtis, 1983). PY is membrane permeant and the concentration of PY required for optimal RNA staining is crucial, due to its reported toxicity (Darzynkiewicz et al., 1986, Darzynkiewicz and Traganos, 1990). Studies conducted by Ladd and colleagues suggested that only at lower than 5 μg/mL was viability of human BM $CD34^+$ haematopoietic cells maintained above 95% (Ladd et al., 1997). They chose a final working concentration of 1 μg/mL, which was verified to be RNA-specific by comparing PY expression in RNase-treated and untreated cells (Ladd et al., 1997). Based on its RNA-specificity and low cytotoxicity at 1 μg/mL, we used PY to separate G_0 and non-G_0 cells according to cellular RNA content, so it would be non-invasive (Figure 5.2). No reduction of cell viability was observed with increased exposure to PY at 15, 30, and 60 minutes, and the stain remained stable over time (Figure 5.1). This staining method was then used in our cell sorting procedure.

Next, we investigated two proliferation markers, Ki-67 and CD71. Ki-67 is the 'gold standard' proliferative index (du Manoir et al., 1991). It is active in all active cell cycle phases (G1, S, G2, M) but is absent from G_0 cells. In interphase, the Ki-67 protein is exclusively detected within the cell nucleus, whereas most of the protein is relocated during mitosis to the surface of the chromosomes (Scholzen and Gerdes, 2000). Ki-67 is also used to estimate the localisation of dormant cells within the BM (Saito et al., 2010). The transferrin receptor CD71 is a proliferation-related surface marker. Overexpression of CD71 has been found in various tumours mediating upregulated iron uptake (Magro et al., 2011, Habashy et al., 2010, Chan et al., 2014). A possible approach to analyse sample proliferation/dormancy status is by Ki-67/CD71 co-expression (Wei et al., 2015, Pallis et al., 2013). Here we used PHA-activated peripheral blood lymphocytes from healthy donors to demonstrate Ki-67/CD71 co-staining to identify dormant cells (Palacios, 1982, Dasgupta et al., 1987). After 72h cell culture, the majority of PHA-stimulated (at 5 μg/mL) cells had $Ki67^+/CD71^+$ expression, indicating active cell cycling, whilst almost all of the unstimulated T cells remained dormant ($Ki\text{-}67^-/CD71^-$). The percentage of $Ki\text{-}67^+$ cells is similar to that of $CD71^+$ cells (Figure 5.3). The Ki-67/CD71 co-labelling method was further used in 5-FU treated AML patient samples. The anti-proliferation drug 5-FU is known to deplete cycling cells but spare LSCs with leukaemia-initiating potential in SCID mice and thus enrich for 5-FU-resistant, dormant leukaemic populations (Terpstra et al., 1996). Based on this, we used 5-FU to enrich for G_0 cells in AML samples (n =

5) and the dormant $CD34^{+}CD38^{-}$/Ki-67^{-}/$CD71^{-}$ fraction increased from 7% to 25% after 24h exposure to 5-FU 300 μM (Figure 5.4; Mean ± SD).

However, the Ki-67/CD71 co-staining method has its limitations. Firstly, the detection of the intracellular antigen Ki-67 requires cell membrane permeabilisation and therefore could potentially compromise CD71 staining, which is based on cell membrane (McClelland et al., 1984). The assessment of marker reproducibility clearly reflected this problem in CD71 stability in fixed cells (Figure 5.5). Moreover, the cell fixation process could damage other important cell surface markers, such as LAPs. It could also damage cell integrity and thus cause cell death. Thus, the usage of Ki-67 should be ruled out from our cell sorting strategies.

In contrast, the advantage of CD71 is that the staining process does not require cell fixation due to its transmembrane locality, and is much simpler. Future studies should consider CD71 as a dormancy marker in living cell sorting. Moreover, the non-invasive staining with CD71 can be combined with Annexin V assays for apoptosis (Pallis et al., 2013), for which cell membrane integrity is essential as this assay is based on detection of phosphatidylserine (PS) translocation between inner and outer leaflet of the cell membrane.

A further step was taken to see the correlation between marker staining (n=8) and a significant link was found between PY and CD71 in unfixed cells (Figure 5.6). This makes sense as both CD71 expression and a high RNA content in cells are indicators of a proliferative (non-G_0) cell cycle status, and the strong

positive correlation between these two markers supports them to be dormancy markers.

Yet, we could not identify a marker with high expression in dormant cells, which would be ideal. The common ground of PY, CD71 and Ki-67 is that they all positively indicate the proliferating population with active nucleic acid synthesis. The dormant fraction was identified through the negative-gating strategy and this could be ambiguous, as it was based on the assumption that if cells were not cycling, then they were dormant. Also, low expression of a marker could indicate poor *in vitro* cell quality rather than physiological dormancy. It is worth further including experiments such as the Annexin V assay (Vermes et al., 1995) and the SA-beta-Gal assay (Kahlem et al., 2004) to rule out apoptotic cells and senescent cells respectively (although senescent cells are large and granular, so maybe do not constitute a problem provided appropriate FSC_{low}/SSC_{low} gating is used). A further search for positive dormancy markers would be beneficial as well.

One possible marker was ALDH, a group of enzymes that are expressed at high levels in stem cells of different tissue origins (Marchitti et al., 2008, Cai et al., 2004, Douville et al., 2009, Armstrong et al., 2004, Corti et al., 2006, Fallon et al., 2003, Hess et al., 2004, Lohberger et al., 2012). And high percentage of LSCs were among the $ALDH^{+}$ populations (Pearce et al., 2005, Ran et al., 2009). One advantage of this marker is that the staining procedure is non-invasive. Using a standard kit, Aldefluor, ALDH can be used in cell sorting to purify and to further characterise $ALDH_{dim}$ and $ALDH_{br}$ populations (Gerber et

al., 2012, de Leeuw et al., 2014). However, in this study, there was no evidence that ALDH expression could indicate dormant cell enrichment in $CD34^{+}CD38^{-}$ AML cells. We therefore discontinued the investigation in ALDH as it is unlikely to be a promising dormancy marker.

To summarise, the current study explored various possible dormancy markers and their correlation in determining the dormant populations in AML. PY and CD71 without the fixation procedure seem to be the most reliable and advantageous markers over the others tested, including Ki-67 and CD71 with fixation. The permeabilisation/fixation process seemed to affect marker reproducibility, and any markers that require fixation are not suitable for cell sorting, as cell viability is inevitably compromised. Some of our results are equivocal. Further search for dormancy markers, especially those that can positively determine cell dormancy, is needed.

CHAPTER 6

MOLECULAR CHARACTERISATION OF DORMANCY IN AML

6.1 Background

For the last few decades, normal and leukaemia haematopoietic stem cells have been under the spotlight of medical research and are well described phenotypically and functionally, as reviewed in (Shlush et al., 2014, Horton and Huntly, 2012, Konopleva and Jordan, 2011, Becker and Jordan, 2011, Lane and Gilliland, 2010, Weissman and Shizuru, 2008, Dick, 2008, Jordan, 2007, Huntly and Gilliland, 2005). However, our knowledge of molecular pathways involved in stem cell dormancy in AML still remains limited. Identification of dormancy regulatory genes would help us to further understand the molecular characterisation of the disease and also enable us to improve anti-cancer targeted therapies.

Dormancy in haematopoietic cells is tightly regulated by cell cycle regulatory genes, such as the Rb proteins, E2F, p53, p21, and p27, at the protein level (Matsumoto and Nakayama, 2013). However, the function and stabilisation of Rb/E2F and p53 are largely dependent on phosphorylation (Matsumoto and Nakayama, 2013, Kitagawa et al., 1995, Giacinti and Giordano, 2006, Broude et al., 2007, Ashcroft et al., 1999), whereas p27 and p21 regulate the cell cycle through 'positional cues' by translocating between the nucleus and the cytoplasm (Blagosklonny, 2002, Fan et al., 2004). We sought to identify genes that regulate dormancy at the gene expression level by qRT-PCR gene expression analysis.

In Chapter 5, we developed a cell sorting strategy using the dormancy marker PY to separate living AML cells into the dormant and cycling compartments

(Section 5.2), so as to further investigate the two subsets in a comparative way. As dormant cells have very low RNA (Guan et al., 2003), it was imperative to ascertain adequate RNA and cDNA for gene expression analysis. The aim of the work in this chapter was to amplify RNA using the Eberwine method (Phillips and Eberwine, 1996, Morris et al., 2011, Morris et al., 2014), and then characterise the molecular properties of both dormant and cycling cells within the same AML samples.

6.2 Identification of Candidate Genes For Cell Dormancy

To determine genes that may play a role in dormancy regulation on the transcriptional level in AML cells, we conducted a comprehensive literature research to select candidate genes. This search was conducted by Avantika Patil, an MSc student I co-supervised. The updated characteristics of these genes are summarised in Table 6.1. Previously described in Chapter 1 (Section 1.2.7), several genes are found to play intrinsic regulatory roles in LSC dormancy at the transcriptional level, including FOXO3 and LKB1; HIF1α and its target genes, HES1, and GATA2 are involved in the extrinsic dormancy regulations. Briefly, the transcriptional factor FOXO3 enforces dormancy in HSC by cell cycle control (Alvarez et al., 2001, Tothova et al., 2007, Martinez-Gac et al., 2004, Kops et al., 2002) and interaction with AKT (Sykes et al., 2011). In AML, approximately 40% cases displayed elevated FOXO3 expression, which serves to maintain leukaemic growth by preventing myeloid maturation and apoptosis (Sykes et al., 2011). A functional connection was

found between FOXO3 and the transcriptional activation of LKB1, which blocks cell cycle progress through activation of AMPK, a protector for LSCs from metabolic stress and DNA damage (Saito et al., 2015a). Along with its role of integrating energy sensing and growth control, studies suggested that LKB1 might play a critical role in enforcing HSC dormancy (Gan et al., 2010, Gurumurthy et al., 2010, Nakada et al., 2010). Together with FOXO3, LKB1 may regulate dormancy through the mTOR pathway (Ito et al., 2009). HIF1α can be activated by signals in the BM. And the accumulation of HIF1α is dependent on the PI3K/Akt/mTOR growth pathway (Pedersen et al., 2008). Higher expression of HIF1α has been found in LSCs compared to normal HSCs (Zhang et al., 2012). The HIF1α target genes, GATA2 and CITED2, were reported to transcriptionally regulate HSC and LSC function (Chen et al., 2007b, Tipping et al., 2009) and play key roles in stem cell maintenance. CITED2 acts as a GATA2 activator in HSC (Saito et al., 2015b). Importantly, GATA2 acts as a molecular entry point into the transcriptional programme which regulates dormancy in HSC and progenitor cells (Tipping et al., 2009).

Considering the evidence documented above that these genes are involved in dormancy control and regulation, we went on to explore gene expression in dormant subsets.

Table 6.1. Candidate genes in this study. (Updated from Avantika Patil, MSc dissertation, 2012)

	Candidate genes					
	LKB1/ STK11	**FOXO3**	**GATA2**	**CITED2**	**HES1**	**HIF1a**
Nature of protein product	Tumour suppressor; Serine/ Threonine kinase	Transcription Factor	Transcription Factor	Transcriptional modulator; co-activator and co-repressor	Transcription Factor	Transcription Factor
Induction	FOXO3	Oxidative stress, absence of survival factors	CITED2	Hypoxia	Activation of notch signalling pathway	Hypoxia; growth factors, cytokines and circulatory factors under normoxia
Protein subcellular localisation	Nucleus, mitochondrion (during apoptosis)	Translocates from cytosol to nucleus upon oxidative stress	Nucleus	Nucleus	Nucleus	Cytoplasm under normoxia, nuclear translocation in response to hypoxia
Expression in HSCs	Highly expressed, enhanced expression in G0 cells	Highly expressed	Expressed in G0 cells	Highly expressed	Highly expressed in G0 cells	Highly expressed
Expression in LSCs	Likely to be over-expressed,	Likely to be over-expressed	Likely to be over-expressed,	Likely to be over-expressed	Likely to be over-expressed	Over-expressed, enhanced expression in G0 cells
HSCs vs LSCs	Not known	Not known	Not known	Not known	Not known	Higher expression in LSCs
Pathways	Energy metabolism, cell polarity, apoptosis, DNA damage response, mTOR	Apoptosis, oxidative stress	Phagocytosis, cell proliferation	Differentiation, stress response	Notch signaling	Stress response, apoptosis, tumour angiogenesis
Interactions	FOXO3, mTOR, AMPK, most likely p53	LKB1, AKT, CITED2	HES1	AML1, HIF1a, FOXO3, HES1	HIF1a FOXO3	Notch signalling, $p16^{Ink4}a$, $p19^{Arf}$, CITED2, HES1
Reference	(Lutzner et al., 2012, Gurumurthy et al., 2010, Saito et al., 2015a, Nakada et al., 2010)	(Miyamoto et al., 2007)	(Tipping et al., 2009, Saito et al., 2015b)	(Chen et al., 2007a, Adams, 2012, Du and Yang, 2012, Korthuis et al., 2015)	(Sang et al., 2008, Sang et al., 2010)	(Du et al., 2012, Wang et al., 2011, Zhang et al., 2012)

6.3 Determination of Housekeeping Genes

To characterise gene expression in AML dormancy, it is important to identify reference genes stably expressed in blood mononuclear cells in both dormant and cycling states. Emerging studies indicated that a series of genes, including TNF receptor-associated protein 1 (TRAP1), 2,4-dienoyl CoA reductase 1 (DECR1), folylpolyglutamate synthase (FPGS), and peptidylprolyl isomerase B (PPIB), could be useful as normalisation genes for qRT-PCR whole blood studies of human disease (Stamova et al., 2009).

RNA was extracted from an equal number (5×10^6) of untreated or activated T cell by PHA or ConA, before 1 μg of RNA was converted to cDNA via reverse-transcription. The qRT-PCR threshold cycle (Ct) values, which are inversely proportional to the amount of target nucleic acid in samples, were measured and plotted in Figure 6.1. T cell activation by mitogens did not significantly affect the Ct values of FPGS ($p = 0.875$ with PHA; $p = 0.35$ with ConA), suggesting that it was stably expressed in T cells in dormant and cycling states. As for PPIB, TRAP1 and DECR1, more qRT-PCR cycles were required to detect dormant cells than activated T cells ($P < 0.05$), indicating a variation of gene expression between the cell cycle states.

Furthermore, an AML gene expression profiling study categorised by risk groups (by Prof Ken Mills's group at Queen's University Belfast, Northern Ireland) indicated that there was a significant increase in DECR1 ($p < 0.001$) and PPIB ($p < 0.01$) expression in the cytogenetically normal group compared to the adverse group (suggesting a variability in patients), whereas it was

borderline (p = 0.16) for TRAP1. No significant difference in FPGS expression was observed between the normal and adverse cytogenetic groups (p = 0.46), suggesting that it is stable across AML cytogenetic groups. Taken together, FPGS was shortlisted as housekeeping genes in this study.

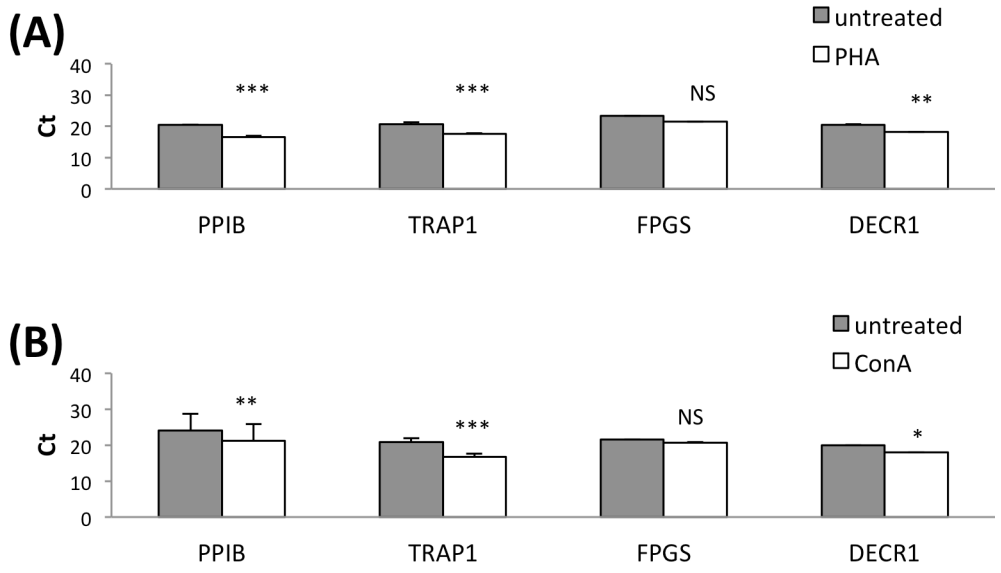

Figure 6.1. Gene detection in human T cells before and after mitogen treatment. Ct values from Real Time PCR for PPIB, TRAP1, FPGS and DECR1 are plotted on the Y-axes, before and after mitogens PHA (A) or ConA (B) treatment. Data are shown as mean ± SD. NS: not significant. * $P \leq 0.05$. ** $P < 0.01$. ***$P<0.005$.

6.4 RNA Amplification

We previously developed a cell sorting strategy using the dormancy marker PY (Chapter 5; Section 5.2), in order to study the molecular properties of dormant and cycling cells comparatively. A small number of cells from each of seven AML samples were sorted into $CD45^{+}CD34^{+}PY^{low}$ (dormant) and $CD45^{+}CD34^{+}PY^{high}$ (cycling) subsets, with a sort count of [$1.76 \times 10^3 – 4.06 \times 10^4$] and [$7.6 \times 10^2 – 3.12 \times 10^4$], respectively (Table 6.2). It is estimated that a

typical mammalian cell contains 10 – 20 pg total RNA (Suslov et al., 2015), and dormant cells have less RNA than cycling cells (Guan et al., 2003). Each of our sorted samples would therefore contain very little total RNA, in the nanogram range and we therefore reasoned that we would need to amplify RNA to generate a sufficient amount of starting material for detection of transcripts by real time PCR. To acquire sufficient total RNA for gene analysis, an RNA amplification step was taken via the 'Eberwine method' (Phillips and Eberwine, 1996, Morris et al., 2011, Morris et al., 2014) which was used to achieve RNA amplification while preserving the abundance relationships among transcripts. The method is detailed in Chapter 2 (Section 2.14.2).

We sought to amplify small quantities of RNA from the sorted patient samples. Three rounds of amplification were performed for each sample (Table 6.2). The spectrophotometric ratio of 260/280, commonly used to predict nucleic acid purity, was measured to assess the purity of RNA. The values of 260/280 were between 1.87 and 2.25. The RNA concentrations derived from each following round (i.e. the second and third round) were compared to the first round, by using the binary logarithm ($\log_2$) of the ratio (Figure 6.2). The amount of RNA had increased logarithmically, with a log ratio of 1.36 – 2.1 from the second round, and 3.17 – 4.15 from the third round. A linear regression was also observed with $R^2 > 0.99$ (Table 6.3).

Table 6.2. PY-stained and FACS-sorted AML samples for gene expression analysis. RNA was amplified by the Eberwine method.

	PY	Sort count	RNA ID	mRNA product					
				Round 1 (ng/µL)	260/ 280	Round 2 (ng/µL)	260/ 280	Round 3 (ng/µL)	260/ 280
AML1	PY-low	2.52×10^4	1	40.5	2	133.4	2.15	516.1	1.97
	PY-high	1.9×10^4	2	48.5	2.08	197.8	2.15	594.5	1.9
AML6	PY-low	7.83×10^3	3	15.5	1.95	48.4	1.97	180.1	1.95
	PY-high	8.6×10^3	4	24.1	2	77.2	1.98	315.2	1.99
AML 26	PY-low	2.8×10^3	5	13.7	1.95	51.6	1.87	211.9	1.92
	PY-high	1.1×10^3	6	15.9	1.9	60.7	1.92	252.1	1.93
AML 28	PY-low	1.76×10^3	7	16.9	1.89	64.4	2.11	249.2	1.88
	PY-high	7.6×10^2	8	10.6	1.98	45.3	1.98	187.7	1.97
AML 30	PY-low	4.06×10^4	9	77	1.88	226.9	1.97	810.2	2.13
	PY-high	3.12×10^4	10	88.3	1.97	226.1	1.92	792.4	2.01
AML 34	PY-low	1.52×10^4	11	20.3	1.9	67.4	1.96	210.1	1.98
	PY-high	2.13×10^4	12	35.6	1.92	132.7	1.88	350.5	1.92
AML 41	PY-low	7.11×10^3	13	12.5	2.09	42.7	2	179.4	1.95
	PY-high	9.16×10^3	14	13.75	1.98	48	1.97	181.2	2.25

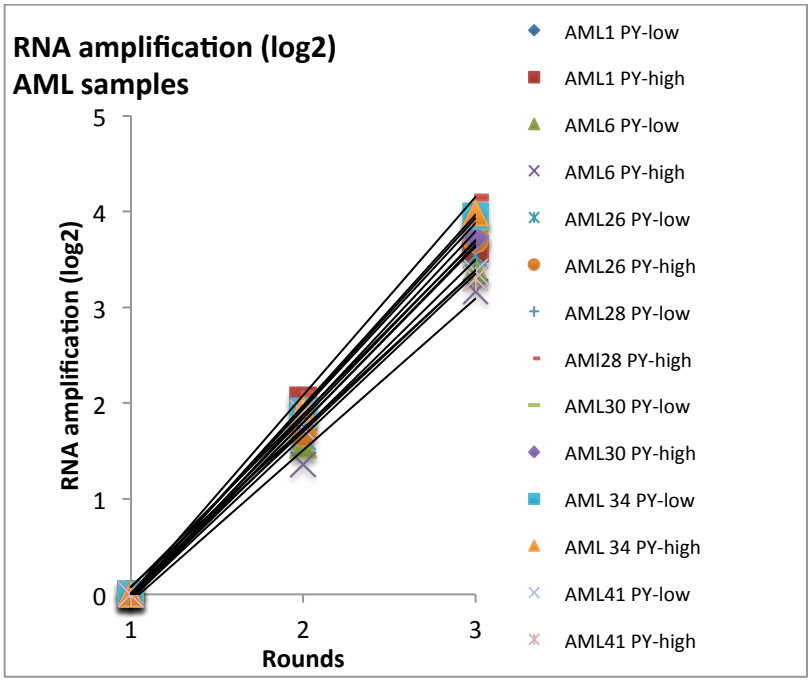

Figure 6.2. RNA amplification following three rounds of the Eberwine method. mRNA was isolated and amplified from PY-stained, double-sorted (PY-low and PY-high) cells from 7 AML samples. Three rounds of amplification were performed. Results shown are in binary logarithm (log_2), derived by the ratio of RNA concentrations between the following (second and third) round and the first round. Round1 is set to 0 (log_2^1).

Table 6.3. Binary logarithm comparison between each following amplification round and round1. R1, round1; R2, round2; R3, round3. R1 log2 value is set to 0.

		$Log_2^{(R2/R1)}$	$Log_2^{(R3/R1)}$	R^2
AML1	PY-low	1.72	3.67	0.99867
	PY-high	2.03	3.62	0.99568
AML6	PY-low	1.56	3.40	0.99778
	PY-high	1.36	3.17	0.99323
AML26	PY-low	1.66	3.54	0.9983
	PY-high	1.68	3.71	0.99704
AML28	PY-low	1.93	3.88	0.99999
	PY-high	2.10	4.15	0.99996
AML30	PY-low	1.77	3.84	0.99799
	PY-high	1.80	3.72	0.99969
AML 34	PY-low	1.91	3.95	0.99967
	PY-high	1.93	3.99	0.99969
AML41	PY-low	1.73	3.37	0.99976
	PY-high	1.90	3.30	0.99778

6.5 Quantification of Gene Expression By qRT-PCR

Following the RNA amplification work, quantitative RT-PCR was used to analyse the expression of 6 genes in the dormant and cycling subpopulations in AML samples. An equal amount (1 μg) of amplified RNA (Table 6.2) was converted to cDNA by reverse transcription, and an equal volume (1 μL) of cDNA was added to each real time reaction. Standard curves were generated using the KG-1a cell line cDNA (concentration [0.78125 - 50 ng/μL]). The indices (slope, R2 and PCR efficiency) of the standard curves are shown in Appendix 4.

Values of gene expression were extrapolated from the standard curve. To make sure the data were in the linear range of detection by qRT-PCR, only those within the standard curves were valid. Normalisation of target gene expression levels was by the housekeeping gene FPGS, which was detected in 12 out of 14 RNA samples (paired RNA samples from AML#28 had undetected FPGS, and was thus discarded). Four candidate genes, FOXO3, CITED2, GATA2 and LKB1 were measurable in the sorted subpopulations. As is shown in Figure 6.3, samples AML26 and AML34 have evidently higher expression of CITED2 and GATA2 in PY^{low} subsets, suggesting that these two genes are worth further investigation in an expanded cohort.

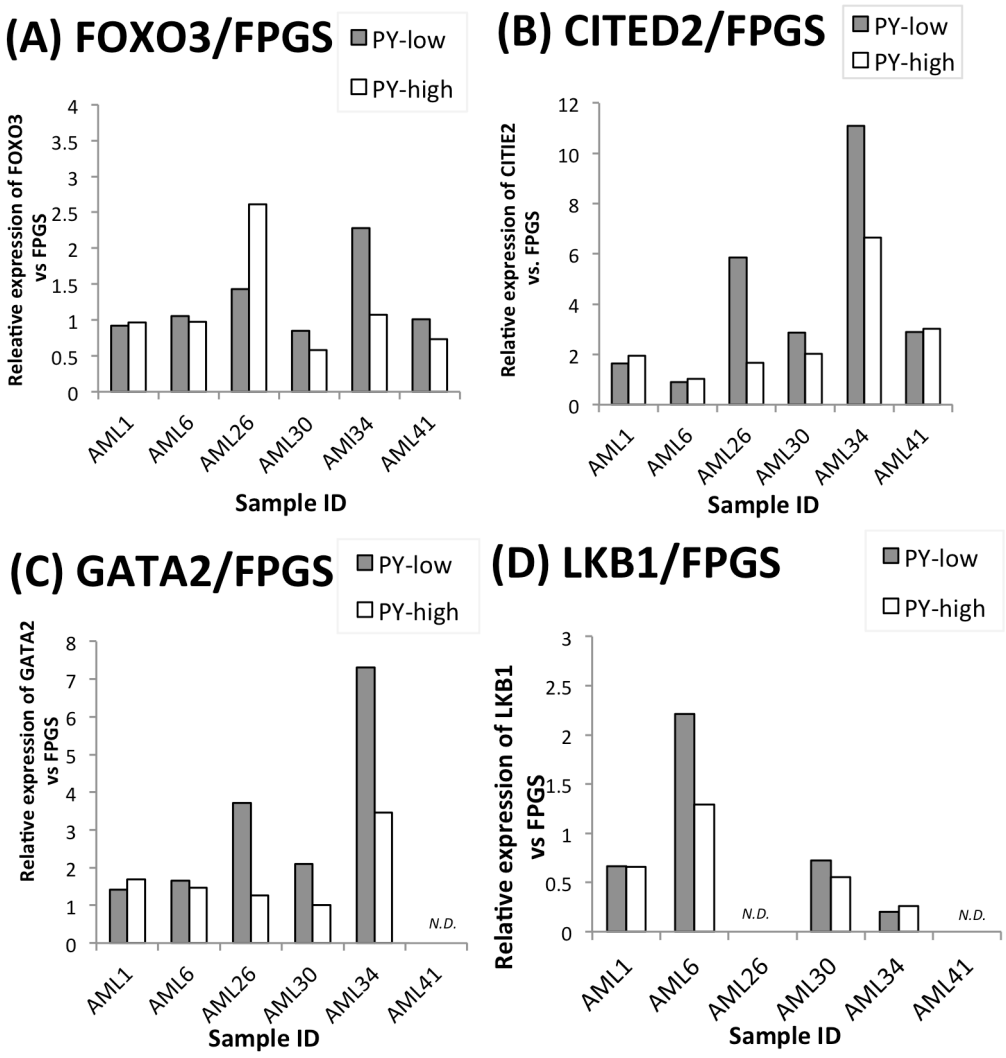

Figure 6.3. Relative expression of candidate genes in the PY-low and PY-high subpopulations after normalisation by the housekeeping gene FPGS. Four genes were measured in AML samples. (A) FOXO3, (B) CITED2, (C) GATA2 and (D) LKB1. The absence of bars indicates undetermined gene expression. *N.D.*, not determined.

6.6 Discussion

Six genes, HIF1a, CITED2, LKB1, HES1, GATA2, and FOXO3 were selected through literature research as possible dormancy regulators in AML on the gene expression level (summarised in Table 6.1). These molecules, comprising growth factors, cytokines, protein kinases, phosphatases, transmembrane

receptors and transcriptional modulators, play pivotal roles in the regulation of the cell cycle, immunity, apoptosis and cell adhesion, and were previously reported to be highly expressed in dormant HSCs or LSCs. We investigated whether dormant cells expressed these genes, and if so whether they were expressed differentially in dormant cells, compared to their cycling counterparts.

We designed a sorting strategy to separate an equal number of cells, i.e. 10% of the $CD45^{+}CD34^{+}$ populations in each sample, into PY-low (dormant) or PY-peak (cycling) fractions (the methods are described in sections 2.11 and 5.2). A small number of cells (up to 4.06×10^{4}) were obtained from each sorted sample (Table 6.2) and due to the low RNA nature of dormant cells, it was important to achieve a quantifiable amount of RNA for gene analysis studies. RNA amplification provides a powerful tool to overcome limitations in the amount of starting materials. The Eberwine method utilises T7 RNA polymerase based amplification procedure that allows isolation of mRNA from as little as a single cell (Morris et al., 2014, Morris et al., 2011). The mRNA products of the first round can be used as the starting point for subsequent rounds of RT and IVT, initiated by random priming (Phillips and Eberwine, 1996). It should be noted that this method strictly amplifies mRNA from the first strand reaction, where the poly-T portion of the T7-oligo(dT) primer selects for mRNA species by binding to the polyA tails. The most abundant RNAs in the cell, ribosomal RNAs, however, will not be involved in this procedure (Morris et al., 2011). Table 6.2 demonstrates the yield of mRNA, but does not reflect the amount of

total cellular RNA that could have been extracted from the sorted PY-high/low cells.

A logarithmical (log2) increase of RNA was observed after each additional round of amplification in patient samples (Figure 6.2). However, as the Eberwine amplification method involves a complex, time-consuming procedure, we kept a vigilant watch on preserving RNA quality and purity. Poor quality RNA samples can lead to spurious real time PCR results. The spectrophotometric ratio of 260/280, commonly used to predict nucleic acid purity, was measured to justify the RNA results. A ratio of ~2.0 is generally the "rules of thumb" as pure for RNA. Adequate 260/280 ratios were obtained in this study, ranging from 1.89 to 2.2 (Table 6.2). However, as some of the ratios are <1.9 (e.g. in sample #28), factors that could affect RNA purity should be looked into. Small pH changes in the solution can cause the 260/280 to vary (Wilfinger et al., 1997). Acidic solutions will lower the ratio by 0.2 - 0.3, while basic solutions will raise the ratio by 0.2 - 0.3. The integrated pH indicator in some of the reagents, such as the Qiagen MinElute Reaction Cleanup kit, safeguarded the optimal pH along the way. The weighted average 260/280 for the four present nucleotides, Guanine (1.15), Adenine (4.50), Cytosine (1.51), and Uracil (4.00), determines the 260/280 of the sample RNA (Leninger, 1975). An RNA purification step was performed at the end of each round of amplification by the MEGAclear transcription clean-up kit, to separate RNA from unincorporated NTPs, enzymes, and buffer components. However, any residual DNA contamination (e.g. co-extracted genomic DNA) may result in a lower ratio, due the presence of Thymine (260/280 = 1.47) instead of Uracil.

And it can serve as a PCR template and confound RNA detection results. Co-extracted proteins (including RNases) are unlikely to be revealed by the 260/280 ratio, as it takes a relatively large amount of protein to be present in the sample to alter this ratio (Glasel, 1995), but protein contamination can cause PCR inhibition. It can also lead to the degradation of RNA template due to the presence of RNases that come from sources such as the skin, saliva, or even breath (Morris et al., 2011). It is therefore imperative that RNase-free techniques be closely observed so that the samples do not degrade, which results in loss of detection of rare transcripts. The washing steps may minimise, if not eliminate, the possibility of carry-over contamination of chemicals (e.g. ethanol, sodium acetate) from the cDNA synthesis process, which may also affect real time PCR results.

Standard curves were set up by using the KG-1a cell line cDNA (concentration 0.78125 - 50 ng/μL). Cut-off points were set for valid data, i.e. any 'absolute' qRT-PCR reading below 0.78125 ng/ μL, or above 50 ng/μL were disregarded. The R^2, slope, and efficiency are used to determine the performance of a PCR reaction (Appendix 4). A 100% efficient reaction will yield a 10-fold increase in PCR amplicon every 3.32 cycles during the exponential phase of amplification ($\log_2^{10} = 3.3219$). Generally, a standard curve with a correlation coefficient of ≥ 0.99, an efficiency between 90 and 110% is considered acceptable according to manufacturer's note (https:// www.thermofisher.com/uk/en/home/life-science/pcr/real-time-pcr/qpcr-education/pcr-understanding-ct-application-note.html#5). A slope of $-3.3 \pm 10\%$ (i.e. -3.6 to -3) reflects an efficiency of $100\% \pm 10\%$. A considerably lower R^2

value (0.88) was observed in the FOXO3 standard curve, which indicates precision problems during setup, such as pipetting, mixing, and plate-setup factors.

An endogenous control (housekeeping gene) was used as an active reference to normalise the amount of target gene mRNA using qRT-PCR. We confirmed that FPGS is stably expressed in dormant and cycling subsets of PB-derived T cells (Figure 6.1) and a gene expression profiling database (by Ken Mills and colleagues) suggested that FPGS expression is unlikely to shift across AML cytogenetic groups. Thus we chose FPGS as a housekeeping gene in this dormancy study. Nevertheless, there may be other endogenous controls available to be explored in the dormancy study. Results from a recent study (Matsuzaki et al., 2015) indicate that the β2-Microglobulin (B2M) can reliably be used as a reference gene for the relative quantification of expression levels in HSCs between active and dormant states, under *in vivo* and *in vitro* conditions.

Next, we performed qRT-PCR gene analysis. The housekeeping gene FPGS and 4 genes of interest, FOXO3, CITED2, GATA2, and LKB1 were measurable in the sorted populations from all AML samples except for sample #28, although mRNA was amplified in this sample (Table 6.2). RNA is chemically unstable and is inherently susceptible to RNase degradation, which could lead to the inhibition of cDNA synthesis. It would be advisable to check the cDNA quality either by regular PCR, or by checking the 260/280 ratio

(pure DNA has a ratio around 1.8) on nanodrop, before the gene expression analysis.

We did not find clearly overexpressed genes in dormancy, although samples #AML26 and AML34 had markedly higher expression of CITED2 and GATA2 in the PY^{low} (dormant) subsets (Figure 6.3). A link between these two transcriptor genes in human haematopoietic cells was recently revealed (Saito et al., 2015b). However, we did not find evidence to support why only 2 out of 6 samples have shown the increase. While FLT3-ITD mutations are found in both AML26 and AML34 (Appendix 1), it is unlikely to be the explanation, as 3 other samples (AML1, AML30 and AML41) also carry the same mutation. Moreover, samples AML26 and AML34 are not cytogenetically in common (Appendix 1). It is however worth further investigating CITED2 and GATA2 expression in dormant leukaemia subsets, in an expanded cohort. And a broader approach, such as gene expression profiling, may help to identify candidate dormancy-regulating genes.

Recently, advances have been made to the Eberwine method. Changes include an adaptation to RNA sequencing, instead of exponential PCR amplification (Saliba et al., 2014). IVT amplifies RNA linearly using T7 RNA polymerase, but is biased toward the 3' end of input transcripts, and each of the RNA amplification rounds leads to a further shortening of the transcript during the second strand synthesis (Morris et al., 2011). Several variants of the method have been developed (Nygaard and Hovig, 2006, Marko et al., 2005). But the IVT protocol still remains labour intensive. Recent developments, such as

CEL-seq (cell expression by linear amplification and sequencing) and MARS-seq (massively parallel RNA single-cell sequencing) (Hashimshony et al., 2012, Jaitin et al., 2014), were designed to compensate the drawback of the IVT procedure. Also, commercial RNA amplification kits designed to extract sub-nanogram range of RNA have recently been made available. But due to their novelty, these kits have not been comprehensively field evaluated for accuracy reproducibility, performance and ease of use. Shanker and colleagues conducted an evaluation for four kits, including SMARTer Ultra Low input RNA (part number 634935); SuperAmp (R&D Systems, Minneapolis, MN, USA); Ovation RNA-Seq System V2 (part number 7102; NuGEN Technologies Incorporated); and SeqPlex RNA (Sigma-Aldrich; R&D Systems). According to their report, no single kit appeared to meet all the challenges of small input material. However, these kits have shown promising performances, and good reproducibility/recovery of data (Shanker et al., 2015). Further investigations are needed before the kits can be used as a routine application for basic and clinical research.

This current study endeavoured to search for genes that regulate dormancy on gene expression level. We have confirmed that FPGS can reliably be used as a housekeeping gene for the relative quantification of expression levels in AML cells across dormant and cycling states. The lack of success to find clearly overexpressed genes in dormancy suggests that a broader approach is necessary to identify candidate genes.

CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

7.1. Study Aims

The residual, dormant leukaemia cells residing in the BM niche are considered responsible for chemoresistance and relapse in AML. The mainstream chemotherapy, i.e. the nucleoside analogue/anthracycline-based regimen is designed to target proliferating cells; therefore dormant chemoresistant leukaemia cells can be spared by traditional chemotherapy. Novel agents with alternative mechanisms of action are needed to target and eradicate the disease-initiating cells. Previously, research in our group has established that mTOR inhibition induces the principal features of dormancy, including low RNA content, low metabolism, and low basal ROS formation in the absence of apoptosis (Pallis et al., 2013). The current study focused on targeting the intrinsic apoptosis pathway, particularly in dormant cells. The aim was to develop *in vitro* methods to mimic the physiological BM niche environment, identify dormant leukaemia cells, and investigate the efficacy of the BCL-2 BH3 mimetics ABT-737 and ABT-199, and the multikinase inhibitor TG02, all of which play a role in targeting members of pro-survival BCL-2 molecules and thereby induce apoptosis in AML cells (Lagadinou et al., 2013, Pan et al., 2014, Konopleva et al., 2006, Goh et al., 2012, Pallis et al., 2012, Pallis et al., 2010). We were keen to find out whether these drugs could overcome dormancy- and niche-mediated chemoresistance in AML and effectively target the dormant subpopulations. We also investigated possible dormancy markers that could help us to distinguish dormant cells from normal cells. And with the aid of FACS sorting techniques, we sought to obtain the dormant and cycling subsets of AML cells and characterise dormancy on the molecular level.

7.2. General Discussion

BM stromal cells protect AML cells from the anti-leukaemic effects of chemotherapy, e.g. daunorubicin and cytarabine (Garrido et al., 2001, Ishikawa et al., 2007, Konopleva et al., 2002a), targeted tyrosine kinase inhibitors (TKIs) such as FLT3 inhibitors (Alvares et al., 2011, Zeng et al., 2009, Dos Santos et al., 2014, Weisberg et al., 2009), and mAb-directed (e.g. anti-CD44) targeted therapy (Chen et al., 2015). Previously, work in NOD/SCID mice by Schubert and colleagues highlighted the potent leukaemogenic and long-term *in vivo* survival potential of $CD34^{+}$, $PKH26^{bright}$ (dormant) AML primary cells, in contrast to the $CD34^{-}$, $PKH26^{dim}$ (cycling) cells (Schubert et al., 2011), thus giving strong evidence and rationale for identifying, characterising dormant AML cells and assessing their chemosensitivity. Using an AML/stroma co-culture model, Alvares and colleagues demonstrated chemoresistance in a dormant subset ($CD34^{+}PKH26^{high}$) to a FLT3 inhibitor, TKI258, and confirmed that a significant number of the dormant cells were leukaemic by fluorescence in situ hybridization (FISH) analyses for leukaemic gene rearrangements (Alvares et al., 2011). These findings stress the possibility that primitive dormant leukaemia cells may be a key feature of the disease and thus a crucial target for therapy.

We developed and optimised a 12-day *in vitro* assay to quantify dormant AML cells labelled with the cell division tracker PKH26 and LAP markers, with and without the addition of drugs. Also, we used the CytoCount control beads to obtain absolute cell number on flow analysis. Our finding that some aberrant LAPs can persist in culture for 12 days on stroma further warrants the use of

this model in the chemosensitivity study. A small subset of $PKH26^{high}$ (non-dividing), LAP^{+} cells, which demonstrated a primitive low FSC/SSC profile on flow analysis, could be identified on Day12. With the aid of FACS sorting, we were able to purify the dormant ($CD34^{+}LAP^{+}PKH26^{high}$) leukaemic subpopulation for further analyses. We found that some of these dormant cells maintained their clonogenic potential, confirming that these cells were indeed dormant rather than being senescent. However, we do not surmise that all $CD34^{-}$ cells are non-leukaemic. Some AML patient samples, namely "CD34-negative AML", do not present CD34, or have less than 1% $CD34^{+}$ cells (van der Pol et al., 2003, Schuurhuis et al., 2013, Almhareb et al., 2011). LSCs from AML samples with mutated NPM were found in the $CD34^{-}$ fraction, with engraftment capacity (Taussig et al., 2010). Likewise, we do not presume that all tested LAP-negative cells are non-leukaemic. A standard panel of LAP antibodies, which have been most frequently detected in AML patient cells (Feller et al., 2013) was used in this study to identify leukaemic cells in AML samples (Table 2.1, Section 2.10, Chapter 2). Nonetheless, the list of LAP antibodies is not exhaustive. Therefore it was possible that some of the tested LAP-negative cells were leukaemic. Using molecular markers (e.g. FLT-ITD) is another useful way to detect leukaemic cells in samples. Studies by FISH and PCR analyses have demonstrated the presence of FLT3-ITD mutations in the non-cycling fraction of the primitive AML compartment and that the involvement of such mutations is heterogeneous, i.e. not all AML samples bore the same mutations (Alvares et al., 2011, Pollard et al., 2006).

Our chemosensitivity data indicated that the efficacy of novel agents (TG02, ABT-737 and ABT-199) in dormant cells cultured on stroma was at least 70% of that against cycled cells, providing a profound advantage over Ara-C, which completely failed to eradicate cells in the dormant fraction. Our data also showed comparable efficacy of ABT-737 and ABT-199 in targeting AML patient cells. When used at the same dose, these two BH3 mimetics showed analogous dose-response patterns. This finding is consistent with other studies, where ABT-199 and ABT-737 were similarly potent in AML samples as monotherapy (Bogenberger et al., 2015). Comparable induction of apoptosis by the two BH3 mimetics was also observed in human multiple myeloma (MM) cell lines and primary cells as monotherapy (Touzeau et al., 2014), and in breast cancer models in combination with tamoxifen (Vaillant et al., 2013). In addition, a preclinical mouse model of lymphoma showed similar responsiveness to ABT-737 and ABT-199 (Vandenberg and Cory, 2013).

However, the targeting of BCL-xL and the subsequent dose-related side effect of transient thrombocytopenia observed in phase I trials has been a major setback to ABT-737 and its oral bioavailable agent ABT-263 (Wilson et al., 2010) and quenched the enthusiasm for further clinical investment of this compound. ABT-199, on the other hand, specifically targets BCL-2 and therefore does not cause platelet deficiency (Souers et al., 2013, Vogler et al., 2013). In a phase II study, ABT-199 monotherapy demonstrated an adequate safety profile and promising clinical activities in patients with relapsed/refractory AML who had limited available treatment options (Konopleva et al., 2014). Yet, due to its lack of affinity for MCL-1, resistance

in leukaemia cells that rely on MCL-1 may limit the use of ABT-199 as monotherapy.

Recent studies indicated the promises to overcome ABT-199 resistance by drug combination. Preliminary work from our laboratory showed that concomitantly targeting MCL-1 with TG02 and BCL-2 with ABT-199 was complementary and enhanced individual toxicities to AML cell lines and patient cells (Abdul-Aziz et al., 2014, Burrows et al., 2014). The most sensitive target of the multikinase inhibitor TG02 is CDK9, a transcription controller that regulates the activation of RP2, reducing transcription rates which in turn drives the selective depletion of short-lived proteins, including MCL-1 and XIAP while sparing the more stable BCL-2 and BCL-xL (Goh et al., 2012, Pallis et al., 2012, Burrows et al., 2014). MCL-1 expression in AML is regulated by STAT5, a direct target of FLT3 (Yoshimoto et al., 2009). And the cytokine-driven phosphorylation of STAT5 is a feature of clones from chemoresistant patients in AML (Rosen et al., 2010). Research in our laboratory showed that MCL-1 downregulation by TG02 was independent of niche cytokines and STAT5, thus may be an important strategy against AML chemoresistance in the BM niche (Pallis et al., 2012). Additionally, the unique spectrum of targets (including CDKs, JAK2, FLT3, etc.) gives TG02 the advantage over single TKI treatment, which is often inadequate to eradicate dormant, chemoresistant AML populations (Alvares et al., 2011, Weisberg et al., 2012). Moreover, JAK2 inhibition was found effective in overriding stromal protection and potentiating FLT3 inhibition in primary AML and cell lines (Weisberg et al., 2012).

Others showed that combining ABT-199 with conventional chemotherapeutic drugs Ara-C or daunorubicin could increase DNA damage, decrease MCL-1 levels and MCL-1 association with BIM, and synergistically induce cell death (Niu et al., 2015). Combinatorial blockade of BCL-2 pathways and the mitogen-activated protein kinase (MAPK) cascade, the major pathway in AML that is activated by upstream mutant proteins such as FLT3, was shown to be synergistic in AML cell lines (Han et al., 2015). Activation of p53 by the novel MDM2 inhibitor Idasanutlin and BCL-2 inhibition could reciprocally overcome leukaemia resistance to apoptosis encountered by using either treatment alone (Pan et al., 2015). Moreover, targeting CHK1 may enhance the cytotoxic effects of ABT-199 on AML cells through downregulation of MCL-1 (Zhao et al., 2015). And ABT-199 compared to ABT-737 resulted in similar sensitisation of the de-methylating agent 5-aza-2'-deoxcytidine (5-Aza) in AML *ex vivo* (Bogenberger et al., 2015).

A further aim of this study was to identify possible markers that could help distinguish dormant cells from proliferating cells. We demonstrated that the RNA-synthesis marker PY and the proliferation marker CD71 could help to identify dormant cells in AML samples in terms of reproducibility and staining stability, and can be used in FACS sorting for living cells. However, these markers could overestimate dormancy, due to the 'negative selection' strategy. Therefore a marker with high expression in dormant cells would be ideal. In our laboratory, a gene expression profiling study in TGF-β-treated, dormancy-induced AML cell lines is underway. So far, the preliminary results have indicated the B cell translocation gene 2 (BTG2) as a promising dormancy

marker. This makes sense, as BTG2 is involved in the regulation of the G_1/S transition of the cell cycle (Duriez et al., 2002, Tsui et al., 2008), and is highly expressed in dormant cells (Struckmann et al., 2004). It has also been shown to enhance deadenylation, and is a general activator of mRNA decay, thereby contributing to gene expression control (Mauxion et al., 2008). Further investigations on BTG2 are being undertaken in our research group.

We sorted AML cells into the dormant (PY-low) and cycling (PY-high) subsets and sought to identify genes that may play a role in dormancy via transcriptional regulation. In order to achieve sufficient starting material for the analysis, the Eberwine method was performed to amplify RNA and we demonstrated a logarithmical (log_2) increase of RNA after each additional round of amplification in patient samples. We have also confirmed the validity of FPGS as a housekeeping gene in AML dormancy study. However, we did not find clearly overexpressed genes in dormant (PY-low) AML cells by qRT-PCR analysis, although emerging evidence suggests the roles of genes that regulate haematopoietic cell dormancy and maintenance on the transcriptional level (Gan et al., 2010, Gurumurthy et al., 2010, Nakada et al., 2010, Chen et al., 2007b, Tipping et al., 2009) and higher expression of certain genes, such as HIF1α, was observed in LSCs (Zhang et al., 2012). A broader approach, such as our on-going gene expression profiling study in dormancy-induced AML, would be helpful to identify candidate genes.

7.3. Conclusions And Future Directions

In conclusion, this study developed *in vitro* methods to identify and characterise dormant AML cells, using combinations of phenotypic markers, LAPs, and dormancy markers. We have provided the evidence that targeting the intrinsic anti-apoptotic pathway by TG02 or the BH3 mimetics can efficiently diminish dormant and chemoresistant AML cells protected by BM cells. Nevertheless, an emergence of reports on chemoresistance to single-agent ABT-199 has led our attention onto the combined mechanisms of action, such as combining BCL-2 and MCL-1 inhibition, or targeting the intrinsic pathway of apoptosis in addition to other survival pathways. Also, we have considered furthering our search for dormancy markers that are positively expressed in non-cycling cells, and the laboratory is currently investigating BTG2. Future work will be carried out to evaluate the expression, stability, and reproducibility of this molecule in dormant and cycling AML cells. Also, a gene expression profiling study will help us to further characterise and understand the molecular mechanisms involved in dormant LSCs in AML. This could lead to the development of new targeted therapies to eliminate MRD, and enhance long-term cures for AML patients.

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Appendices

1. AML patient sample information.

Table 1. Patient demographics in this project.

AML sample	Sample ID	BM or PB	Fresh or frozen	Age (years)	Sex	FLT3 mutation	Aberrant surface markers	Cytogenetics
AML 1	AM608	PB (LP)	frozen	19	M	ITD	no aberrant phenotype, but HIGH CD33 with +/-CD13	Normal
AML 2	MG603	BM	frozen	64	F	WT	CD34+, CD7	Complex
AML 2R	MG618	BM	frozen	65	F	WT	CD34+, CD7	Complex
AML 3	TB601	PB	frozen	71	M	ITD	CD34+, CD7	Normal
AML 4	MB596	PB	frozen	67	F	ITD	CD34+, CD7	46XX del(2)(q11q21)der(7)t(1;7)(q2q22),add(11)(q2)[9]
AML 4R	MB609	PB	frozen	68	F	WT	CD34+, CD7	46XX del(2)(q11q21)der(7)t(1;7)(q2q22),add(11)(q2)[9]
AML 5	TM582	BM	frozen	42	M	ITD	CD34+, CD19	Trisomy 11, MLL
AML 6	KG536	BM	frozen	40	F	WT	CD34+, CD7	Complex
AML 7	AR444	BM and PB	frozen	74	F	WT	CD34+, CD7	Normal
AML 8	HV643	PB	fresh	83	M	WT	no aberrant phenotype	No result
AML 9	SR644	PB	fresh	65	M	WT	no aberrant phenotype	Normal
AML 10	RG645	BM	fresh	64	M	WT	no aberrant phenotype	Normal
AML 11	PB658	PB	fresh	47	M	ITD	no aberrant phenotype	No result
AML 12	KD638	PB	fresh	87	M	WT	CD33+, CD117+ MPO+ CD34- HLADR- CD13-	Normal
AML 13	CP615	PB	fresh	70	F	ITD	no result	Normal
AML 14	DA604	BM and PB	frozen	65	M	WT	no result	Normal
AML 15	CR594	BM and PB	frozen	38	M	ITD	no result	No result
AML 16	JR593	BM and PB	frozen	78	M	ITD	no result	Normal
AML 17	MK583	PB	frozen	65	F	WT	no result	No result
AML 18	SS574	PB	frozen	68	M	ITD	no result	Normal
AML 19	MB572	PB	frozen	70	F	WT	no result	Normal/complex hybridised; chromosomes 2, 9 and 21 abnormalities
AML 20	EB567	BM and	frozen	82	F	ITD	CD7; CD34-	Normal

		PB						
AML 21	DH557	BM and PB	frozen	76	M	ITD	no result	Normal
AML 22	PB522	BM and PB	frozen	64	F	WT	no result	No result
AML 23	AR452	PB	frozen	74	F	WT	CD34+ CD33-, CD117+	Normal
AML 24	PL367	BM	frozen	50	M	ITD	CD34+, CD7	Normal
AML 25	HM365	BM	frozen	79	M	WT	no aberrant phenotype	Normal
AML 26	MB297 B	PB	frozen	39	M	ITD	no result	Normal
AML 27	EP264	BM and PB	frozen	72	F	ITD	no result	No result
AML 28	DR256	PB	frozen	73	F	WT	no result	No result
AML 29	VS251	PB	frozen	54	F	ITD	no result	Normal
AML 30	DW252 B	PB	frozen	56	M	ITD	no result	Normal
AML 31	AA245	PB	frozen	30	F	WT	CD56; CD34-	Complex
AML 32	LE 15-1881	PB	frozen	19	no infor-mation	WT	no result	No result
AML 33	BK 15-1363	BM and PB	frozen	56	F	WT	no aberrant phenotype	11q23
AML 34	MB 15-1146	PB	frozen	68	no infor-mation	ITD	no result	+8, +11
AML 35	JL 15-0428	PB	frozen	44	F	WT	CD34+, CD2	inv(16)
AML 36	JJ 15-0421	PB	frozen	42	M	WT	CD34+CD33-, CD117+	inv(16)
AML 37	TK 15-0343	PB	frozen	41	F	WT	no aberrant phenotype	t(8;21)
AML 38	SW 14-9397	PB	frozen	63	F	ITD	no result	Normal
AML 39	DF 14-8792	PB	frozen	65	M	WT	CD34+CD33-, CD117+	Inv(16)
AML 40	SR 14-8779	PB	frozen	69	F	ITD	CD34+, CD117+, CD7	Complex
AML41	MM 14-8549	PB	frozen	76	F	ITD	No result	No result

LP: leukapheresis product

2. Protocol for the ALDEFLUOR™ ASSAY

(source: www.stemcell.com)

1.Label one “test” and one “control” tube for each sample to be tested. Place 1 mL of the adjusted cell suspension into each “test” sample tube.

2. Add 5 µL of ALDEFLUOR™ DEAB Reagent to the “control” tube. Recap control tube and DEAB vial immediately.
NOTE: ALDEFLUOR™ DEAB is provided in 95% ethanol. Recap immediately to prevent evaporation.

3.Add 5 µL of the activated ALDEFLUOR™ Reagent per milliliter of sample to the first sample “test” tube. Mix and immediately transfer 0.5 mL of the mixture to the DEAB “control” tube.
NOTE: the ALDH enzymatic reaction begins immediately upon addition of the activated substrate to the cell suspension. It is imperative that an aliquot of the ALDEFLUOR™-reacted cells be added to the DEAB control tube without delay.

4.Add control and substrate solutions as described in steps 2 and 3 above for each sample to be tested.

5.Incubate “test” and “control” samples for 30 to 60 minutes at 37°C (do not exceed 60 minutes).
NOTE: Optimal incubation times may vary between different cell types. For suggestions on optimization of ALDEFLUOR™ staining conditions for non-hematopoietic cells, cultured cells, and cell lines, refer to the Technical

6.Following incubation, centrifuge all tubes for 5 minutes at 250 x g and remove supernatant. Resuspend cell pellets in 0.5 mL of ALDEFLUOR™ Assay Buffer and store the cells on ice or at 2 - 8°C.
NOTE: If immunophenotyping is to be performed, add and incubate the antibodies after step 6. To prevent efflux of the ALDEFLUOR™ product it is important that the antibody incubation is performed in ALDEFLUOR™ Assay Buffer. Whenever possible keep the cells chilled (2 - 8°C or on ice) to slow down the product efflux.

3. Reagents list for the Eberwine amplification method

Table 2. Key reagents used in the RNA amplification

Name	Company	Catalogue number	Comments
dATP	Invitrogen	55082	
dGTP	Invitrogen	55084	
dCTP	Invitrogen	55083	
dTTP	Invitrogen	55085	
T7-Oligo(dT) primer	Ambion	AM5712	
RNasin	Promega	1006205	
Second strand buffer, 5x	Invitrogen	10812-014	
DNA ligase	Invitrogen	18052-019	
Rnase H	Invitrogen	18021-071	
DNA polyerase I	Invitrogen	18010-017	
T4 DNA polymerase	Invitrogen	18005-017 or 18005-025	
QIAquick PCR Purification Kit	Qiagen	28106	
Glycogen	Invitrogen	10814-010	
Sodium acetate, 3M	Fluka Biochemika	71196	
Random Primers	Invitrogen	58875	
Ambion MEGAscript T7 kit	Invitrogen	AM1334	
Superscript III Reverse Transcriptase	Invitrogen	18080-044	in kit comes with first strand buffer (Y02321) and 0.1M DTT
MEGAclear Kit	Ambion	1908	
Nuclease free water	Ambion	AM9937	
MinElute Reaction Cleanup kit	Qiagen	28206	

4. Real Time PCR efficiency parameters

Table 3. The indices of the qRT-PCR standard curves.

Gene	Slope	R^2	PCR Efficiency (%)
FPGS	-3.51	0.99	92.90
HES1	-3.79	0.95	83.53
GATA2	-3.79	0.98	83.64
FOXO3	-3.16	0.88	107.22
CITED2	-3.83	0.98	82.51
LKB1	-3.69	0.99	86.50
HIF1a	-4.19	0.96	73.30