





Regulation of Cell Survival in Acute Myeloid Leukaemia

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To Jesus Christ, the healer of the nations and to my mother, Stephanie and father, Philip who have given me all the love, support, encouragement, and opportunity to study leukaemia

How much better it is to get wisdom than gold!
And to get understanding is to be chosen above silver.
Proverbs 16:16

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Abstract

Acute myeloid leukaemia (AML) is a clonal disease of myelopoiesis characterised by poor survival due to frequent relapse. The cytokines interleukin-3 (IL-3) and granulocyte macrophage-colony stimulating factor (GM-CSF) are soluble glycoproteins that bind cell surface receptors on haemopoietic cells to stimulate their biological effects including cell survival and proliferation. Cancers cells usurp cytokine signalling pathways via various mechanisms to evade apoptosis and deregulate proliferation, two fundamental hallmarks of the cancer phenotype. Within a given patient's leukaemia there are smaller sub-populations of cells that possess the unique ability to self-renew and initiate tumour formation, termed "leukaemia stem cells" (LSCs). LSCs possess robust survival properties, are resistant to chemotherapy and over-express the IL-3 receptor α chain, however therapies directed specifically against LSCs are currently lacking. This body of work examines the cytokine receptor signalling events transduced by the IL-3/GM-CSF receptor active in acute myeloid leukaemia blasts and LSCs with the aim of discovering novel avenues for therapy.

In this thesis four targets for AML therapy are investigated and developed:-

(1) Firstly, we demonstrate feasibility of LSC targeting as a therapeutic strategy by demonstrating efficacy in vivo and in vitro of a blocking monoclonal antibody 7G3 directed to the extracellular domain of the IL-3 receptor CD123 which is over-expressed on AML stem cells.

(2) Secondly, we show that in the majority of AML patients, a distinct serine in the IL-3 receptor beta chain is constitutively phosphorylated and is required for cytokine-mediated survival. In a search for druggable kinases that may be responsible for this phosphorylation we isolated phosphatidyl inositol 3-kinase with novel activity as a *protein kinase* and show that it is linked to cell survival.

(3) We demonstrate that osteopontin (*OPN*) is a functionally relevant gene regulated by the serine-survival pathway that mediates survival of $CD34^{+}CD38^{-}CD123^{+}$ leukemic stem/progenitors. Furthermore, increased expression of OPN is associated with poor prognosis in normal karyotype AML.

(4) Finally, we identified a small molecule kinase inhibitor PIK-75 that is capable of the dual targetting of both PI3K and CDK9 in AML in vivo and in vitro. It is suggested that the simultaneous targeting of different molecules converging on a cell survival pathway represents a novel therapeutic avenue.

Author Declaration

This work contains no material that has been accepted for the award of any other degree or diploma in any University or other tertiary institution to Daniel Thomas, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Powell, JA, Thomas D, Barry, EF, Kok, C, McClure, BM, Tyskin, A, To, LB, Brown, A, Lewis, ID, Herbert, K, Goodall, GJ, Speed, TP, Asou, N, Jacob, B, Osato, M, Haylock, DN, Nilsson, SK, D'Andrea, RJ, Lopez, AF and Guthridge, MA. (2009) Expression profiling of a hemopoietic cell survival transcriptome implicates osteopontin as a functional prognostic factor in AML. *Blood*. **114**(23):4850-4870.
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Joint-Author Contributions

Contributions to publications included in this thesis by individual co-authors are outlined below.

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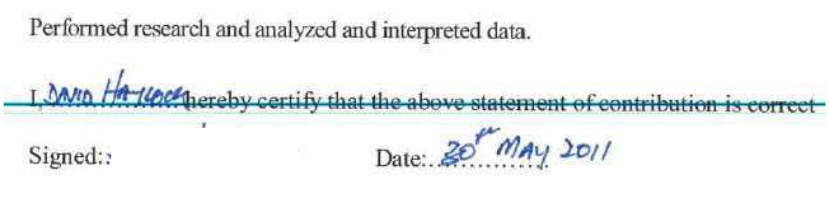

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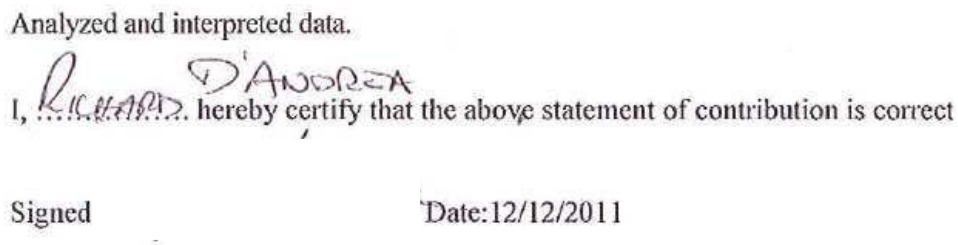

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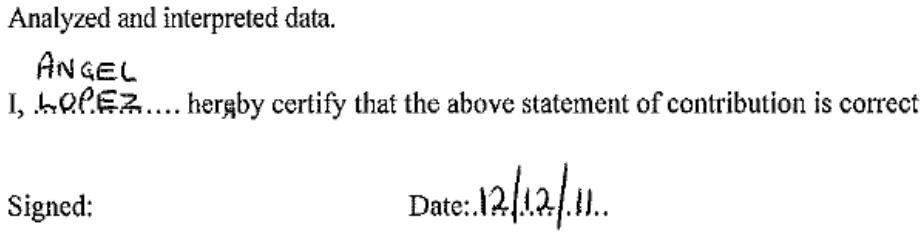

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Abbreviations

4HT	4-hydroxy tamoxifen
AML	acute myeloid leukemia
ATP	adenine triphosphate
βc	GM-CSF and IL-3 receptor beta subunit
BM	bone marrow
Cdk9	cyclin-dependent kinase 9
CTD	carboxy terminal domain of RNA polymerase II
CI	confidence interval
CLL	chronic lymphocytic leukemia
CPM	counts per minute
CSL	Commonwealth Serum Laboratories
FAB	French-American-British
FACS	fluorescence-activated cell sorting
FDM	factor-dependent myeloid
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
GMRα	granulocyte-macrophage colony stimulating factor receptor alpha chain
hour	h
IC50	half maximal inhibitory concentration
IL-3	interleukin-3
ITD	internal tandem duplication
LSPCs	leukemic stem and progenitor cells
mAb	monoclonal antibody
min	minute
MNCs	mononuclear cells
ND	not determined
NS	not significant
OPN	human osteopontin gene
pAb	polyclonal antibody
PtdSer	phosphatidylserine
PtdIns	phosphatidylinositol

PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PIP	phosphatidylinositol phosphates
PH	pleckstrin homology
RAH	Royal Adelaide Hospital
SD	standard deviation
SEM	standard error of the mean
TKIs	tyrosine kinase inhibitors
WCC	white cell count
wt	wild-type

Chapter 1

Introduction

1.1 Summary

Acute myeloid leukaemia (AML) is a rapidly growing cancer arising in the bone marrow that is characterised by an accumulation of immature myeloid precursors that exhibit deregulated survival and proliferation (Collin, 1995). AML affects both young and old with the highest incidence in patients over 60 yrs. Despite improvements in supportive care and allogeneic stem cell transplantation, the prognosis for most patients has not improved for three decades and current therapy consists of induction chemotherapy followed by consolidation with cytarabine, anthracycline +/- etoposide (Bradstock *et al.* 2005; Miyawaki *et al.* 2005). Prognosis is predictable in part by cytogenetic abnormalities within the leukaemic clone, however a large proportion of patients over 60 have a normal karyotype and are therefore difficult to stratify (Zaidi *et al.* 2008). New strategies for treatment with larger therapeutic index are required to improve outcome in this patient group.

A number of landmark discoveries with the last decade have accelerated research in AML. It has been established that within each patient's leukaemia there are smaller sub-populations of cells that differ in biological properties but are cytogenetically identical. These cells possess the unique ability to self-renew and initiate tumour formation and are termed "leukaemia stem cells" (LSCs) (Bonnet and Dick, 1997; Mazurier *et al.* 2004). LSCs possess robust survival properties and being intrinsically resistant to chemotherapy, similar to normal stem cells, are likely responsible for the common phenomenon of disease relapse. LSCs have been shown to over-express certain cell surface markers including the interleukin-3 (IL-3) receptor α chain CD123 (Jordan *et al.* 2000), however therapies directed specifically against LSCs are currently lacking. It is postulated but not proven that similar to normal haematopoietic stem cells, LSCs require an ideal niche in the bone marrow that may regulate some of their intrinsic properties (Lane *et al.* 2009).

The cytokines interleukin-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) are soluble glycoproteins that bind cell surface receptors on myelopoeitic progenitor cells to stimulate their biological effects including cell survival and proliferation. Cancer cells usurp cytokine signalling pathways via various mechanisms to evade apoptosis and

deregulate proliferation, two fundamental hallmarks of the cancer phenotype (Hanahan and Weinberg, 2000). Hence there is much interest to cancer biologists in studying the nature and activation of cytokine receptor signalling both in AML and in AML cancer stem cells.

Cytokines bind to specific receptors on the cell membrane with high affinity, facilitating dimerization of signalling receptor subunits which then function to transduce a signal, intially through JAK2 and Src family tyrosine kinases that closely associate with the proximal portion of the receptor. More recently serine/threonine kinases have been demonstrated to also phosphorylate cytokine receptor but in conditions distinct from that observed with tyrosine phosphorylation (Guthridge *et al.* 2006; Miyakawa *et al.* 2000). The GM-CSF receptor is a heterodimer that comprises a major binding subunit (GMRα) and a common signalling subunit (βc). The structure of the GM-CSF receptor has recently been determined and demonstrates a hexameric complex consisting of two GM-CSF molecules, 2 GMRα chains and 2 βc chains (Hansen *et al.* 2008). Interestingly the structure demonstrated an intertwining βc dimer formation that has not been seen in other cytokine receptor families to date. This unique configuration appears to separate 2 cytoplasmic domains of βc and their associated JAK2 tyrosine kinase molecules more than 100 Å apart and may serve to closely regulate activation in the absence of GM-CSF binding (Hercus *et al.* 2009).

A domain in the proximal cytoplasmic portion of βc comprising Ser585 and Tyr577 has been shown to be necessary and sufficient for survival and proliferation signalling in primary haematopoietic cells (Guthridge *et al.* 2006; Guthridge *et al.* 2004; Guthridge *et al.* 2000). Constitutive rather than cytokine-regulated Ser585 phosphorylation has been demonstrated in blasts from 13 patients with acute myeloid leukaemia (Guthridge *et al.* 2006). This novel observation prompted further study into the nature of Ser585 signalling in AML presented in this thesis, searching for both upstream and downstream molecules that are active in this pathway and may be potentially druggable.

One such molecule commonly activated and mutated in cancer which is regulated by GM-CSF/IL-3 and linked to survival signalling is phosphoinositide 3-kinase (PI3K) (Cantley, 2002; Guthridge and Lopez, 2007; Zhao *et al.* 2006). PI3K is recruited to cytokine receptors through SH2-domain binding to phosphorylated tyrosine residues although other

mechanisms of recruitment are increasingly recognised. Localisation of PI3K in proximity to the plasma membrane leads to the phosphorylation of phosphatidyl inositol phosphates and the subsequent docking of pleckstrin homology domain proteins such as Akt to signal a proliferation and/or survival response (Manning and Cantley, 2007). In addition to this lipid kinase activity, PI3K has long been recognised to possess intrinsic protein kinase activity (Foukas and Shepherd, 2004a). The function and substrates of this protein kinase activity are not understood but evidence indicates it serves as a molecular switch to negatively regulate lipid kinase signalling (Foukas *et al.* 2004).

Downstream of PI3K, Bcl-2 family members such as Mcl-1 and Bcl-2 are regulated by the GM-CSF receptor to effect cell survival (Chao *et al.* 1998). AML cells over-express Mcl-1 (Kaufmann *et al.* 1998), a short-half life protein that is tightly regulated and associated with resistance to chemotherapy and some targeted therapies (Kaufmann *et al.* 1998; van Delft *et al.* 2006). The regulation of Mcl-1 expression and its relationship to PI3K signalling in response to cytokine is poorly characterised but both molecules remain lead targets for anti-leukaemic drugs.

The object of this thesis is to study the GM-CSF/IL-3 receptor signalling pathway in AML in order to reveal potential targets for therapy. Because βc Ser585 phosphorylation is deregulated in the majority of primary human AMLs and can promote deregulated survival of leukemic cells, I have sought to identify potential therapeutic avenues by which constitutive Ser585 signalling can be blocked with a view toward developing targeted therapies for the treatment of AML in the future. The work presented in this thesis identifies four new targets (one extracellular and three intracellular) with the potential to block Ser585 signalling in AML and uncouple deregulated survival programs in leukemic stem and progenitor cells. Key discoveries :-

(1) Firstly we demonstrate feasibility of LSC-targeted therapies by demonstrating efficacy *in vivo* and *in vitro* of a blocking monoclonal antibody 7G3 directed to the extracellular ligand-binding domain of the IL-3 receptor, CD123, on AML stem cells (Chapter 3).
(2) Secondly we show that in the majority of AML patients, Ser585 of the IL-3/GM-CSF receptor is constitutively phosphorylated and is required for cell survival. We

have shown that the *protein kinase* activity of phosphatidyl inositol 3-OH kinase (PI3K) is responsible for Ser585 phosphorylation and that inhibition of the protein kinase activity of PI3K results in the induction of apoptosis in AML cells (Chapter 4).

(3) We demonstrate that osteopontin (*OPN*) is a functionally relevant downstream target of the Ser585-survival survival pathway that mediates survival of $CD34^{+}CD38^{-}CD123^{+}$ leukemic stem/progenitors and that increased expression of *OPN* is associated with poor prognosis in normal karyotype AML (Chapter 5).

(4) Finally we demonstrate the effective dual targetting of both PI3K and CDK9 using PIK-75 kinase inhibitor in AML in vivo and in vitro (Chapter 6).

1.2 Acute Myeloid Leukaemia

1.2.1 Clinical Characteristics

Acute myeloid leukaemia (AML) is a haematological malignancy characterised by an accumulation of clonal immature myeloid cells (blasts) most often arising in the bone marrow that display deregulated proliferation, survival and maturation arrest. There is a concomitant suppression of the normal cellular elements of the marrow and so patients often present with symptoms and signs of bone marrow failure such as anaemia, thrombocytopenia and leukopaenia. Unlike most other cancers, a diagnosis of acute myeloid leukaemia is a medical emergency as patients can present with a high burden of disease (up to 10^{13} leukaemia cells) and may rapidly succumb to infection, life-threatening bleeding, thrombosis, hyperviscosity or tumour lysis syndrome if treatment is not begun urgently. The World Health Organisation currently defines acute myeloid leukaemia as the presence of greater than 20% blasts (precursor or progenitor cells) in the bone marrow or blood with some evidence of myeloid differentiation (cytoplasmic granulations, uptake of myeloid stains such as Sudan black or positive immune markers specific for the myeloid lineage) (Vardiman *et al.* 2009).

AML has an incidence of 2-3 per 100 000 per annum in children rising to 15 per 100 000 in older adults (Yamamoto and Goodman, 2008). The incidence in Australia has been relatively stable over the last 3 decades. Known risk factors for AML include previous

chemotherapy, radiation exposure and exposure to various chemicals such as benzene containing organics but in the majority of cases no direct cause can be found. Current treatment consists of induction therapy which consists of conventional chemotherapeutic drugs cytarabine (otherwise known as Ara C, an anti-metabolite) and an anthracycline (idarubicin or daunorubicin which work by intercalation with DNA). Remission is defined as a reduction in the number of leukaemic blasts to less than 5% of cells in bone marrow and currently 70-80% of patients routinely achieve remission although rates are better in younger adults (Bradstock *et al.* 2005) (Figure 1.1). Thereafter 3-4 consolidation cycles of chemotherapy, often consisting of high dose cytarabine, are given in order to remove slower cycling cells that may not have been targeted in the initial induction phase. At present, treatment for AML is unsatisfactory as most patients will eventually relapse despite encouraging initial complete remission rates (Tallman *et al.* 2005) (Figure 1.1). Relapsed leukaemia has been shown to be less sensitive to conventional chemotherapy and may harbour new mutations (such as loss of p53 tumour suppressor) and cytogenetic abnormalities as well as upregulation of drug efflux transporter systems such as P-glycoprotein (Wuchter *et al.* 2000). Overall 5 year survival rates for older adults remain less than 10%, for younger adults between 30-40% (Tallman, 2008). Some younger patients may be eligible for an allogeneic transplant if a suitable matched donor (sibling or unrelated) is available (Koreth *et al.* 2009) and there is good evidence for an immunological graft versus leukaemia affect in AML (Craddock, 2008).

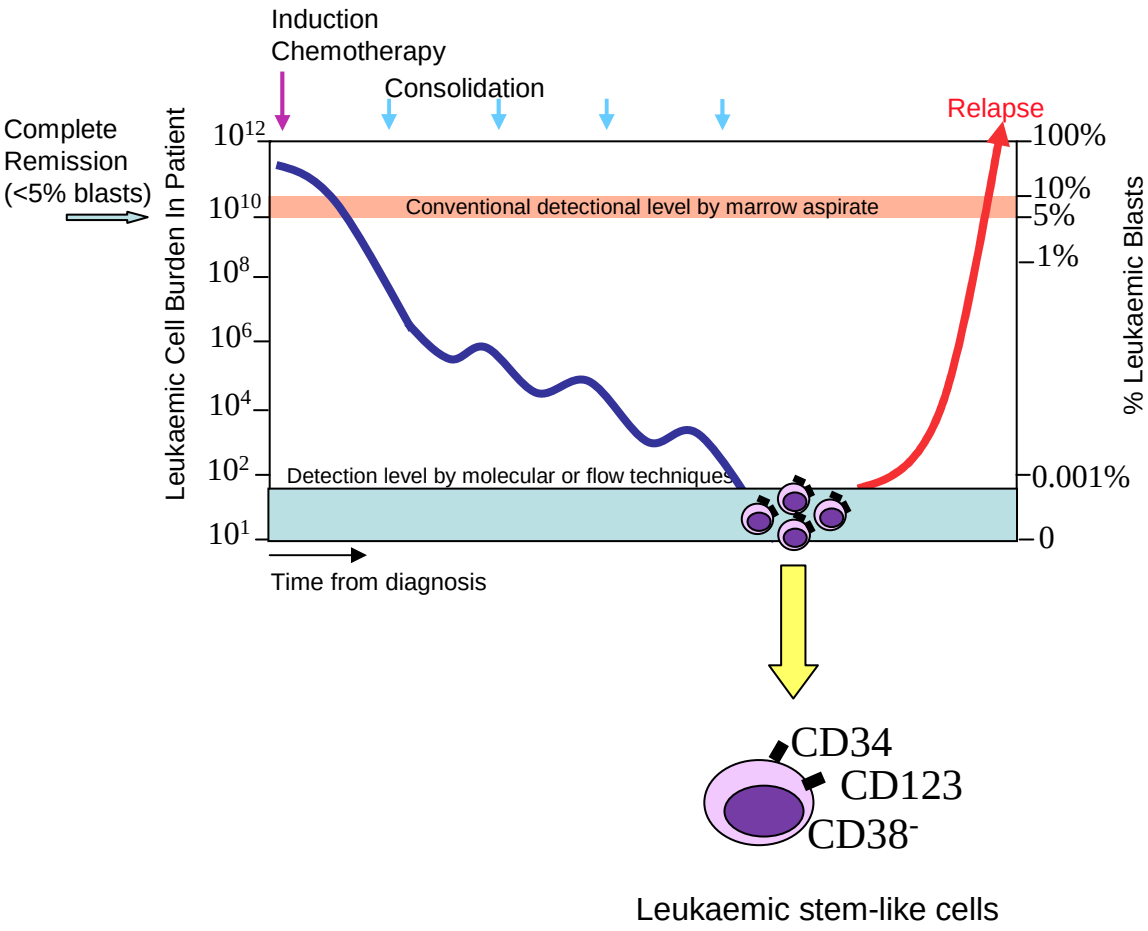

Figure 1.1: Relapse after complete remission in a typical AML patient. Patients present with a haematological emergency, often with more than 10^{11} blasts in their body in bone marrow, blood, spleen and sometimes extra-medullary infiltration. Conventional treatment consists of induction therapy with cytarabine, anthracycline +/- etoposide and approximately 80% of patients under 60 enter complete remission. Complete remission is crudely defined as less than 5% of leukaemic blasts in bone marrow by microscopy but is a gross under-representation of the total leukaemic load detected by more sensitive techniques such as real time PCR or flow cytometry. Most patients are treated with 3-5 cycles of consolidation therapy but this does not prevent relapse in the majority. The high frequency of disease relapse is attributed to the existence of long-lived leukaemic stem cells that can repopulate an entire tumour in NOD-SCID mice, are slowly dividing and show resistance to chemotherapy. These cells are enriched in a sub-population that express CD34, CD123 and are CD38 low to absent (Bonnet and Dick, 1997).

1.2.2 AML Classification and Prognosis

AML can be classified according to the cytogenetic lesions detected in the initial blast population at diagnosis (Figure 1.2). Certain cytogenetic abnormalities confer a favourable prognosis including core binding factor AML inv16 or t(8;21) and acute promyelocytic leukaemia t(15;17) while other abnormalities including the presence of complex chromosomal breaks, monosomy 7, monosomy 5 exert a very poor prognosis on outcome (Mrozek *et al.* 2001). Normal karyotype AML exerts an intermediate prognosis but overall survival rates are still poor (Figure 1.3). Microarray profiling of 285 cases of AML has revealed new subgroups including some with normal karyotypes that have clinically significant associations with long term outcome (Valk *et al.* 2004). This underscores the notion that (perhaps more than other cancer types) AML is a heterogeneous disease in terms of both biology and underlying genetic lesions. The development of targeted therapy for AML, unlike that for CML which is a single oncogene disease, is challenging. Such therapies will either need to be tailored for the specific molecular abnormalities proven to be present in a given patient's leukaemia (such as using Flt3 tyrosine kinase inhibitors in cases of Flt3-ITD mutation or all-trans-retinoic acid in t(15;17) AML) or alternatively, biological targets need to be discovered that are common to all types of AML regardless of molecular lesions. The over-expression of CD123 in AML compared to normal $CD34^{+}CD38^{-}$ haematopoietic stem cells is a potential example of the latter but even this is not universal(Jordan *et al.* 2000).

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1.2.3 Cytokine Receptor Mutations in Acute Myeloid Leukaemia

A number of growth factor receptors (Flt3, c-kit and c-fms) that normally exert a proliferative and survival function in haematopoiesis have been found to be mutated in AML. Furthermore, signalling proteins downstream of cytokine receptors including N-ras (Needleman *et al.* 1986), Jak2 (Kralovics *et al.* 2005a; Kralovics *et al.* 2005b), Jak3 (Walters *et al.* 2006), SHP2 (Mohi *et al.* 2005) are occasionally mutated and constitutively active in leukaemia. No oncogenic mutation has ever been confirmed in the IL-3/GM-CSF/IL-5 receptor in humans (Freeburn *et al.* 1996) however a number of constitutively active mutant forms of β_c have been described in cell culture that render haematopoietic cells factor-independent and give rise to a pre-leukaemic

myeloproliferative disorder when reconstituted in mouse bone marrow (Jenkins *et al.* 1995; McCormack and Gonda, 1997).

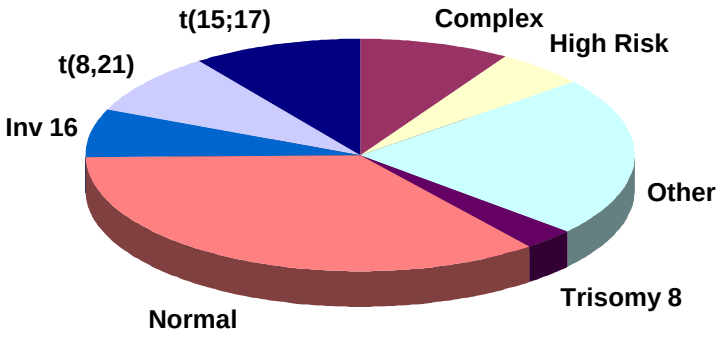
The most common mutations found in AML occur in the Flt3 receptor with an incidence of approximately 30% across all AML subtypes and over 70% in normal karyotype AML (Kottaridis *et al.* 2001). Flt3 mutations comprise either i) internal tandem duplications resulting in extra-chain length at the juxta-membranous region, or ii) point mutations in the kinase domain or iii) mutations in the extracellular domain (Small, 2006). Both ITD and tyrosine kinase mutations have been shown to act as "driver mutations" in mouse models and have been demonstrated to confer factor-independent growth in growth factor-dependent cell lines and constitutive tyrosine kinase activation (Frohling *et al.* 2007) . Occasionally rare point mutations are found that do not appear to cause factor independence and these are termed "passenger" mutations (Frohling *et al.* 2007). Many cases of AML without FLT3 driver mutations display over-expression of Flt3 and secrete Flt3 ligand creating an autocrine loop that may contribute to leukemogenesis (Carow *et al.* 1996; Small, 2006; Zheng *et al.* 2004).

Less commonly c-kit, also a tyrosine kinase receptor also expressed on haematopoietic stem cells, is found to be mutated in AML (Cairoli *et al.* 2006). A number of point mutations including D816 mutations in the second intracellular kinase domain and internal tandem duplication mutations in the juxtamembrane domain have been discovered, predominantly in core-binding factor AML series, and the presence of the mutations appear to activate tyrosine kinase activity and affect patient outcome (Beghini *et al.* 2004; Cairoli *et al.* 2006). In addition, n mutations in c-fms have been found in some cases of AML and chronic myelomonocytic leukaemia (CMML) but the true prevalence and significance of these mutations is unclear (Ridge *et al.* 1990).

Surprisingly, high throughput sequencing of the tyrosine kinase domains of 85 tyrosine kinase genes in 188 patient samples of AML without c-kit or FLT3 mutations has failed to reveal any major new tyrosine kinase mutations (Loriaux *et al.* 2008). This suggests that AML, may not always be driven by any single oncogenic tyrosine kinase and this finding is consistent with growth factor signalling abnormalities that we have observed in patient

samples involving constitutive activation of serine/threonine kinase signalling pathways(Guthridge *et al.* 2006; Mohi *et al.* 2004).

A Cytogenetic Abnormalities in Adults with AML age < 60



B Cytogenetic Abnormalities in Adults with AML age > 60

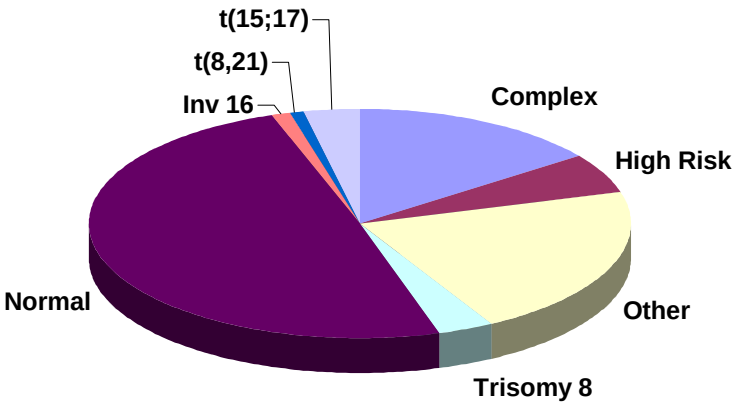


Figure 1.2: Proportions of cytogenetic subgroups in AML. Patients (A) under 60-yrs and (B) over 60 yrs. Note the large proportion of cytogenetically normal AML in patients over 60 (~50%) who poorly tolerate standard chemotherapy and for which targetted therapies are needed.

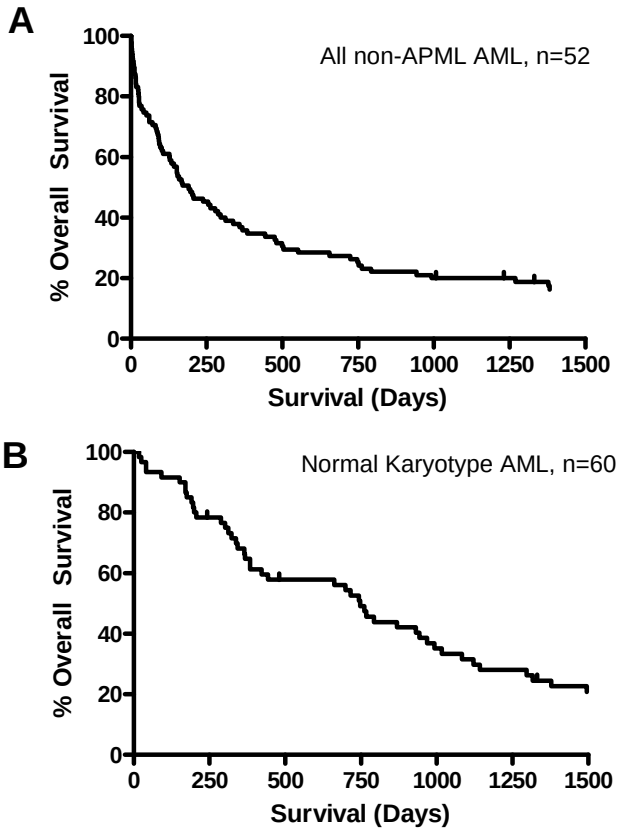


Figure 1.3: Overall survival of AML patients treated with induction chemotherapy at the Royal Adelaide Hospital. (A) Kaplan-Meier Log Rank analysis of all AML patients who underwent standard induction chemotherapy and for which RNA was available for analysis at the Royal Adelaide Hospital from 1998 to 2008, excluding APML(M3) (n=52). Median survival 191 days with no plateau at 5 years, less than 30% alive at 5 years indicative of ongoing relapse in the majority of patients. Similar survival curves are seen in other treatment centres worldwide. (B) Kaplan-Meier survival curve for normal cytogenetic AML patients after induction chemotherapy treated at either Royal Adelaide Hospital 1998-2008 or Kunamoto University School of Medicine, Japan 1987-2003. Patients with acute promyelocytic leukaemia are excluded from analysis. Again no plateau is observed at 5 years, median survival 748 days. This data was generated by the author to analyse the effect of Osteopontin expression on patient outcome and the data for each patient is available in the Chapter 5, Supplementary material, Table S1.

1.2.4 Evidence for Leukaemia Stem Cells

A major breakthrough in our understanding of leukaemia (and cancer in general) occurred in 1997 with the publication of the first in vivo evidence for cancer stem cells (Bonnet and Dick, 1997). The leukaemia stem cell model was originally put forward in an effort to explain intra-tumour heterogeneity observed within most patient samples. Based on the current understanding of haematopoiesis, it was posited that in a similar fashion, each AML population consists of a hierarchy of cell populations that differ in their self-renewal capacity and their ability to reconstitute a *bonafide* leukaemia in an engrafted animal. To varying degrees, this model has proven invaluable in identifying certain sub-populations within a given AML that possess distinct properties from the bulk of the leukaemic blasts. In a number of patient samples, enrichment for the ability to engraft NOD-SCID mice was consistently found to be present in the population that expressed CD34+CD38- immune markers, a similar immunophenotype to that present on normal haematopoietic stem cells (Bonnet and Dick, 1997). These cells were designated "leukaemic initiating cells" and by virtue of their ability to reconstitute AML in serially engrafted immunocompromised mice. were partially overlapping but not identical with cells present in *in vitro* AML colony forming assays. Clonal tracking of these cells by lentiviral integration site patterns revealed vast heterogeneity in the ability of AML cells to give rise to a leukaemia in mice following serial transplantation with some cell populations giving rise to a leukaemic population only in the first (primary) transplant; while others were able to reconstitute AML not only in the primary transplant, but also the secondary and tertiary transplants (Hope *et al.* 2004). What was remarkable about the early studies of Bonnet and Dick was that leukemic initiating cells were extremely rare constituting <1% of the bulk AML. Other publications have revealed similar frequencies of leukaemia stem cells although the actual frequency appears to depend on the particular tumour model being studied and the heterogeneity present within each tumour (Guo *et al.* 2008; Yilmaz *et al.* 2006). Murine Eμ–myc lymphomas for instance display no real heterogeneity and show near 100% engraftment and hence a "leukaemia stem cell" sub-population cannot be defined in this tumour model (Kelly *et al.* 2007).

The expression levels of various other antigens on these CD34+CD38- AML stem cells have been studied to look for unique markers that may define the leukaemic stem cell as

opposed to normal haematopoietic stem cells. Interestingly the IL-3 receptor α subunit (CD123) is over-expressed almost exclusively on leukaemia stem cells compared to their normal non-transformed CD34+CD38- counterpart (Jordan *et al.* 2000). CD123 is overexpressed frequently on bulk AML populations (Florian *et al.* 2006; Graf *et al.* 2004) and the degree of expression has been correlated with long term prognosis (Riccioni *et al.* 2004; Testa *et al.* 2002). Thus, CD123 may represent a unique marker for leukemic stem cells and consequently a useful target for monoclonal antibody therapy in AML as CD123 over-expression is a consistent finding regardless of underlying cytogenetic abnormalities present (Jordan *et al.* 2000; Testa *et al.* 2002). The laboratory of Angel Lopez and Mark Guthridge has developed a monoclonal antibody, 7G3 to CD123 that binds with high affinity and prevents IL-3 induced receptor activation(Sun *et al.* 1996) and data regarding its potential role in AML LSC targeting is present in Chapter 3.

Leukaemic stem cells also have been reported to express a splice variant of CD44, CD44v, a receptor for hyaluronan and osteopontin, that is upregulated compared to normal haematopoietic stem cells. An activating monoclonal antibody to CD44, H90, can induce differentiation of primary AML blasts and can block leukaemia engraftment and leukaemia stem cell homing in murine xenotransplantation models of human AML (Jin *et al.* 2006).

Other work has shown that leukaemia stem cells exhibit several properties that are present in normal stem cells including i) robust survival (Hope *et al.* 2004), ii) self-renewal (Bonnet and Dick, 1997), iii) slow proliferation rates and quiescence (Hope *et al.* 2004), iv) increased resistance to chemotherapy(Ishikawa *et al.* 2007; Nervi *et al.* 2009; Zeng *et al.* 2009) and v) binding to specialised niche environments in the bone marrow stroma (Ishikawa *et al.* 2007; Jin *et al.* 2006). These studies underscore the importance of studying survival /quiescence signalling pathways in leukaemic stem cells in order to develop novel targets to interfere with leukaemic stem cell survival, proliferation and self-renewal. Overcoming apoptosis has long been recognised as a critical hallmark of cancer (Figure 1.4) and the data presented is mainly focussed on elucidating and interfering with the survival pathways active in AML.

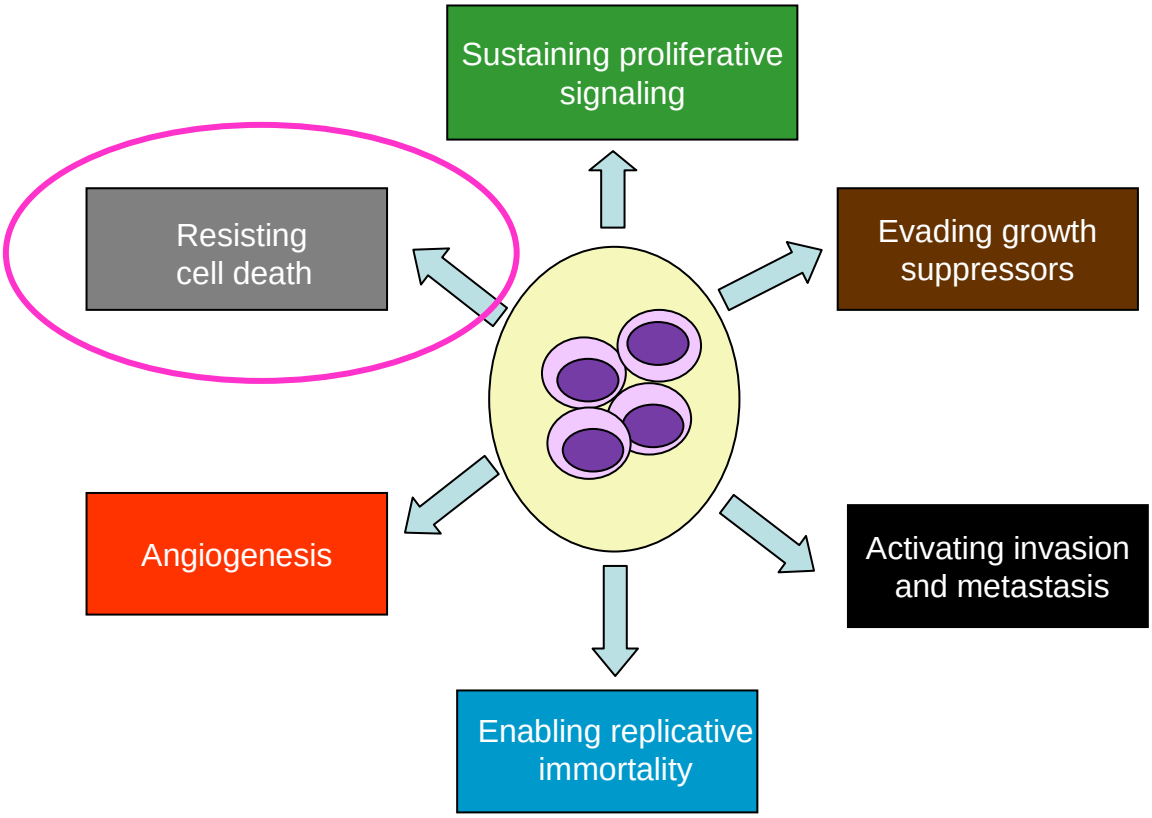


Figure 1.4: Model showing the key determinants of tumourigenesis as outlined by Weinberg and Hanahan in their original Cell review (Weinberg and Hanahan, Cell, 2000, revised 2011). Cell survival or the resistance of cell death (circled) is a critical property of the leukaemia phenotype and a critical determinant of the longevity and resistance to chemotherapy apparent in leukaemic stem cells. The work in this thesis is principally aimed at the problem of overcoming cell survival in AML.

1.2.5 Emerging Therapies for AML

For more than 3 decades, outcomes have not changed significantly for patients with AML compared to the progress seen in multiple myeloma or chronic myeloid leukaemia(Tallman *et al.* 2005). However, the development of monoclonal antibody technology and discovery of molecular lesions in AML that are theoretically druggable in patients has renewed hope (Burnett and Knapper, 2007) (Table 1). More recently, the discovery of leukaemia stem cells has stimulated a new repertoire of targets, some of which are explored in this thesis, and offer complementary approaches to anti-metabolite chemotherapy aimed at rapidly dividing blasts (Majeti, 2011).

There is no one specific marker for leukaemia that is not also present on normal haematopoiesis but the majority of AMLs express CD33, a myeloid progenitor marker. Targeted therapy directed against CD33 in the form of a humanized monoclonal antibody I195 has been demonstrated to convert acute promyelocytic leukaemia patients who were in haematologic but not molecular remission into molecular remission (Jurcic *et al.* 2000)but when added to chemotherapy showed no clear benefit (Feldman *et al.* 2005). Currently, an immunoconjugate of anti-CD33 IgG_4 humanized antibody combined with calicheamicin chemotherapy, gemtuzumab ozogamicin is under phase III clinical trial (Burnett and Knapper, 2007). A study of GO monotherapy in older patients with AML in first relapse showed an improves overall remission rate of 28% with 13% complete response (Sievers *et al.* 2001). However severe liver toxicity including the increased occurrence of veno-occlusive disease has hampered clinical use (Larson *et al.* 2005). It would appear that CD33 is not expressed at high levels on CD34+CD38- leukaemia stem cells (Taussig *et al.* 2005) and the activity of anti-CD33 mAb against LSCs remains unknown but appears limited.

The frequent occurrence of FLT3-ITD mutations in AML that are associated with a moderately poor prognosis (Kottaridis *et al.* 2001) and can drive factor-independent growth in vitro (Brandts *et al.* 2005) has prompted investigation into the clinical efficacy of FLT3 tyrosine kinase inhibitors. There are now over 13 in development with two, CEP-701 (lestaurtinib) and PKC-412, in phase III clinical trials. Both of these are indolocarbazoles which are derivatives of staurosporine and are not entirely selective for

the FLT3 kinase (Burnett and Knapper, 2007). Thus far monotherapy trials have been disappointing with partial transient responses probably limited by pharmacokinetic factors (Knapper *et al.* 2006; Smith *et al.* 2004; Stone *et al.* 2005). For example, CEP-701 is extensively protein bound in humans and only patients for which plasma could inhibit FLT3-ITD positive cell lines correlated with a clinical response (Levis *et al.* 2006).

In theory, AML patients harbouring c-kit mutations, as occurs in 30% of inv16 patients (Paschka *et al.* 2006), may benefit from tyrosine kinase inhibitors directed against c-kit. Azacitidine, a demethylating and cytosine analog, has been useful in prolonging disease free survival in elderly patients with myelodysplasia or low blast count AML but is unlikely to impact on the bulk of de novo AML cases (Fenaux *et al.* 2010).

In conclusion, new approaches to AML treatment are desperately needed. New insights into the mechanisms of self-renewal and survival of AML stem cells and a search for targets differentially expressed on critical sub-populations is likely to be more fruitful in the years to come.

1.3 Haematopoiesis

In order to properly study the dysregulation of survival, proliferation and differentiation observed in leukaemic states, one must also have a deep understanding of normal blood homeostasis. Haematopoiesis refers to the formation of all the various cell types present in the bone marrow and peripheral blood. This process involves an extremely high cell turnover (> 10^{13} cells produced per day) in order to maintain steady-state blood counts as the half-life of the majority these cells is relatively short (neutrophils 6 hours, platelets five days, red cells 3 months). A decrease in the number of mature blood cells may result in disease such as infection, uncontrolled bleeding or impaired oxygen delivery. All blood cells are ultimately derived from haematopoietic stem cells. These haemopoietic stem cells are relatively rare (one in 20 million bone marrow cells) and have the ability to i) self-renew and ii) regenerate all lineages of haematopoiesis. Haematopoiesis is regulated by soluble factors, called cytokines, that were discovered when bone marrow cells were grown in a semisolid matrix containing medium that had been conditioned by various cell

lines resulting in colonies of granulocytes and macrophages (Nicola, 1994a). Identification of the active factors present in the culture medium led to the discovery of cytokines such as granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor (Lopez *et al.* 1983).

In addition to hematopoietic cells, the bone marrow also comprises non-haematopoietic cells including fibroblasts, osteoclasts and endothelial cells which are responsible for providing the necessary bone marrow microenvironment permissive for haematopoiesis. The bone marrow microenvironment is important not just as a structural platform for blood cell production but also provides active signals to nurture and regulate haematopoiesis. Examples of cytokines involved in physiological stromal–stem cell interactions include stem cell factor, osteopontin, certain chemokines and Ang-1. Cleavage of stem cell factor by matrix metalloproteases in response to G-CSF to allow migration of stem cells into the peripheral circulation (Heissig *et al.* 2002; Kollet *et al.* 2006). Osteopontin is a heavily glycosylated and phosphorylated soluble factor present in the endosteal surface of bone marrow. Recent studies have highlighted the importance of osteopontin as a niche stem cell regulator with anti-proliferative effects (Haylock and Nilsson, 2006; Nilsson *et al.* 2005). Stromal cell-derived factor (SDF-1) is a chemokine that causes stem cell migration across the endothelial cell barrier to the extravascular space in bone marrow where stem cells engage with a number of cell adhesion molecules that allow cell-matrix and cell-cell interaction (Dar *et al.* 2006). Ang-1 signalling from osteoblasts through Tie2 appears to have a niche role in keeping haematopoietic stem cell in the quiescent state (Arai *et al.* 2004). The extent to which these or similar factors also regulate AML stem cells remains to be determined but is an area of ongoing research (Lane *et al.* 2009).

1.3.1 Stochastic versus Instructive Models of Haematopoiesis

Haematopoietic cells can be grouped into a hierarchical system of classification according to their ability to self-renew (pluripotent stem cells), divide and differentiate. Pluripotent stem cells, termed haematopoietic stem cells (HSCs) lie at the top of this hierarchy and are characterised by their ability to self-renew and give to rise all the cellular elements of the blood in bone marrow ablated animals. Progenitor cells the immediate progeny of stem

cells and represent a transient population characterised by intense proliferation and a more restricted repertoire of differentiation fates.

There has been some debate regarding the mechanisms of stem cell renewal versus stem cell differentiation and the role that cytokines play in regulating this process. There is much evidence for a *stochastic* model of differentiation, which states that a given stem cell will differentiate down a particular cell fate according to an essentially random process and that cytokines exert merely a supportive role for cells already committed to a particular lineage(Ogawa, 1999). In support of this model paired progenitor cells obtained from mice treated with 5-fluorouracil show a range of lineage potentials and combinations of cell fate even when cultured in identical conditions. Other studies have shown that the pro-survival gene bcl-2 can rescue the monocyte/osteoclast defect in M-CSF receptor deficient mice giving rise to fully functioning monocytes suggesting that the M-CSF receptor itself was playing no role in deciding the fate of these cells; rather its survival function could be rescued by another pro-survival pathway (Lagasse and Weissman, 1997). Furthermore chimeric cytokine receptor studies have shown that a number of receptors can substitute for the Epo receptor to produced erythrocyte differentiation if they are expressed in erythroid progenitor cells (Goldsmith *et al.* 1998; Socolovsky *et al.* 1998a; Socolovsky *et al.* 1998b).

In contrast to this model, the instructive model of haematopoiesis draws on evidence that cytokines can indeed alter the differentiation fate of some progenitor or stem cells. GM-CSF can induce differentiation of FDCP1 cells to mature macrophages and neutrophils (Just *et al.* 1991) and ectopic expression of the Epo receptor can induce erythroid specific genes such as glycophorin A and beta globin (Chiba *et al.* 1993; Liboi *et al.* 1993). Certain portions of cytokine receptor cytoplasmic domains have been linked to such differentiation-type events but no one signalling molecule has ever been linked specifically to an instructive signal from a cytokine receptor. To date most cytokine receptors utilise a common pool of signal transduction sub-units such as Ras, Raf, PI3K, STAT, Shc, Grb2 suggesting that any differences between cytokine receptors may be secondary to quantitative rather than qualitative differences in the signalling machinery utilised.

An interesting report shows evidence for an in vivo instructional role for the GM-CSF receptor. By using a common lymphoid progenitor than normally differentiates down the lymphoid lineage, exogenous expression of the GM-CSF receptor or the IL-2 receptor resulresult in a switch to the myeloid lineage (Kondo *et al.* 2000). Furthermore the granulocyte and macrophage specifying activity appeared to originate from different portions of the same receptor cytoplasmic tail suggesting that the instructional role was connected with specific differentiation signals. The switch from lymphoid to myeloid lineage could only occur in a very early pre-B cell (Kondo *et al.* 2000). In a more mature B cell the instructional role of GM-CSF was lost. Indeed, it may be that both stochastic and instructive models of haematopoiesis apply to varying degrees at different junctions of the haematopoietic tree. The plasticity between lymphoid and myeloid lineage fate is also reflected by the infrequent occurrence of undifferentiated and biphenotypic leukaemias bearing both lymphoid and myeloid markers on the same blast cell. Recent evidence using human stem cells expressing a mixed-lineage leukaemia (MLL) fusion gene show that leukaemia initiating cells in this model retain both myeloid and lymphoid lineage potential and remained responsive to microenvironmental cytokines (Barabe *et al.* 2007).

1.4 Cytokines

1.4.1 Structural Classification of Cytokines

Cytokines are small soluble proteins ranging from 10 to 50 kDa that can exist as either monomers (e.g. GM-CSF) or dimers (IL-5, growth hormone). The structures of a number of cytokines has been determined including interleukin-8 (a chemokine), GM-CSF and growth hormone (Nicola, 1994b). They are divided into four major groups according to X-ray crystallography and predicted structure based on amino acid sequence similarity. Group 1 cytokines contain four alpha-helical bundles and include GM-CSF, G-CSF, erythropoietin, thrombopoietin and many of the interleukins. Group 2 cytokines consist of a long chain beta-sheet and include tumour necrosis factor (TNF) alpha and beta, CD40, Fas ligand and members of the fibroblast growth factor (FGF) family of cytokines. Group 3 cytokines consist of short chain alpha/beta structures and include epidermal growth factor (EGF), insulin, and insulin-like growth factor (IGF-1). Group 4 cytokines have

mosaic structures consisting of combinations of structures in groups 1 – 3 including the neuregulins and hepatocyte growth factor (Nicola, 1994b).

1.4.2 Biological Activities of Group 1 Cytokines

Group 1 cytokines (otherwise known as haematopoietic growth factors) can stimulate four major biological responses when they bind to cell surface receptors:- proliferation, differentiation, survival and functional activation. Not all cells will elicit the same four responses when treated with cytokine and these differences may depend on the lineage and maturational stage of the cell as well as the strength of stimulus. For instance, when a neutrophil is stimulated with GM-CSF an enhancement of survival and functional activation is observed (Lopez *et al.* 1983) but there is no proliferation or differentiation because neutrophils are already terminally differentiated and do not divide.

1.4.3 Proliferation

Cytokines stimulate the proliferation of progenitor cells, moving them from G_0 in the cell cycle into G_1 (growth phase) followed by DNA synthesis (S phase) and then through to complete mitosis (M). The proliferative response can be measured by number of assays (thymidine incorporation, BrdU incorporation, CFSE staining (Lyons and Parish, 1994)) and has shown to be due to a real increase in the number of cell divisions occurring rather than simply prolonged survival on an apoptotic background. The concentration of cytokine required to stimulate proliferation differs between cell lineage and differentiation stage: macrophage progenitors are more sensitive than erythroid progenitors and granulocyte progenitors to proliferate in response to varying concentrations of murine GM-CSF (Metcalf *et al.* 1980).

Synergy is also observed when varying combinations of cytokines are utilised to stimulate colony formation and proliferation. Synergy between GM-CSF and stem cell factor (SCF) for example has been demonstrated (Baker *et al.* 1994) or thrombopoietin, stem cell factor and IL-11 in megakaryocyte colony formation (Broudy *et al.* 1995). Other combinations

of cytokines such as M-CSF and GM-CSF appear to antagonise each other but the mechanism is not clear (Metcalf *et al.* 1992).

1.4.4 Survival

Survival is a critical biological response induced by haematopoieic cytokines. Studies with Bcl-2 family members knockout mice and myelodysplastic syndrome in which there is increased cellularity in the bone marrow but increased apoptosis (Dzhagalov *et al.* 2007; Raza *et al.* 1995) underscore the crucial role of survival signalling in the regulation of haematopoiesis. Growth factor induced survival is virtually synonymous with the prevention of apoptosis via the intrinsic pathway and is associated with a number of metabolic and signalling events that are distinct but often required for sustained proliferation. A number of cell-lines are dependent on growth factors for maintaining viability and withdrawal of factor from the medium leads to cell death by apoptosis (Williams *et al.* 1990). Very low concentrations of cytokine (in the femptomolar range) have been shown to enhance the survival of mature granulocytes and eosinophils in the absence of detectable cell proliferation(Begley *et al.* 1986). On the other hand, higher concentrations of cytokine are able to promote both cell survival as well as cell proliferation(Burgess and Metcalf, 1980; Guthridge *et al.* 2006). In some systems, a cytokine at high concentrations can exert a survival function in the absence of proliferation (e.g. thrombopoietin when given to erythroid progenitors)(Kaushansky *et al.* 1995).

1.4.5 Differentiation

Differentiation refers to the process by which a cell becomes more restricted in developmental potential. Differentiation of stem cells results in the production of cells which have a reduced capacity for self-renewal and are more restricted in ultimate cell fate decisions. In more mature cells that are already committed to a particular lineage, differentiation (or maturation induction) is associated with morphological changes, changes in the expression of cell surface markers and increased expression of lineage specific genes such as GATA-1(Yu *et al.* 2002) (erythroid) and Pu.1 (myeloid) (DeKoter *et al.* 1998).

Differentiation appears to be tightly coupled to proliferation. A number of experiments have demonstrated that differentiation commitment is irreversible and restricted to cells that are in cycle. Studies with oncogenes such as Ras have observed transformation and unrestricted proliferation in some cell types while in others (such as PC12 cells) differentiation responses have occurred (Bar-Sagi and Feramisco, 1985).

1.4.6 Functional Activation

Group 1 cytokines can stimulate a variety of cellular functions in mature haemopoietic and and even some non-haemopoietic cells. Relevant examples include G-CSF induced increase in neutrophil mobility, priming of macrophages to lipopolysaccharide by GM-CSF and chemotaxis of eosinophils by interleukin-5 (Nicola, 1994a).

1.4.7 Biological Properties of GM-CSF

GM-CSF was first identified as an activity present in mouse lung conditioned medium that could stimulate the production of granulocyte and macrophage colonies in soft agar from bone marrow cells (Sheridan and Metcalf, 1973). Human GM-CSF was identified from placenta-conditioned media in 1979 subsequently purified (Burgess *et al.* 1977; Nicola *et al.* 1979) and lead to the determination of amino-acid sequence, cloning and production of recombinant protein (Cantrell *et al.* 1985; Gasson *et al.* 1984; Miyatake *et al.* 1985; Sieff *et al.* 1985; Wong *et al.* 1985a; Wong *et al.* 1985b).

In terms of haematopoiesis, the major physiological role of GM-CSF appears to be in the induction of emergency myelopoiesis in response to inflammatory stimuli such as bacterial infection or major tissue injury. GM-CSF deficient mice fail to illicit appropriate increases in granulocytes, monocytes and eosinophils when challenged with various microbial pathogens but this defect is marginal in some contexts(Scott *et al.* 2000) suggesting a degree of redundancy via other inflammatory cytokines that can stimulate leucocytosis (Nishinakamura *et al.* 1995; Nishinakamura *et al.* 1996a).

Otherwise in steady-state haematopoiesis GM-CSF appears to be redundant and myelopoiesis can be maintained by other factors, most likely M-CSF and G-CSF (Dranoff *et al.* 1994; Dranoff and Mulligan, 1994). However, GM-CSF is required for steady-state

pulmonary homeostasis in which it plays a critical role in lung macrophage differentiation and function (Dranoff *et al.* 1994). Mice deficient in GM-CSF suffer lung pathology resembling the human disease pulmonary alveolar proteinosis in which quantities of pulmonary surfactant (phospholipid) and are not efficiently phagocytosed and recycled. This defect is haematopoietic in origin and can be rescued by bone marrow transplantation (Nishinakamura *et al.* 1996b) and depends on a particular form of alveolar macrophage that requires GM-CSF for production via the induction of PU.1 myeloid specific transcription factor (Shibata *et al.* 2001). Patients with pulmonary alveolar proteinosis have been discovered to have neutralising antibodies to the GM-CSF receptor or missing components of the GM-CSF receptor (Dirksen *et al.* 1997; Seymour *et al.* 1998).

GM-CSF can stimulate the *in vitro* proliferation, differentiation, survival and functional activation of cells in the myeloid lineage (Metcalf, 1994) and at high concentrations stimulate the proliferation of erythroid and megakaryocyte progenitor. GM-CSF has a number of other properties related to immune function (Monroy *et al.* 1990). It is produced by T cells, macrophages, endothelial cells, bone marrow stromal cells, fibroblasts, basophils and mast cells at sites of inflammation. At sub-nanomolar concentrations it can enhance microbicidal activity of neutrophils and macrophages, increase oxidative metabolism and phagocytic activity including antibody-dependent cell-mediated cytotoxicity, increase expression of adhesion molecules and other cytokines such as IL-1, G-CSF and M-CSF and prime macrophages to activation by lipopolysaccharide(Metcalf, 1994).

A subset of myeloid dendritic cells can be produced by GM-CSF in vitro but GM-CSF is not exclusively involved in this process (Vremec *et al.* 1997). GM-CSF also inhibits mast cell differentiation (Welker *et al.* 2001) and inhibits lymphoid development from CD34+ haematopoietic stem cells (Vremec *et al.* 1997) .

1.4.8 Role of GM-CSF in human disease

GM-CSF is involved in the pathology of a number of inflammatory and allergic diseases and is implicated in the biology of various haematological malignancies. GM-CSF mRNA and protein has been detected in the fluid of affected joints from arthritis patients

(Williamson *et al.* 1988) and around orthopaedic implants (Al-Saffar *et al.* 1996). Administration of GM-CSF worsens arthritis in several mouse models (Campbell *et al.* 1997)and GM-CSF deficient mice are protected from developing joint inflammation (Campbell *et al.* 1998). Other autoimmune diseases including glomerulonephritis and multiple sclerosis have been shown to have some degree of GM-CSF dependence (Kitching *et al.* 2002; McQualter *et al.* 2001).

Asthmatic patients have increased levels of mRNA to GM-CSF and IL-5 in lung tissue (Broide *et al.* 1992; Broide and Firestein, 1991) and greatly increased numbers of eosinophils during acute exacerbations (Egan *et al.* 1996). Given the unique role of GM-CSF, IL-3 and IL-5 in promoting eosinophil production (Nishinakamura *et al.* 1996a) it has been proposed that targeting these cytokines in particular may benefit long term steroid dependent asthma patients (Ramshaw *et al.* 2001).

Several haematological malignancies utilise GM-CSF as an autocrine survival factor. Juvenile myelomonocytic leukaemia, a rare form of myeloproliferative/myelodysplastic syndrome in children, exhibits cells that are dependent on GM-CSF for survival (Iversen *et al.* 1996). Similarly chronic myelomonocytic leukaemia in adults has shown to have some dependency on GM-CSF in xenograft studies with a GM-CSF antagonist (Ramshaw *et al.* 2002).

Acute myeloid leukaemia blast populations express receptors for GM-CSF, IL-3 and IL-5 to varying degrees (Budel *et al.* 1990). Expression of the receptor does not always correlate with autocrine cytokine production or biological response such as proliferation when blast cells are stimulated with cytokine, consistent with the heterogeneity often observed in AML subtypes and the range of genetic abnormalities (Piao *et al.* 1989).

1.5 GM-CSF Receptor Signalling

1.5.1 Structure of GM-CSF Receptor

The receptors for GM-CSF, interleukin-3 and interleukin-5 consist of an α subunit which is unique for each cytokine and a common beta subunit β_c (Gearing *et al.* 1989; Hayashida

et al. 1990; Kitamura *et al.* 1991; Tavernier *et al.* 1991). The α subunit is the major ligand binding subunit (K_d ~ $5x10^{-9}$M) but on its own cannot transduce a signal, at least in haematopoietic cells (Muto et al., 1995; Robb et al., 1995) (Figure 1.5). The β_c converts a ligand bound α chain to a high affinity receptor (K_d ~ $5x10^{-11}$M) and is responsible for the majority if not all signal transduction (Bagley *et al.* 1997; Hayashida *et al.* 1990; Kitamura *et al.* 1991).

Many of the members of the haemopoietin receptor family have been shown to share receptor subunits. For example gp130 is the common subunit for the IL-6, CNTF, cardiotrophin, oncostatin-M, LIF and IL-11 receptors and IL-2γ is shared by the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15 (Nicola, 1994a).

In mice there are two forms of the beta subunit, β_c and another IL-3 specific analogous beta sub-unit designated β_{IL-3} (Hara and Miyajima, 1992; Metcalf, 1994). In terms of cross-reactivity, human GM-CSF cannot bind to murine GM-CSF receptor but crosstalk between human and mouse GM-CSF α chains has been demonstrated (Metcalf *et al.* 1990).

The α and β subunits are both members of the class I cytokine receptor superfamily as defined by sequence comparisons and structural homology (Bazan, 1990) possessing four conserved cysteines in the first fibronectin Type III-like repeat and the characteristic WSXWS motif in the second repeat. Mutagenesis studies in both growth hormone and Epo receptors suggest the WSXWS motif is required for proper folding of the extracellular structure of the receptor (Baumann *et al.* 1996; Baumgartner *et al.* 1994; Hilton *et al.* 1996).

The crystal structure of the GM-CSF receptor has now been determined(Hansen *et al.* 2008) and reveals a hexameric complex consisting of 2 GM-CSF molecules, 2 GMRα chains and 2 βc chains. The crystal structure confirms the intertwining βc dimer structure observed previously in the isolated βc molecule (Rossjohn *et al.* 2000). This is an unusual configuration and serves to keep the 2 βc cytoplasmic domains more than 100 Å apart.

Such a degree of separation is likely to prevent unbound transphosphorylation and activation of the receptor (Hansen *et al.* 2008).

The hexameric complex reveals 3 sites of interaction similar to the structure of the growth hormone:growth hormone receptor complex. The solution of the growth hormone receptor crystal structure revealed one growth hormone molecule binding two identical growth hormone receptor subunits, providing compelling evidence for ligand-induced dimerization as a mechanism for receptor activation (De Vos *et al.* 1992). This paradigm had already been established for the receptor tyrosine kinase family (Schlessinger *et al.* 2000) and indicated that binding of growth hormone to the receptor occurred in a sequential manner, first through site 1 on growth hormone binding one subunit and subsequently through site 2 binding the other receptor subunit. The sequential mechanism of binding is well supported by the difference in surface area between the two interfaces, the first interface being considerably larger (De Vos *et al.* 1992). A region of interaction between the two subunits that did not involved ligand was also apparent consisting of residues in the membrane proximal domains of each receptor chain and which is considered to contribute to stabilisation of entire complex upon ligand binding. Other cytokines have been proven to bind their receptors in a manner similar to growth hormone with one molecule of cytokine binding two receptor chains (Aritomi *et al.* 1999; Aritomi *et al.* 2000; Livnah *et al.* 1996; Livnah *et al.* 1999).

Site 1 interaction between GM-CSF and GMRα is consistent with functional studies such that helix D of the cytokine binds the hinge region defined by domains 1 and 2 of GMRα. Site 2 is an interaction between helix A of GM-CSF binding both βc chains: the A-B and E-F loops of domain 1 from βc chain 1 and the B-C and F-G loops of domain 4 on the second βc chain. Mutagenesis studies had previously established the importance of Tyr 365, His 367, Ile368 and Tyr421 of βc as being critical for cytokine binding and receptor activation (Woodcock *et al.* 1994; Woodcock *et al.* 1996). This composite interaction site is interesting because in other cytokine receptor complexes there is usually interaction with just 1 receptor chain at site 2. An essential glutamate at position 21 of GM-CSF (Lopez *et al.* 1992) is analogous to Glu^{22} in IL-3 (Bagley *et al.* 1995) and Glu^{13} in IL-5 and is involved in this site 2 interaction. This therefore indicates that site 2 is an attractive target

for the development of biologicals or small molecules that can simultaneously block the action of all three cytokines.

The site 3 interaction involves the GMRα chain and domain 4 of βc independent of ligand through a hydrophobic interface. This may be the mechanism of the strong interaction between GMRα and βc detectable in biochemical and functional studies in the absence of ligand. The GM-CSF receptor but not the IL-3 or IL-5 receptor has also been shown to exist as a pre-formed complex in the absence of ligand binding (Woodcock *et al.* 1997). GM-CSF receptor α and β subunits can be co-immunoprecipitated in the absence of ligand and mutations in the α subunit that abolish GM-CSF binding and can be compensated for by over-expression of β_c. The significance of this preformed complex is not clear but may explain the rapid association of GM-CSF to monocytes and eosinophils relative to IL-5 or IL-3 (Woodcock *et al.* 1997).

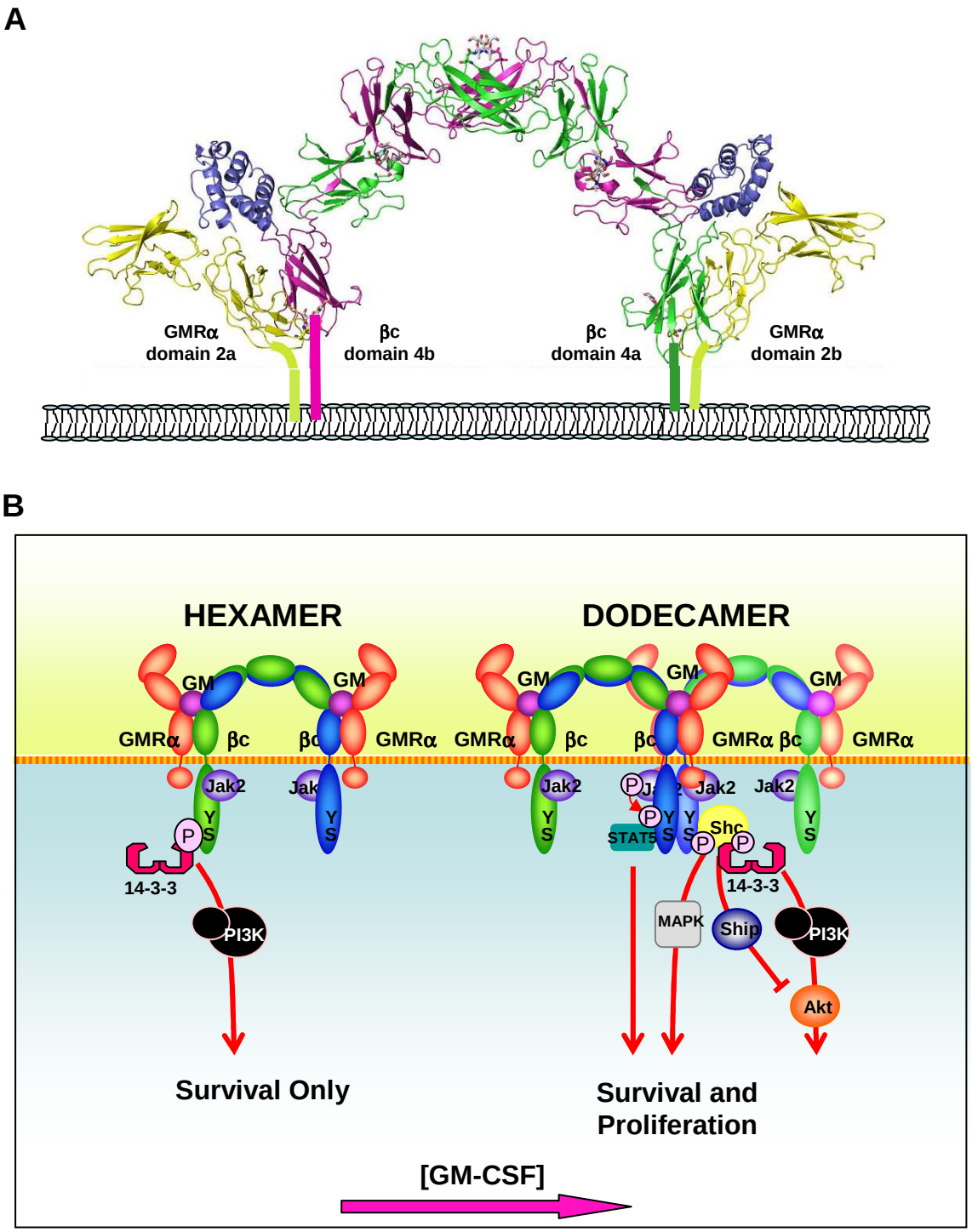


Figure 1.5: Crystal structure and signalling pathways of the GM-CSF receptor ternary complex. Cartoon ribbon picture showing the hexamer complex as it would sit on a cell surface. One monomer of βc is shown in green and the other in magneta. GM-CSF is shown in dark blue and GMRα in yellow. Labels denote the protein domains interacting with the cell membrane. This figure was prepared with PyMOL and adapted from original published by Hansen et al with thanks. (B) Model showing signalling pathways activated by GM-CSF in low cytokine (hexamer) to effect cell survival versus high cytokine conditions (dodecamer complex formation) to signal proliferation and survival (see Guthridge et al, 2006). Low picomolar cytokine results in Ser585 phosphorylation of βc and recruitment of 14-3-3 and activation of PI3K. Nanomolar concentrations of cytokine result in activation of the JAK2 tyrosine kinase, Tyr577 phosphorylation of βc, the recruitment of a PI3K signaling complex via Shc and the activation of canonical PI3K lipid signaling via AKT to promote cell proliferation and survival. This model was adapted from Hercus et al, 2009 presented in Chapter 5.

1.5.2 Signal Transduction

Signal transduction refers to the complex process by which interaction of a cytokine with its cognate receptor initiates intracellular signalling that in turn generates a specific biological response. A major drive behind investigations inot the mechanisms regulating growth factor and cytokine receptor signalling has beent eh growing recognition that cancer is a disease that arises from deregulated intracellular signalling (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). One of the promises of signal transduction research is that it will lead to the development of new generation targeted therapies with the selective ability to block deregulated cytokine and growth factor signalling pathways in cancer. The first success stories have already entered the clinic where tyrosine kinase inhibitors such as imatinib and dasatinib have shown remarkable activity in CML patients (Druker *et al.* 2006; Kantarjian *et al.* 2007). However, these early successes have been followed by a number of disappointing outcomes in clinical trials where small molecule tyrosine kinase inhibitors against FLT3 and EGFR have shown limited success in haematological and solid tumors respectively, see section 1.2.5. (Carter *et al.* 2005; Knapper *et al.* 2006). A major goal of the projects undertaken in this thesis is to identify tyrosine kinase-*independent* pathways involved in oncogenic transformation that can be interfered with in AML.

Signal transduction is a dynamic process and not surprisingly involves rapid, usually reversible post-translational modifications of signalling proteins such as phosphorylation, ubiquination, methylation and addition of lipid moieties. Various other lipids such as sphingosine and phosphatidylinositols and divalent cations such as Ca^{++} as well as reactive oxygen species function as important transient secondary messengers (Hunter, 2000). Post-translational modifications of proteins can function in a number of ways. Firstly they can alter the biochemistry of the protein resulting in a conformational change that may, for example, activate or inhibit an enzymatic function. Secondly they can serve to anchor a protein to a particular compartment in the cell (such as farnesylation of Ras). Thirdly, and not least importantly, post-translational modification such as phosphorylation is critical in facilitating protein-protein interactions. A landmark observation, first realised in the study

of phosphotyrosine interacting partners, was the discovery of Src-homology-2 (SH2) domains as conserved domains of large signalling molecules that allow high affinity binding of the signalling molecule to phosphotyrosine motifs present on the binding partner (Pawson and Scott, 1997). The biochemistry of many signaling proteins, especially adaptor proteins such as Grb2 (Schlaepfer and Hunter, 1996), IRS-1 and Gab2 (Gu *et al.* 1998), could be explained by the protein-protein interaction domains predicted by examination of their primary amino acid sequence. This led to the notion that many signalling molecules consisted of multiple domains of modular building blocks that connect enzymatic function to specific spatio-temporal events via various interaction domains (Pawson and Scott, 1997).

Many such other domains have been subsequently discovered using bioinformatic tools and biochemical validation. These included pleckstrin homology domains which bind inositol phosphates (Lemmon, 2007), WW and SH3 domains which bind proline-containing segments (Ilsley *et al.* 2002), and PDZ domains (Nourry *et al.* 2003).

1.5.3 Jak-STAT Pathway

There are four members of the Jak kinase family, Jak1, Jak2, Jak3 and Tyk2 (Firmbach-Kraft *et al.* 1990; Harpur *et al.* 1992; Wilks *et al.* 1991; Witthuhn *et al.* 1994). Jak 1 and Jak2 are activated by GM-CSF where as Tyk2 is predominantly activated by cytokines that utilise gp130 as their principal signalling subunit and Jak3 specifically associates with the γ_c chain of the IL-2 cytokine receptor family (Miyazaki *et al.* 1994; Russell *et al.* 1994). A number of in vivo studies have confirmed the functional importance of the Jak kinases in cytokine biology. A loss-of-function mutation in the Drosophila homologue of Jak, Hopscotch, results in loss of proliferation and lethality while a gain of function leads to formation of melanocytic tumours and hypertrophy of haematopoietic organs (Harrison *et al.* 1995). Jak3 deficient mice show lymphoid hypoplasia (Nosaka *et al.* 1995; Thomis *et al.* 1995) and mutations in Jak3 have been found in rare patients with severe combined immunodeficiency (Macchi *et al.* 1995).

The STAT group of proteins are major group of substrates for the Jak kinases. They were first discovered as transcription factors in involved with interferon signalling. Currently

there are 8 members (Akira *et al.* 1994; Barry *et al.* 1997; Hou *et al.* 1994; Hou *et al.* 1995; Wakao *et al.* 1994). A range of biological responses is associated with STAT transcriptional activation. Examples include induction of acute phase response genes by STAT3 (Akira *et al.* 1994; Wegenka *et al.* 1994) and mammary gland milk proteins by STAT5 (Welte *et al.* 1994). The ability of STATs to translocate to the nucleus is dependent on tyrosine phosphorylation and dimer formation and mutations in either SH2 domain abolish dimer formation (Ihle, 1996; Ihle and Kerr, 1995).

Activation of the GM-CSF receptor results in the tyrosine phosphorylation of a variety of classes of signalling molecules including kinases such as Jak2, Src and phosphatidylinositol-3 kinase (Welham *et al.* 1994a); adaptors such as Grb2 (Okuda *et al.* 1997), IRS-2 (Welham *et al.* 1997), Cbl (Hanazono *et al.* 1996; Odai *et al.* 1995) Shc (Inhorn *et al.* 1995; Sato *et al.* 1993); guanine nucleotide exchange factors such as Vav (Hanazono *et al.* 1996; Inhorn *et al.* 1995; Matsuguchi *et al.* 1995); phosphatases such as SHP-2 and SHIP (Inhorn *et al.* 1995; Itoh *et al.* 1998; Odai *et al.* 1997; Okuda *et al.* 1997; Welham *et al.* 1994b); and transcription factors such as Stat5 (Itoh *et al.* 1998; Mui *et al.* 1995). According to studies with the GM-CSF receptor and other similar cytokine receptors it is likely that Jak2 kinase is responsible for much of this phosphorylation (Quelle *et al.* 1994). Jak2 associates with the receptor via a proline-rich motif termed box 1 in the membrane proximal portion of the GM-CSF receptor. Binding of GM-CSF to the receptor to form a high order complex results in bringing together two β_c subunits from adjacent hexamers allowing trans-phosphorylation of the receptor by neighbouring Jak2 kinase (Hansen *et al.* 2008). There are eight tyrosine residues in the cytoplasmic portion of β_c, six of which are also present in the mouse receptor. Studies in other receptor systems have shown that phosphotyrosine residues can serve as docking sites for the binding of signalling molecules that contain Src-homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Pawson and Scott, 1997). All six tyrosines appear to mediate STAT5 binding to varying degrees (Gobert *et al.* 1996). Studies utilising dominant negative forms of STAT5 have discovered a number of genes that are transcribed following dimerization and translocation of STATs to the nucleus including pim-1, oncostatin-M and Id-1 (Yoshimura *et al.* 1996).

1.5.4 Ras-MAPK Pathway

The Ras/Raf /MAPK pathway historically associated with cell proliferation is activated in response to GM-CSF and is linked to three tyrosine residues, Y577, Y612 and Y695, present in the cytoplasmic domain of the receptor (Sato et al., 1993). Y577 has been shown to associate with the phosphotyrosine binding domain containing adaptor Shc (Itoh *et al.* 1996; Okuda *et al.* 1997; Pratt *et al.* 1996). Tyrosine 577 and 612 are required in some studies for the association of grb-2, another adaptor that contains an SH2 domain. Other work has demonstrated a ternary complex between β_c, Grb2 and Shc (Lanfrancone *et al.* 1995) suggesting a mechanism by which Grb2 via recruitment of Sos, a guanine nucleotide exchange factor, could result in the activation of Ras and downstream MAPK signalling pathways. Deletion mutants of β_c have shown a requirement of residues 626 – 763 for full activation of Shc, Ras, Raf and MAPK indicating Y695 and Y750 may be required (Sato *et al.* 1993).

1.5.5 PI-3K Pathway

The phosphoinositide 3-kinase (PI3K) family of enzymes catalyse the phosphorylation of the 3' hydroxyl subunit of phosphoinositides thereby producing lipid secondary messengers adjacent to the the cell membrane (Katso *et al.* 2001). There are three classes of PI3K enzymes but only class I and II transmit signals downstream of cytokine receptors. Class I can be further divided into class IA and class IB based on structural and functional differences (Cantley, 2002). Class IA enzymes are associated with signalling from tyrosine kinase receptors and haemopoietin receptors including the GM-CSF receptor. They consist of a catalytic subunit, p110 and a regulatory subunit, p85. There are three isoforms of p110: α, β and δ. Interestingly p110δ is expressed predominantly in leukocytes (Seki *et al.* 1997).

The p85 regulatory subunit possesses two SH2 domains through which it can recruit the catalytic domain to an activated receptor presumably via phosphotyrosine residues (Figure 1.6). However, p85 does not simply function as an adaptor but also regulates the catalytic activity. Once bound to a phosphotyrosine, the inhibitory action of p85 on p110 catalytic activity is relieved and so p85 has a dual function in bringing the enzyme in close

proximity to lipid substrate and also to activate its lipid kinase activity (Yu *et al.* 1998b; Yu *et al.* 1998a).

In addition to binding p85, p110 binds the small oncogenic GTPase Ras via a Ras-binding domain located in between the p85 binding domain and kinase domain (Figure 1.6). The significance of direct p110 binding to Ras has been unclear as binding affinity is relatively weak and there is little evidence for an interaction of endogenous Ras and PI3K in vivo. However, recently mice with mutations in the Ras binding domain of p110α isoform have been generated which show defects in growth factor proliferation signalling and are highly resistant to Ras induced tumourigenesis (Gupta *et al.* 2007).

The Class 1B enzyme, PI3K γ is similar to Class 1A proteins but does not have a p85 binding domain. It has an N-terminal domain similar in sequence to the pleckstrin homology domain of Rho-GAP that may function to tether the kinase to the cell membrane or lipid secondary messengers. Rather than activation by growth factor receptors, PI3Kγ is activated through stimulation of G protein coupled receptors including chemokine receptors. The G protein complex Gβγ can bind a unique regulatory subunit p101 which appears to play a similar role to p85 although cannot directly regulate catalytic activity. In a mouse model of peritonitis, treatment with specific small molecule PI3Kγ inhibitor, inflammation was significantly decreased as well as neutrophil infiltration demonstrating the importance of this pathway in chemokine inflammatory signalling (Hirsch *et al.* 2000)

Ever since its discovery (Kaplan *et al.* 1987) PI3K has been known to demonstrate serine kinase activity in addition to its lipid kinase activity (Figure 1.6) (Hiles *et al.* 1992). Initial reports identified autophosphorylation sites in p85 (Ser608) (Dhand *et al.* 1994a; Dhand *et al.* 1994b; Foukas *et al.* 2004; Geltz and Augustine, 1998), p110β (Ser1070)(Czupalla *et al.* 2003), p110δ (Ser1039)(Vanhaesebroeck *et al.* 1999) and p110γ (Ser1101)(Czupalla *et al.* 2003) which are wortmannin-sensitive indicative of intrinsic protein serine kinase activity. The serine kinase activity of PI3K is regulated by growth factors (including PDGF and insulin)(Foukas *et al.* 2004) and according to a number of reports appears to negatively regulate lipid kinase activity (Beeton *et al.* 2000; Czupalla *et al.* 2003; Dhand *et al.* 1994a) suggesting a dichotomy of function. Furthermore, the mechanisms by which

the serine kinase activity is activated appear to distinct from that which regulation of lipid kinase activity and are likely to be phosphotyrosine independent (Foukas and Shepherd, 2004a). For example, tyrosine phosphorylated YXXM motif peptides activate the lipid kinase activity of PI3K but have no effect on the serine kinase activity (Layton *et al.* 1998). More recently, other PI3K-serine kinase substrates have been identified and include p101(Bondev *et al.* 1999), insulin receptor substrate 1 (IRS1)(Lam K *et al.* 1994; Tanti *et al.* 1994), PDE3B phosphodiesterase (Rondinone *et al.* 2000), eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) (Foukas and Shepherd, 2004b), MEK1(Bondev *et al.* 1999) and H-Ras (Foukas and Shepherd, 2004b). A recent report identified a role for the serine kinase activity of PI3K in agonist-dependent β-adrenergic receptor (βAR) internalization via its ability to phosphorylate Ser61 of non-muscle tropomyosin (Naga Prasad *et al.* 2005). However, in contrast to the plethora of reports examining lipid signalling downstream of PI3K, little is known regarding the functional roles of the protein kinase activity of PI3K (Backer, 2005; Foukas and Shepherd, 2004a). The relationship of PI3K protein kinase signalling in cytokine receptor signal transduction has not been investigated.

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Phosphatidylinositol-3 kinase (PI3K) is activated in response to GM-CSF, similar to many other cytokine receptors, although there is no obvious consensus binding motif (such as YXXM as in the PDGF receptor) for recruitment to a receptor complex (Jucker and Feldman, 1995; Sato et al., 1993; Welham et al., 1994). There is recent evidence that PI3K may also be recruited to the receptor complex through 14-3-3 adaptor protein (Barry *et al.* 2009; Guthridge *et al.* 2004) and/or GMRα cytoplasmic domain (Perugini *et al.* 2010).

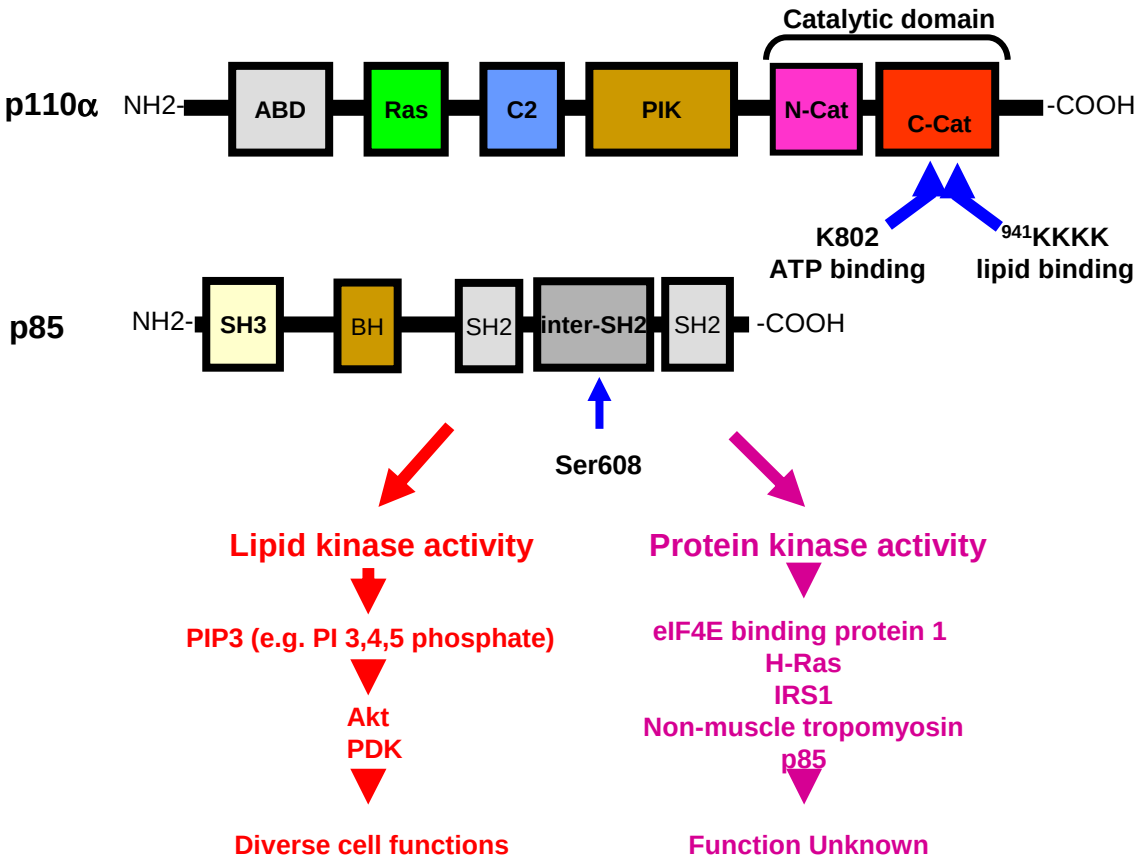

Figure 1.6: Domain structure of PI3K showing functional protein domains and downstream targets of lipid and protein kinase activity. PI3K consists of 4 catalytic isoforms: p110α,p110β,p110δ and p110γ. Pictured above is the domain structure of p110α, the most ubiquitous and frequently mutated isoform in cancer. It consists of an actin-binding domain (ABD), ras binding domain (Ras), coiled domain (C2), and a split catalytic domain (N-Cat and C-Cat). p85 consists of an SH3 domain, Bcr-homology domain (BH), and two SH2 domains split by an inter-SH2 domain. This segment undergoes autophosphorylation on Ser608 by intrinsic PI3K protein kinase activity (see Chapter 4). Whilst the lipid kinase substrates and pathway is well studied, the function and identify of protein kinase substrates which include IRS-1 and tropomyosin are not well characterised.

1.5.6 Proliferation

Deletion mutants of β_c have revealed that the multiple functions transduced by the GM-CSF receptor are mediated by multiple pathways generated from distinct domains. A membrane proximal region of 35 amino acids is required for proliferation in Ba/F3 and CTL cells but this domain alone is unable to support long term survival (Itoh *et al.* 1996; Sakamaki K *et al.* 1992; Sato *et al.* 1993). This region contains the conserved motif Box 1 which can associate with Jak2 (Quelle *et al.* 1994)and a number of studies using deletion mutants of Box 1 or a dominant negative form of Jak2 indicate that Jak2 activation is both necessary and sufficient for inducing cell proliferation (Itoh *et al.* 1996) however the proliferation response transduced in these cell lines was transient. These results imply that proliferation and survival functions are not necessarily overlapping and that other signalling pathways beyond Jak2 activation are required for long term GM-CSF mediated survival.

Mutations of tyrosines in β_c indicate a high degree of redundancy and no single tyrosine residue has been found to be critical for mediating any of the known biological activities of GM-CSF, unlike some other members of the haemopoietin receptor family where specific tyrosines have been linked directly to a signalling pathway and function (Hemmann *et al.* 1996). Mutation of Y750 in Ba/F3 cells does result in a decreased viability in serum free conditions but studies in other cell lines have shown little difference. We have previously shown that a GM-CSF receptor with all eight tyrosines mutated to phenylalanine show a defect in proliferative response but no defect in viability (Guthridge *et al.* 1998). Similar results have been shown by other groups (Itoh *et al.* 1998; Okuda *et al.* 1997) and detailed studies with other receptors in the haemopoietin family including the EPO and TPO receptors have shown that phosphotyrosines are dispensible for a number of cytokine mediated functions including survival and proliferation (Drachman and Kaushansky, 1997; Klingmuller, 1997). These results are intriguing and indicate the existence of other signalling strategies in the case of the GM-CSF receptor.

1.5.7 Survival

A number of reports have indicated that the survival function of GM-CSF can be dissociated from its proliferative function and that such survival signals arise from a domain distal to the box 1 region (Itoh *et al.* 1996). Truncation mutants comprising only box 1 and 2 were sufficient to allow transient proliferation of cytokine-dependent cells but it was noted the cells were not viable after several days (Inhorn *et al.* 1995; Kinoshita *et al.* 1995). Deletion experiments revealed a region between amino acids 544 and 763 that was required for cytokine dependent survival (Kinoshita *et al.* 1995). In the absence of serum, the entire domain was necessary for survival function. However, in the presence of serum a region 544-626 was shown to be sufficient to promote cell survival. Substitution of Y577 and Y612 by mutagenesis to phenylalanine, the only tyrosines in this region (Kinoshita *et al.* 1995), did not affect the cell survival function conferred by this domain, again suggesting phosphotyrosine-independent mechanisms of signalling.

A number of receptors have been shown to activate PI3K resulting in the production of phosphatidylinositol phosphates which in turn can activate Akt/PKB and other signalling pathways associated with survival function (Manning and Cantley, 2007). GM-CSF stimulation can activate PI3K and Akt which in turn regulate a number of pro-survival and apoptotic pathways by phosphorylation of specific substrates. In response to IL-3, Akt has been shown to phosphorylate Bad, a BH3-only pro-apoptotic molecule implicated in cytokine-mediate survival pathways (Datta *et al.* 1997; del Peso *et al.* 1997). Phosphorylation of Bad allows binding of 14-3-3 proteins thereby sequestering Bad in the cytoplasm preventing association with mitochondrial membranes and activation of an apoptotic program (Datta *et al.* 1999). Akt can also stimulate other pro-survival pathways including the phosphorylation of the forkhead transcription factor, FKHRL1 (Brunet *et al.* 1999) preventing the induction of pro-apoptotic BH3 only proteins such as Bim (Dijkers *et al.* 2000). A third target of Akt likely to be involved in cytokine survival signaling is MDM2. MDM2 is phosphorylated on Ser166 and Ser186 by Akt which promotes translocation to the nucleus where it negative regulates p53 and prevents transcription of Puma and Noxa (Mayo and Donner, 2001; Zhou *et al.* 2001).

Detailed studies of the region in β_c required for cell survival in the laboratory of Angel Lopez and Mark Guthridge revealed a phosphoserine motif, ^{582}HSRSLP^{587} where serine 585 is phosphorylated that can bind the 14-3-3 family of proteins (Stomski *et al.* 1999). Mutation of this motif to alanine when expressed in CTL-EN cells revealed an inability of the receptor to bind 14-3-3 and decreased PI3K and Akt activation after cytokine stimulation (Guthridge *et al.* 2000). Furthermore, cell viability of this mutant form of the receptor was dramatically reduced when plated in low serum conditions. The observation that tyrosine phosphorylation of the receptor and activation of JAK2, STAT5, Erk and Jnk in response to cytokine were unchanged while Akt activation was virtually abolished provided a direct link between this motif, Akt activation and cell survival (Guthridge *et al.* 2000).

Further study using point mutations of Ser585 and the adjacent upstream tyrosine, Y577, retrovirally transfected into primary haematopoietic cells revealed important insights as to the mechanisms utilised by this domain of the receptor to regulate proliferation and survival functions (Guthridge *et al.* 2006). Mutation of Ser585 alone in low serum conditions resulted in a cell survival defect consistent with previous data. Mutation of Ser585 and Y577 alone and in combination when tested in 10% serum conditions revealed that these two residues were necessary and sufficient to induce survival, proliferation and colony formation in the presence of GM-CSF in these cells. This suggested a model whereby the phosphorylation of two residues in close proximity recruited distinct adaptor proteins (Shc via Y577 and 14-3-3 via Ser585) to signal a proliferation and survival response. However it was not clear whether both residues were coupling to the same downstream pathway in a redundant fashion (as has been observed with multiple phosphotyrosines coupling to STAT5 activation) or whether each residue via recruiting a specific adaptor could actually signal in a distinct non-redundant way.

Careful dose-response experiments examining Ser585 and Y577 phosphorylation after ligand stimulation shed more light on this question and led to the concept of a binary switch comprising Y577 and Ser585 which couples the strength of the cytokine stimulus to distinct biological outcomes (Guthridge *et al.* 2006). When examined in a number of leukaemic cell lines and mononuclear cells from normal blood donors it was found that very low doses of cytokine (1pM) could induce serine 585 phosphorylation in the absence

of tyrosine phosphorylation. A higher concentrations of GM-CSF (around 100 pM) Ser585 phosphorylation was less pronounced and tyrosine phosphorylation was observed to be occurring, suggested a dynamic response of these two phosphorylation sites according to level of stimulus. Furthermore, the changes in phosphorylation of these two residues with concentration correlated with previous data showing a difference in the concentration of cytokine required to induce a survival response (~ 1 pM) and the concentration required to induce a proliferation response (>10 pM). It was proposed that these two residues can cooperate at higher doses to signal cell survival and proliferation while at lower doses can work independently to trigger cell survival alone (via Ser585) when growth conditions are not optimal (Guthridge *et al.* 2006).

Leukaemic blasts exhibit deregulated survival and proliferation responses compared to normal haematopoiesis. Our preliminary work with primary AML patient samples indicated that in terms of GM-CSF receptor signalling, Ser585 phosphorylation was constitutive and did not show downregulation in response to exogenous cytokine (Guthridge *et al.* 2006). This result is consistent with perturbed activation of the Ser585 pathway in AML and describes a unique insight into understanding the nature of cytokine receptor signalling in this disease. A search for the upstream kinase(s) responsible for Ser585 phosphorylation in AML and downstream transcriptional targets of this pathway is presented in Chapters 4 and 5.

There is still much to be determined regarding the interaction with receptor proximal events such as activation of JAK2 and PI3K and downstream control of apoptosis. In addition to the role of PI3K/Akt in restraining the pro-apoptotic functions of BH3-only containing proteins such as Bad, there is evidence that GM-CSF and IL-3 regulate the protein levels of pro-survival proteins. Bcl-2, Bcl-x_L, Mcl-1, Bcl-w and A1 can all inhibit apoptosis induced by cytokine withdrawal and there is evidence that GM-CSF and IL-3 can regulate Mcl-1 (Chao *et al.* 1998), Bcl-2 and Bclx_L. Mcl-1 is a short half life protein that undergoes post-translational modification (Schwickart *et al.* 2010) and binds principally to pro-apoptotic Bak to prevent apoptosis. When bound by anti-apoptotic Mcl-1, Bak together with Bax are held in an inactive conformation. BH3-only proteins such as Bad, Puma, Bim sense and integrate the loss of survival signals in a cell and bind pro-survival proteins, releasing Bak and Bax to initiate the apoptotic cascade (Cory and

Adams, 2002). Mcl-1 and $Bclx_L$ are unique in that they can directly bind Bak (Willis *et al.* 2005). (Figure 1.7). Mcl-1 in particular is expressed in myeloid progenitor cells being required for haematopoietic stem cell survival (Opferman *et al.* 2005) and is upregulated in AML and linked to relapse (Kaufmann *et al.* 1998). It is a key target of cancer therapies as it appears to be associated with both chemotherapy resistance (Wuilleme-Toumi *et al.* 2005) and resistance to Bcl-2 targeted therapies in clinical development such as ABT-737 (van Delft *et al.* 2006).

The mechanism by which GM-CSF and IL-3 regulate Mcl-1 transcription and protein stability is not clear. Inhibitors of RNA polymerase II activity have been shown to have an immediate effect on the levels of Mcl-1 in the cytosol, presumably because of its normal rapid turnover. A number of CDK9 inhibitors such as SNS-032 and flavopiridol effect a rapid reduction in Mcl-1 levels and have been shown to block RNA polymerase II transcription through their effects on transcription elongation pausing (Bensaude *et al.* 1999) (Figure 1.8). These inhibitors have been shown to have moderate effects on haematological malignancies in vitro and in vivo and offer hope toward the development of Mcl-1 targeted therapy (Blum *et al.* 2010; Conroy *et al.* 2009).

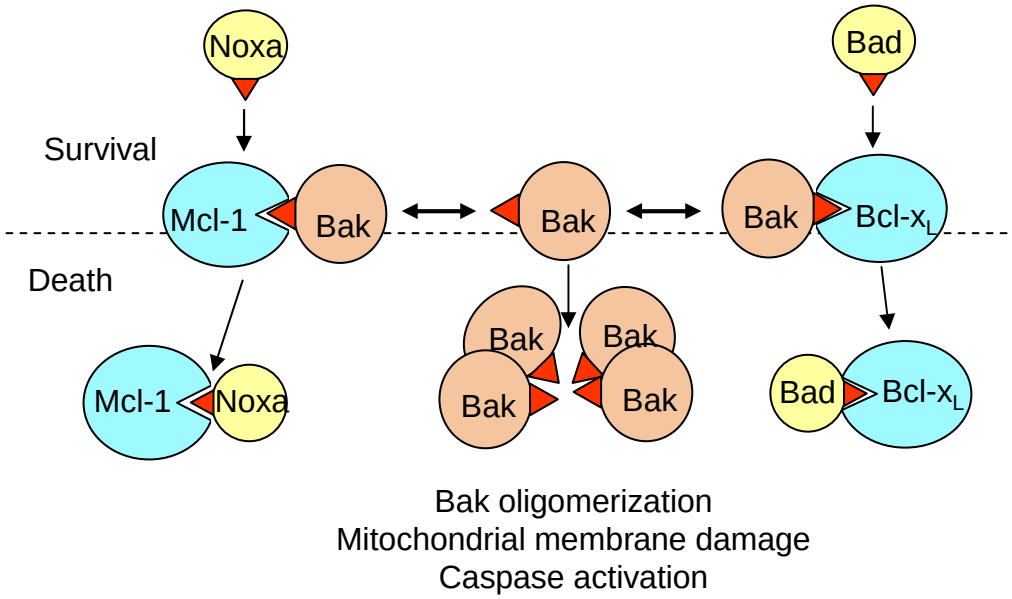

Figure 1.7: Model showing the role of Bcl-2 pro-survival family members directly binding pro-death protein Bak. The intrinsic pathway of apoptosis is activated by unhindered oligomerization of Bak and resulting pore formation on the outer mitochondrial membrane leading to decreased mitochondrial membrane potential and release of Apaf-1 and cytochrome C. The pro-apoptotic action of Bak is held in check by pro-survival family members Mcl-1 and Bcl-$_{XL}$ which have been demonstrated to directly bind to Bak (Willis et al, 2005).

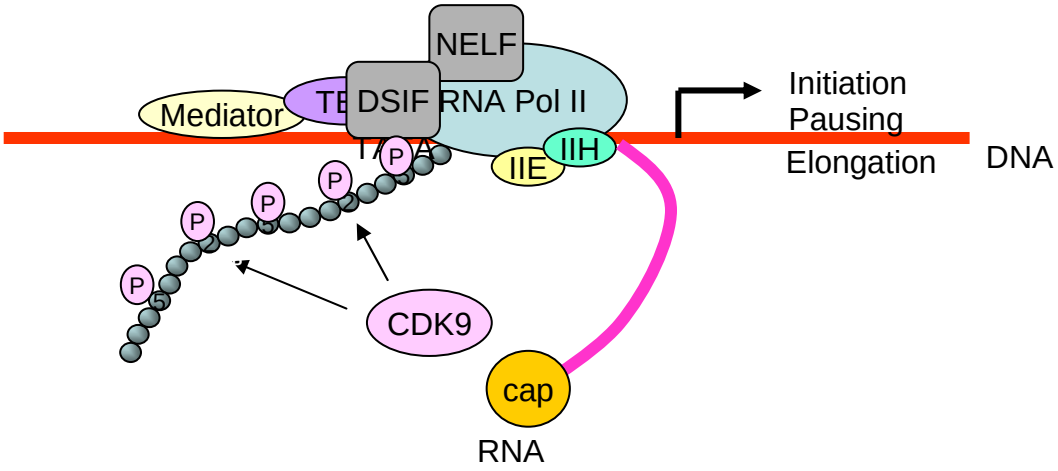

Figure 1.8: Cartoon showing RNA polymerase II and the role of CDK9 in elongation pausing. CDK9 is a serine/threonine kinase that forms part of the TEFb complex (comprising CDK9 and cyclin T1,T2 or K) that is responsible for phosphorylation of Serine 2 of a repeat seven amino-acid sequence (heptad) sequence in the C-terminal domain of RNA Polymerase II (CTD). Phosphorylation of both Ser 2 and Ser 5 are required for transcription initiation and elongation by RNA Pol II. Quiescent cells such as primary T lymphocytes are characterized by an almost complete absence of Ser 2 CTD phosphorylation. Yeast cells in stationary phase are phosphorylated on CTD Ser5 but not on Ser2. Rapid activation of gene transcription is possible upon stimulation after addition of nutrients and phosphorylation of Ser2 by CDK9.

1.5.8 Differentiation

A region comprising amino acids 525 – 610 is required for a myeloid cell differentiation response demonstrated in M1 cells (Smith *et al.* 1997). This region overlaps the segment required for cell survival. The significance of a differentiation event in a cell line is uncertain and may not necessarily reflect the in vivo scenario. Furthermore it is unlikely to represent a truly instructive signal (Kondo *et al.* 2000).

1.6 Conclusions and Implications for Further Research

The search for successful treatments that induce durable remissions in acute myeloid leukaemia remains a major focus for cancer research, both for young and elderly patients and certain cytogenetic subgroups such as normal karyotype AML. While the development of anti-CD33 mAb and Flt3 tyrosine kinase inhibitors have been disappointing as monotherapy to date they have highlighted the complexities and challenge of treating AML compared to chronic myeloid leukaemia. High resolution mapping of the molecular lesions in AML has defined novel subgroups with useful prognostic information and definitive evidence for a leukaemia stem cell population in xenograft experiments has stimulated research therapy directed against cancer stem cells.

To this end, further investigation into the nature of cytokine signalling that is deregulated in AML is likely to be fruitful in both the understanding leukaemogenesis and new target discovery. This thesis is focussed on the GM-CSF and IL-3 receptor and downstream signalling events transduced by these cytokines in AML cell-lines and patient samples. The over-expression of the IL-3 receptor α chain on leukaemic stem cell enriched populations compared to normal bone marrow stem cells and the constitutive phosphorylation of serine 585 of the IL-3/GM-CSF receptor βc in AML are important observations that underpin the research presented. The central role for PI3K in cytokine receptor survival signalling and the over-expression Mcl-1 in AML indicates these downstream molecules are attractive

targets. The cancer stem cell model has emphasised the importance of microenvironmental factors including IL-3 and osteopontin as potential microenvironmental regulators within the leukemic stem cell niche.

These findings are extended herein by [1] developing a FACS-based protocol for isolation of human AML stem and progenitor cells to show proof of principle in targeting the IL-3 receptor α chain [2] identification of the kinase involved in phosphorylating Ser585, [3] profiling genes regulated by Ser585 and expressed in AML with biological and clinical significance, and [4] investigating a kinase inhibitor that targets Mcl-1 by dual blockade of CDK9 and PI3K as a candidate for AML therapy (for summary see Figure 1.9).

By studying cytokine receptor signalling pathways in the relevant primary cells *in vitro* and *in vivo* that it is hoped the course of this disease can ultimately be altered.

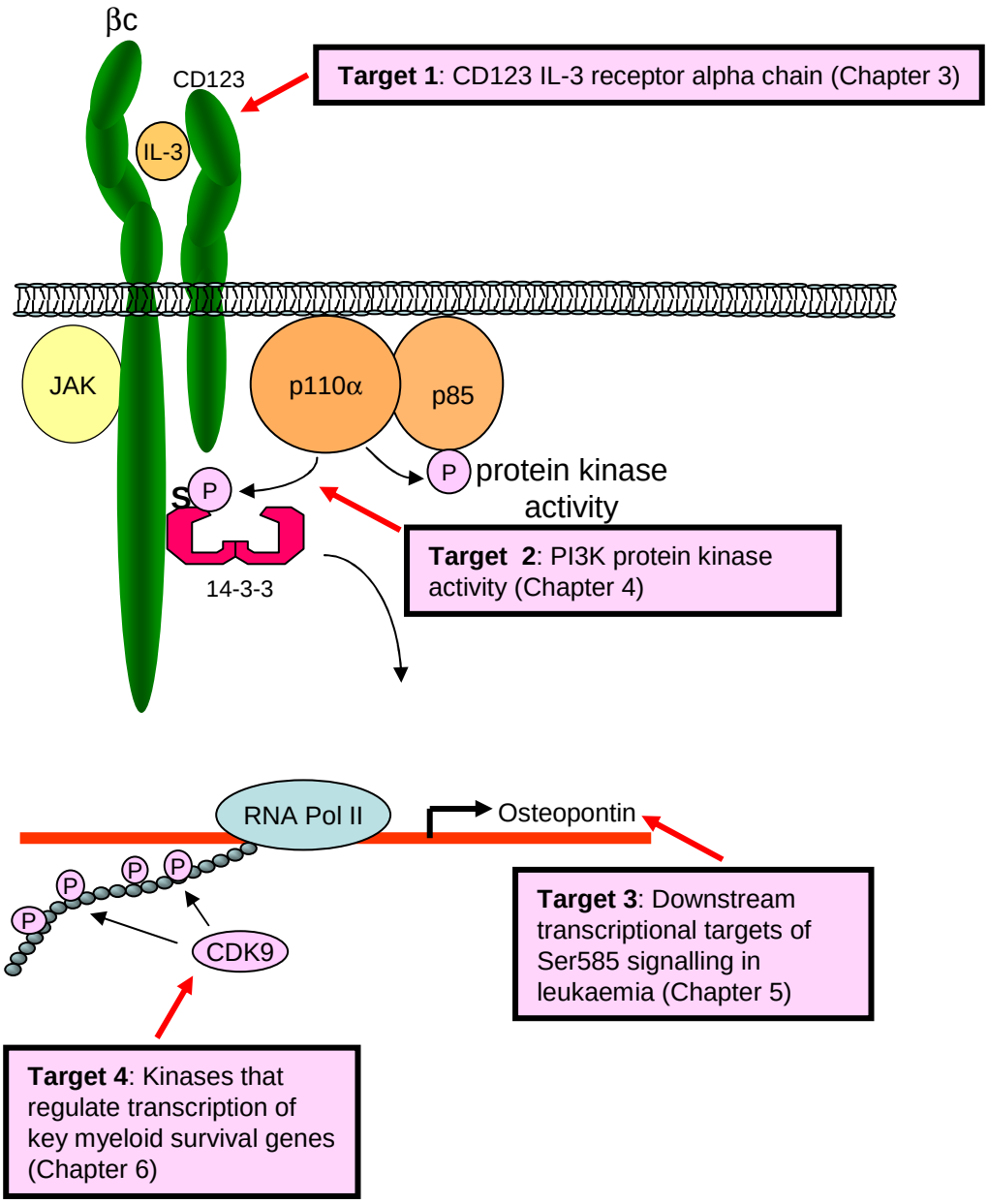

Figure 1.9: Diagram summarizing targets for AML investigated in this thesis: [1], CD123 or IL-3 receptor alpha chain on AML stem cells inhibited in vivo by a blocking monoclonal antibody 7G3 (Chapter 3); [2] the protein kinase activity of PI3K (Chapter 4); Osteopontin, a secreted transcriptional target of IL-3/GM-CSF receptor Ser585 signalling (Chapter 5); and CDK9, an additional target of selective PI3K inhibitor PIK-75 that regulates mcl-1 transcription (Chapter 6).

Chapter 2

Materials and Methods

2.1 Tissue Culture

MV4-11, HL60, KG-1 and FDCP-1 cells were cultured in 10% FCS/RPMI. U937 cells were maintained in 10% FCS/IMDM. HEK293T cells were maintained in DMEM with 10% FCS and were transfected with using lipofectamine (Invitrogen) in 0.5% fetal calf serum (FCS; JRH Laboratories) and DMEM for 4h. Factor-dependent myeloid (FDM) cell lines were generated by HoxB8 transformation as previously described and cultured in DMEM/10% FCS with 0.25 ng/ml IL-3 (Ekert *et al.* 2004).TF-1 factor-dependent cell line was cultured in 10% FCS/RPMI with 2ng/ml GM-CSF and additional HEPES buffer (5ml per 500ml). Primary murine hemopoietic progenitor cells were isolated from the BM of SV129 mice as previously described (Guthridge *et al.* 2006). Lineage negative (Lin^-) cells were isolated by negative selection using a Lineage Cell Depletion Kit (Miltenyi Biotec).

2.2 Primary Acute Myeloid Leukaemia Cells

Apheresis product, bone marrow, or peripheral blood samples were obtained from patients with AML and one patient with CML. Patient samples were collected after informed consent according to institutional guidelines and studies were approved by the Royal Adelaide Hospital Human Ethics Committee, RAH Protocol No. 041009 “Dysregulation of the GM-CSF receptor-mediated survival signalling pathway in haematological malignancies.” The same ethics protocol was amended and approved for “use of blood & bone marrow samples earmarked for discard” RAH Protocol No. 041009a for isolation of normal CD34+ cells used in Chapter 5. A 24-hour on call system was established such that [1] all apheresis products suspected or confirmed to be AML would be notified by haematology day centre nursing staff by mobile phone at the time of the procedure and the product collected promptly or next morning; [2] the bone marrow procedure nurse would notify all suspected or confirmed relapses of AML by phone and sample left at theatre for prompt collection; [3] a tissue bank of all BM samples stored in the liquid nitrogen facility established in the Division of Haematology was made available for AML research projects. Diagnosis was made using cytomorphology, cytogenetics and leukocyte antigen expression (Division of Immunology) and evaluated according to the French-American-British classification. All normal karyotype samples were genotyped for AML prognostic markers

NPM and FLT3-ITD and FLT3 kinase domain D835 point mutations. Mononuclear cells were isolated by Ficoll-Hypaque density-gradient centrifugation and resuspended in PBS containing 0.1% human albumin (CSL). We found the best conditions to freeze were 1-2x10^8 cells/ml in 20% FBS/IMDM after Ficoll-Hypaque centrifugation. For leukaphereis products approximately 300 vials of 2x10^8 cells each were frozen down in one session. For BM samples, depending on total cell no., approximately 2-12 vials were stored at similar concentrations.

2.3 Consumer Relations

An important feedback mechanism was established with both nurses and patients at the Royal Adelaide Hospital through regular patient education sessions at the Leukaemia Foundation and frequent clinical meetings. Patients and staff were informed about the results of the research and the significance for leukaemia.

2.4 SDS-PAGE and Western Blotting

TF-1 cells were factor-deprived in RPMI containing 0.5% FCS for 12 h and then stimulated with different GM-CSF concentrations at 37º for 10min before lysis in NP-40 lysis buffer. Mononuclear cells from normal donors and AML patients were isolated by Ficoll-Hypaque density-gradient centrifugation, washed and resuspended in PBS containing 0.1% human albumin (CSL) and stimulated for 10min with GM-CSF. Cells were lysed in NP40 lysis buffer (10mM Tris [pH 7.4], 137mM NaCl, 10% glycerol with 1% Nonidet P-40 in an inhibitor cocktail containing 10mM β-glycerol phosphate, 1mM phenylmethylsulfonyl fluoride, 10mM NaF, 10mM Na orthovanadate, 4.5 U/ml aprotinin (Sigma) and 1μg/ml leupeptin (Sigma) and subject to immunoprecipitation and Western blot analysis. The β_c subunit was immunoprecipitated using 1 μg of 1C1 and 8E4 anti- β_c mAb; p85 and various isoforms of p110 were immunoprecipitated with Anti-p85 pAb (Upstate) at 1:1000, anti-p110 alpha (Cell Signalling), anti-p110β pAb (Santa Cruz), anti-p110δ mAb A-8 (Santa Cruz), anti-p110γ clones H1/641 at 1 in 500 (Jena Bioscience). Anti-myc (9E10) was used at 1:1000; anti-Flag anti-HA mAb HA7 (Sigma) was used at 1:10,000. Anti-phospho-Ser473Akt, anti-phospho-Ser21/9GSKα/β, anti-phospho-ERK,

anti-phosphotyrosine 4G10 and anti-Ckl (Cell Signalling) were used at 1:1000 and anti-phospho-Ser608 were used at 1:500. Affinity-purified phospho-Ser585 of β_c pAb was used at 1:500; affinity-purified phospho-Tyr577 of β_c pAb was used at a dilution of 1:1000.

2.5 Production of recombinant intra-cytoplasmic portion of βc (βic).

A cDNA for the intracellular domain of the βc subunit of the GM-CSF and IL-3 receptor (gene symbol CSF2RB) encoding amino acids 445-881 was cloned into pET28a with 6xHis tags at both N- and C-termini. Bacterial cultures (400ml) were induced with 0.1mM IPTG for 2hr. Harvested bacteria were lysed in 8M Urea, 0.1M NaH_2PO_4, 0.01M Tris, pH 8.0 with 1mM PMSF and bound to NiNTA resin for 1hr at RT. After washing resin in 8M urea /0.1M NaH_2PO_4/0.01M tris pH 7.0, pH 6.3, pH 5.9 and pH 7.0 using an econo-column (Biorad), protein was eluted with 500mM imidazole in 8M Urea, pH 7.0. Eluted protein was dialized at 4^oC in 50mM Tris-Cl pH 7.4, 300mM NaCl 1mM EDTA, 10% glycerol, 10mM DTT.

2.6 Purification of βc Ser585 Kinase

Primary AML MNCs ($3x10^8$) from patients were lysed in a hypotonic buffer (20mM Tris-Hcl pH 7.4, 0.5mM EDTA, 0.5mM EGTA, 10mM βME, 5% glycerol) containing 2mM NaF and Complete Mini EDTA-free protease inhibitor cocktail (Roche). The lysate was then subjected to centrifugation at 16,000g for 10min followed by ultracentrifugation of the supernatant at 186,000g for 1h. The clarified lysate was then subjected to fast protein liquid chromatography (FPLC) on a Superdex 200PC 3.2/30 column (Amersham Biosciences). Chromatography was performed using a running buffer (Tris-Cl, pH 7.5, 200mM NaCl, 0.1mM EDTA and 10mM β-ME) and a flow-rate of 40µl/min and 40µl fractions were collected.

2.7 Protein Kinase Assays

Protein kinase activity was examined in i) aliquots of eluted fractions following chromatography of primary AML samples, ii) p85 and p110 immunoprecipitates or iii) purifed recombinant p110 catalytic subunits of PI3K. Reaction mixtures comprised of 50μM Ser585 peptide, 50μM Kemptide or 0.5μg of recombinant beta subunit cytoplasmic domain (βic) in kinase buffer (50mM Hepes pH 7.4, 5mM EDTA, 10mM $MnCl_2$, 0.250mM dithiothreitol (DTT), 0.02% Tween 20) with 0.25μCi[γ-^{32}P]ATP, 1μM cold ATP. Reactions were incubated at 30ºC for 30 min and aliquots examined for ^{32}P-labelled peptide on phosphocellulose filters (Whatmann, P81) and liquid scintillation counting. For βic kinase assays, reactions were stopped by adding 2xSDS load buffer followed by SDS-PAGE and autoradiography.

2.8 Lipid Kinase Assays

For PI3K lipid kinase assays, cells were lysed in NP-40 lysis buffer (137 mM NaCl, 1.0% NP-40, 10% glycerol, 50 mM Tris-HCl, pH 7.4) containing 10mM β-glycerol phosphate, 1mM phenylmethylsufonylfluoride, 10mM NaF, 10mM Na orthovanadate, 4.5 U/ml aprotinin (Sigma), and 1 mg/ml leupeptin (Sigma) and immunoprecipitated proteins were examined for PI3K lipid kinase activity using phosphatidyl inositol and 0.25μCi[γ-^{32}P]ATP as substrates. Immunoprecipitates were washed three times in NP40 lysis buffer followed by 3 washes with 1 x kinase buffer (20mM Hepes, pH 7.5, 5mM $MgCl_2$, 1mM EGTA) and supernatants then removed. Assays were performed in a total volume of 100μl consisting of 30μl protein A pellet, 20μl of 1x kinase buffer, 50μl of phosphatidyl inositol (PtdIns) and phosphatidyl serine (PtdSer) mix, 2μl cold ATP at 2.5mM and 1μl γ^{32}P ATP incubated at room temperature for 20 minutes. The reaction was stopped with 100μl of 1M HCl and lipids extracted by addition of 200μl chloroform/methanol (1:1 v/v) and 500μl of 2M KCl saturated with chloroform. Samples loaded onto a thin layer chromatography (TLC) plate pre-dipped in potassium oxalate and dried overnight. Plate is run in TLC tank with chloroform/methanol/acetic acid/water 43:38:5:7 v/v after 2 hrs of equilibration. The PtdIns/PtdSer mix is stored in chloroform/methanol 95:5 v/v 10mg/ml PtdIns (Sigma) and

5mg/ml PtdSer (Sigma) dried down under nitrogen stream and resuspended in 400µl lipid resuspension buffer (20mM Hepes, pH 7.5, 5mM $MgCl_2$, 1mM EGTA) and sonicated for 1min on ice.

2.9 Cell Survival and Proliferation Assays

Cell survival was determined by either trypan blue exclusion, annexin V-FITC (Roche) staining or counting viable cell number in reference to Flow Count Fluorospheres (BD Biosciences). Cell proliferation was determined by BrdU incorporation, using the *in situ* cell proliferation kit (Roche).

2.10 Transfection of primary AML cells with siRNA

CD34+ cells were purified from AML MNCs using CD34 MicroBeads according to the manufactures instructions (Miltenyi Inc). CD34+ cells were transfected with 50nM BLOCK-iT fluorescent oligo and 50-150nM of either GC-control siRNA or scrambled *OPN* siRNA or *OPN* siRNA (Invitrogen)(siRNA oligonucleotide sequences are presented in Supplementary Table S2). Survival was determined by annexin V-alexa 568 staining (Roche). Viable cell number was assessed using Flow-Count Fluorospheres (BD Biosciences).

2.11 Transfection of TF-1 AML cell line

TF1.8 cells were transfected by electroporation (1000µF at 300V) using a Gene Pulser II (Biorad) aiming for a time constant of 12-16msec. $5x10^6$ cells per mammalian electroporation 0.4cm cuvette were resuspended in RPMI without serum and incubated with a total of 8µg DNA per cuvette which included at least 2µg of eGFP reporter construct. Following electroporation cells were rapidly transferred using a Pasteur pipette to 9ml of normal media with 10% serum but without antibiotics. Transfection efficiency was measured at 12-24hrs using FACS analysis and ranged from between 5 to 45%. Dead cells were removed using Ficoll-Hypaque density-gradient centrifugation and cells were replated in various biological assays at $2x10^5$ live cells/ml.

2.12 Purification of AML Stem/Progenitor Cells

For sorting of $CD34^+CD38^-CD123^+$ primitive AML cells, mononuclear cells were washed 3 times in IMDM/10% FCS then stained for 30 min at 4°C in 2 ml IMDM/10% FCS with 80 µl each of CD34-FITC, CD38-PE-Cy7 and CD123-PE antibodies (BD Biosciences, San Jose, California, USA). The $CD34^+CD38^-$ cells were sorted by staining with CD34-APC and CD38-PE antibodies (Becton, Dickinson and Company) at 4°C for 1 hour.

Several practical steps minimised cell loss and biological error due to human factors:

(i) Cells were immediately plated into assays following FACS purification after only one centrifugation which minimised loss of cells due to tube transfer or breakdown from repeat resuspension. The volume of resuspension media was calculated to 80% of total cell count sorted by FACS. This reproducibly gave us $2x10^5$ cells/ml without cell wastage. ie a FACS count of 1000,000 events was resuspended in 4mls to give final $2x10^5$ cells/ml.
(ii) Resuspended cells were then checked for quantity without trypan blue using 30µl cell suspension volume with phase contrast
(iii) Cells were plated in 96-well round-bottomed plates in volumes of 200µl. Drugs were added with final concentration was less than 0.5% DMSO carrier which did not discernibly affect viability.
(iv) Flow-count flourosphere beads were utilised to quantitate subtle changes in the number of surviving sorted cells over time in 200µl assay volumes such that bead to cell ratio approached 1:1. Each well was analysed by 300µl of media and 100µl beads to minimise dilution/pipetting errors.

2.13 AML Colony Forming Assays

Primary AML cells were plated in MethoCult at 100,000 cells/ml with 100pM IL-3 and 100pM GM-CSF and blast colonies (>50 cells) were counted at 3 weeks. Colony assays were performed on mouse bone marrows by

2.14 Intracellular FACS of AML Stem/Progenitor Cells

TF-1, bulk and sorted primary AML cells were stimulated with 20ng/ml IL-3 plus 20ug/ml IgG2a or 7G3 for 1hr (A). (B) Sorted subpopulations were incubated with 150ug/ml 7G3 or IgG2a for 30 min on ice before simulation 1nM IL-3 for 15 min. Cells were fixed with BD Cytokix Buffer (BD Biosciences), methanol permeabilized, and stained with anti-phopshoSTAT5 (BD Biosciences) or isotype control. Cells were then analysed using a FACSCalbur flow cytometer (BD Biosciences).

2.15 AML Engraftment

Animal studies were performed under the institutional guidelines approved by the University Health Network/Princess Margaret Hospital Animal Care Committee and the Animal Care and Ethics Committee of the University of New South Wales, Adelaide University Ethics Committee and the IMVS Ethics Committee. Human AML or normal BM cells were incubated with PIK-75 or vehicle (DMSO) for 2-3h in RPMI/10% FCS following which 5-10x10^6 cells were intravenously injected into irradiated (250–350cGy) NOD/SCID mice as previously described (Jin *et al.* 2009). Engraftment was measured at 4-6 weeks by quantifying the percentage of hCD45+ cells in the BM by flow cytometry (Jin *et al.* 2009). HL-60 cells (1x10^6) were engrafted into sub-lethally irradiated (275cGy) NOD/SCID mice and after 2 weeks mice were intra-peritoneally injected with either PIK-75 or vehicle alone. A 70%PEG400 vehicle or a 65% w/v cyclodextrin vehicle were used for the engraftment and toxicity experiments. Blood counts were determined by a SYSMEX XE-2100 hematology analyser.

2.16 Nuclear Run-on

Nuclear run-on experiments were performed as previously described (Guthridge *et al.* 1996) using ^{32}P-α UTP labelled nuclei. Briefly, 10^7 MV4-11 cells were lysed in 10mM Tris-HCl, pH 7.4, 10mM NaCl, 3mM $MgCl_2$, 0.5% NP40 and 2mM PMSF at 4°C and

nuclei isolated by centrifugation. Run-on reactions were performed in 5mM Tris-HCl pH 8.0, 2.5mM $MgCl_2$, 150mM KCl and 2.5mM DTT containing 100 μCi ^{32}P-UTP, 0.5 mM ATP, CTP, GTP and 40U RNAsin at 30°C for 1h in the presence of either DMSO (vehicle) or inhibitor. Trizol-extracted ^{32}P-labelled RNA was hybridized to 1μg Mcl-1 or 18S rRNA cDNAs immobilized to nylon filters overnight at 42°C (Guthridge *et al.* 1996). The filters were then washed and exposed to phosphoimager screens and signals quantitated using ImageQuant.

2.17 Luciferase Reporter Assays.

HEK293T cells were co-transfected with constructs expressing the Renilla luciferase gene under the control of a thymidine kinase promoter (pRL) and a construct consisting of the mouse Mcl-1 promoter upstream of the Firefly luciferase gene (Chao *et al.* 1998). Cells were then treated with DMSO (vehicle) or inhibitors for a further 20h following which cell lysates were made and reporter gene activity was determined by the dual luciferase assay system (Promega) as previously described (Guthridge *et al.* 2004). Results are presented as ratios of Firefly/Renilla activities in arbitrary units.

2.18 Kinase Binding Assays

Affinity of PIK-75 for specific purified kinases was measured using a competition assay (Ambit Biosciences, CA). The assay was performed essentially as described (Fabian *et al.* 2005) by combining DNA-tagged kinase, immobilized ATP and PIK-75. The ability of PIK-75 to compete with the immobilized ATP was measured via quantitative PCR of the DNA tag.

Chapter 3

Monoclonal antibody-mediated targeting of CD123 (IL-3 receptor α chain) eliminates human acute myeloid leukaemic stem cells

3.1 Summary

Acute myeloid leukaemia (AML) is a cancer of the myeloid compartment of the bone marrow predominantly affecting older adults with current 5 yr survival rates less than 30%. While conventional chemotherapeutic approaches can effect a complete remission (< 5% blasts in bone marrow) in up to 80% of patients under 60, the majority will still relapse within 3 years with chemoresistant disease (Tallman, 2008; Tallman, Gilliland, & Rowe, 2005).

An emerging body of work pioneered by Bonnet and Dick predicts that leukaemia stem cells (LSCs) are the key to understanding post-treatment relapse and chemoresistance. LSCs form the root or stem of a cellular hierarchy and by definition are the only cells capable of self-renewal while still being able to generate rapidly proliferating progenitors and terminal leukaemic blasts. Since the proposal of the cancer stem cell model in AML, a number of other cancers including chronic myeloid leukaemia, glioma, colon carcinoma, lung, breast and pancreatic cancer have been demonstrated to have a cancer stem cell (CSC) component to varying frequencies and biological properties depending on the assay being interrogated (Clarke et al., 2006).

The principal obstacle to achieving cure in the majority of adult malignancies is failure to eradiate a minor population of chemoresistant tumor cells, leading to relapse of the disease. AML serves as a paradigm in this regard, where current treatments consist of high dose combination chemotherapy regimens that are able to induce remissions in the majority of cases. However, remissions are rarely sustained and, for the most part, this treatment is not curative. An emerging body of evidence indicates that a small population of chemoresistant LSCs may lie at the heart of relapse in AML. Therefore, if cure is to be achieved in AML, and by extension other cancers in which CSCs underlie disease relapse, it is essential to develop new treatments that specifically eliminate LSCs and CSCs.

New generation targeted therapies offer hope in overcoming the shortfalls of conventional cytotoxics, and several anti-cancer monoclonal antibody (mAb) therapies are now in routine clinical use and many more in clinical trials. Prior to the commencement of these

Doctoral studies, most of these mAbs target antigens that are expressed on the bulk tumor cell population (such CD20 in non-Hodgkin's lymphoma, HER2 in breast cancer and CD33 in AML), rather than CSCs. Thus, the clinical picture emerging from mAb therapies in cancer is that, while they can be highly effective in reducing tumor burden and/or remission, this does not necessarily translate into cure.

As outlined in the Introduction (Chapter 1), the overall aim of these PhD studies was to find and develop new targets in AML, especially therapies that interfere with the survival of persistent leukaemia stem or initiating cells likely to be responsible for driving tumour growth *in vivo* and that might be responsible for the persistence of residual disease and relapse. In order to show that a potential anti-LSC therapy is able to target a certain cancer sub-population one first needs to be able to isolate cancer stem cells from patients and develop relevant biological assays using these cells.

CD123 (IL-3α receptor subunit) is over-expressed on the surface of the majority of AML LSCs (Jordan *et al*. 2000). While the precise functional role of CD123 in AML LSCs remains to be determined, it is known to be a potent regulator of cell survival and proliferation in haemopoietic progenitors. Thus CD123 may represent a unique therapeutic target in AML amenable to antibody blockade. We have a specific CD123 monoclonal antibody 7G3 that binds to CD123 with high affinity and blocks IL-3-mediated signalling and biological responses (Sun *et al*. 1996). We therefore sought to examine the potential of 7G3 for selectively binding AML LSCs and blocking IL-3-mediated survival and proliferation.

Thus the overall Aim of this Chapter was to examine the therapeutic potential of targeting the CD123 IL-3α receptor using a blocking monoclonal antibody both *in vitro* and in *in vivo* pre-clinical mouse xenograft models. In order to embark on these studies it was necessary to develop a number of key methods to isolate, purify, and functionally analyse AML LSCs. In the initial part of this chapter, the development of suitable methods is summarised and the results of treating purified LSC populations with IL-3 and 7G3 are presented. In the latter part of this chapter these methods were applied in collaborative studies to explore the therapeutic potential of the 7G3 antibody.

This work important in establishing the methods used to test other potential AML therapeutics presented in later chapters and was an important contribution to the publication of the first-in-kind monoclonal antibody directed against AML-stem cells, Jin et al (2011).

3.2 Significance

The cancer stem cell model has radically changed our approach to developing new therapies in cancer and leukaemia. However very few candidate anti-cancer stem cell specific therapeutic approaches have been demonstrated at the time of writing and few laboratories and/or clinical haematologists have access or expertise in culturing and purifying cancer stem cells from patient samples. This chapter summarizes methods developed to isolate and culture AML $CD34^{+}C38^{-}CD123^{+}$ cells obtained from patients at diagnosis. These methods were then used to demonstrate pre-clinically efficacy of a first-in-kind monoclonal antibody, 7G3, directed against CD123 IL-3 receptor α chain overexpressed on AML stem cells. This work was performed as part of a collaboration with John Dick (Toronto) who originally described the cancer stem cell model in AML and Richard Lock (Children's Cancer Institute Australia) who has developed NOD-SCID AML engraftment models with the AML patient samples collected and characterised in this thesis.

3.3 Development of a method to isolate AML stem/progenitor cells using FACS

In order to perform analysis of the status of the IL-3/GM-CSF receptor in AML we first required a consistent source of primary patient samples that were clinically annotated, collected at diagnosis prior to any treatment and had sufficient numbers and quality of cells after thawing. The Royal Adelaide Hospital is a large tertiary central hospital that treats the majority (>70%) of new AML patients in South Australia. We established a 24-hr on-call link with leukapheresis nurse in the Haematology Day Centre and bone marrow biopsy nurse who would alert us of any possible or confirmed cases of AML. A project-specific consent form and ethics approval was obtained from RAH Ethics Committee and protocols

to collect 2 x 10ml samples in EDTA from bone marrow and 500ml leukapheresis bags in acid-citrate dextrose left overnight on cell-rocker (for details see Chapter 2).

We found that the time taken to freezing of fresh marrow or leukapheresis material was a strong determinant of cell viability after thawing. We also found much within patient variability even in vials frozen on the same day and AML stem/progenitor cell yields were optimal after an overnight culture following thawing. AML stem/progenitor cell yields were enriched after a repeat Ficoll-Hypaque centrifugation prior to cell sorting. We also noted AML stem/progenitor cells had improved survived for when cultured in low serum (0.5%) compared to 10% serum (data not shown). These observations led to all experiments on LSPCs performed in this thesis were cultured in 0.5% serum IMDM.

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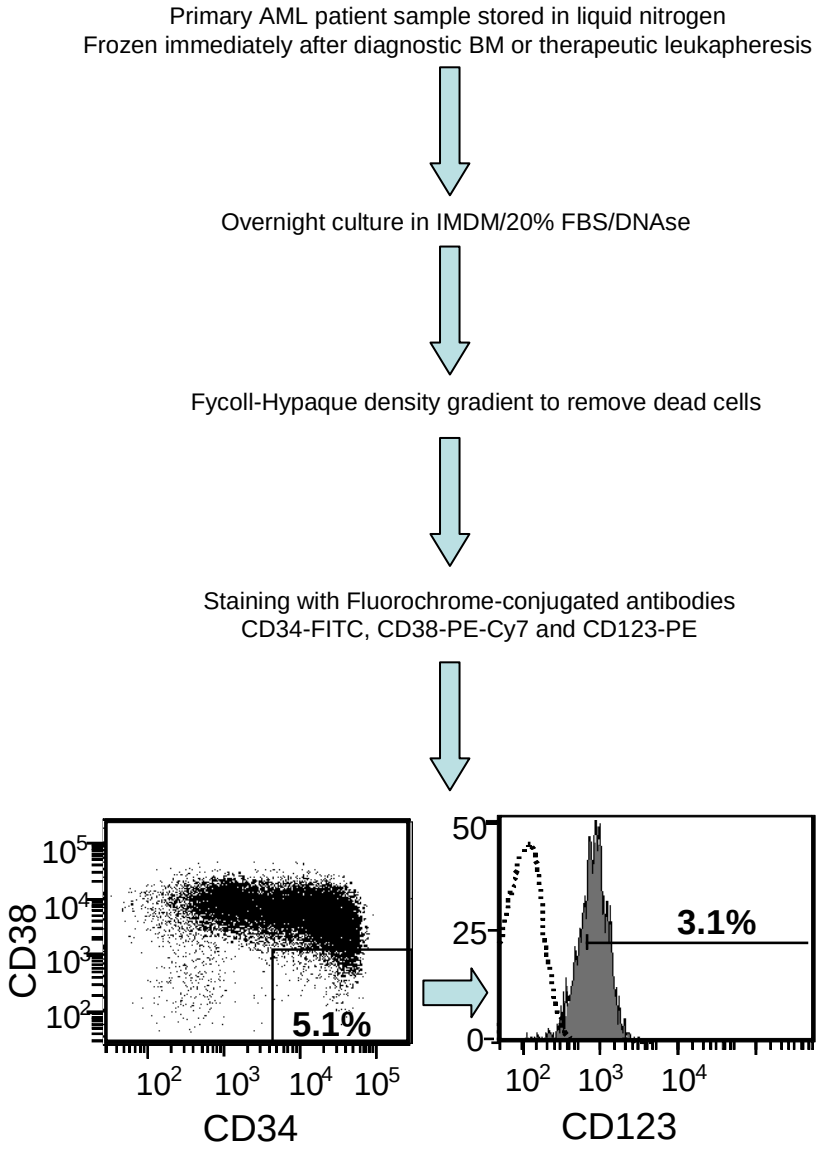

Figure 3.1: FACS sorting strategy for isolation of CD34+CD38-CD123+ AML stem/progenitor cells. For sorting of CD34+CD38-CD123+ primitive AML cells, mononuclear cells were washed 3 times in IMDM/10% FCS then stained for 30 min at 4℃ in 2 ml IMDM/10% FCS with 80 µl each of CD34-FITC, CD38-PE-Cy7 and CD123-PE antibodies (BD Biosciences, San Jose, California, USA). On an average sort between 2-8x10^5 cells were isolated. For patient details see Table 1 in publication Linquin et al, 2009.

3.4 7G3 mAb directed against CD123 inhibits survival of AML CD34$^+$CD38$^-$ cells

The IL-3 receptor consists of a ligand-specific alpha subunit, CD123, and a common beta signalling subunit, βc. A number of studies report overexpression of CD123 on AML blasts, CD34+ leukaemic progenitors and AML-LSCs in comparison with normal haematopoietic stem cells (Jordan *et al.* 2000; Testa *et al.* 2002). The high expression of CD123 appears to have biological significance as AML blasts proliferate in response to IL-3 treatment *in vitro* and some AMLs secrete IL-3 suggesting an autocrine proliferation loop. High CD123 expression levels are also associated with high blast counts and lower remission rates (Testa *et al.* 2002). However, the biological significance of CD123 over-expression and IL-3 signalling in AML-LSC populations is not known.

Very little is understood regarding the molecular regulation that controls AML-LSC survival or self-renewal. For example, whilst IL-3 may be an important survival factor in bulk AML blasts in some patients, it may not be as important at the stem cell level. Likewise, a specific role for IL-3 in homing or autocrine survival signalling of AML stem cells has not been investigated. The mAb 7G3 raised against IL-3 receptor alpha/ CD123 possesses excellent IL-3R neutralizing properties and can inhibit IL-3 induced proliferation (Sun *et al.* 1996). Thus 7G3 represents an ideal tool to study the biological significance of IL-3 in regulating AML LSC function.

Firstly, we wished to test the effect of adding exogenous 7G3 and IL-3 or both on purified AML CD34$^+$CD38$^-$ populations and measure cell survival. For the majority of patients, only a small number of AML CD34$^+$CD38$^-$ cells ranging from $1x10^5$ to $8x10^5$ could be obtained from a given cell sorting procedure. This was due to a number of variables and including i) the overall CD34 and CD38 expression and the interaction of these markers on a given patient's AML - which for most patients this population was less than 2% of bulk (Bonnet & Dick, 1997; Jin et al., 2009); ii) limits on speed and time to access cell sorter (less than 8hrs per day); and iii) percentage of viable AML cells after thawing (usually <50% of bulk). For each sort, a purity check confirmed expression of CD34 and CD123 (>95%) and low to absent CD38.

Four AML patient samples consistently gave sufficient $CD34^+CD38^-$ subpopulation cells for survival analysis (Figure 3.2). In the absence of IL-3, cell survival as measured by annexin V staining was low at 72 hrs (median 22%, ranging from 10 to 60% annexin V negative). 3 out of 4 AMLs showed an increase in cell survival after addition of IL-3, suggesting IL-3 promotes a survival response in LSC enriched sub-populations (Figure 3.2). In two patients, the addition of 7G3 mAb in the absence of IL-3 resulted in diminished cell survival at 72hrs. This is in keeping with endogenous IL-3 production by cells within the $CD34^+CD38^-$ sub-population that functions as an autocrine survival loop. In 3 out of 4 samples, the addition of 7G3 completely blocked the effect of IL-3 on cell survival, confirming that 7G3 has biological activity in purified $CD34^+CD38^-$ AML cells.

The effect of IL-3 on proliferation was measured by BrdU incorporation. However, unlike AML cell-lines no evidence of BrdU uptake could be demonstrated in AML $CD34^+CD38^-$ cells in vitro (data not shown) in the same conditions for which cell survival was measured. This data suggests the increased cell survival observed in the presence of IL-3 (Figure 3.2) is not primarily due to an effect on cell proliferation.

In conclusion, these *in vitro* studies suggest IL-3 has both pro-survival and autocrine biological effects on AML $CD34^+CD38^-$ subpopulations enriched for AML stem cell activity. The dependence on IL-3 is heterogeneous and the pro-survival effect is modest. The data is limited by the few numbers of samples analysed and the short term course of the assay. IL-3 receptor blocking antibody 7G3 can inhibit IL-3 induced survival of purified populations of AML CD34+CD38- cells.

3.5 7G3 blocks phospho-signalling in AML $CD34^+CD38^-$ cells

The data above suggested that similar to bulk AML blasts, $CD34^+CD38^-$ subpopulations could respond to exogenous IL-3 and cell survival blocked by CD123 targeting mAb. This implies a functional receptor signalling complex on the surface of AML $CD34^+CD38^-$ cells. However, functional analysis of AML LSC signalling in response to exogenous cytokine has not been reported. Because of the scarcity of these cells, standard methods of

detection such as Western blotting could not be employed to interrogate downstream signalling in this population by IL-3. Therefore, in order to further demonstrate that AML $CD34^+CD38^-$ subpopulations could signal in response to IL-3 we needed to develop methods to assay intracellular signalling in small populations of cells. We used standard phospho-flow protocols developed by BD Biosciences and found detectable ERK and STAT5 but not AKT phosphorylation shifts in response to IL-3 (Figure 3.3). In bulk AML cells and TF-1 GM-CSF dependent cell-line, consistent phosphor-STAT5 phosphorylation could be observed in response to IL-3. In 4 out of 4 AML $CD34^+CD38^-$ subpopulations analysed, a phospho-STAT5 response was clearly observed. There was no clear correlation between IL-3 induced STAT5 phosphorylation and cell survival in response to IL-3. This would indicate that the presence of a functional receptor does not necessarily translate into a biological response in AML $CD34^+CD38^-$ cells. Similar findings have been observed in AML bulk blasts (Irish *et al.* 2004; Budel *et al*. 1990; Piao *et al*. 1989).

The addition of 7G3 completely blocked IL-3 induced STAT5 phosphorylation in 4 out of 4 $CD34^+CD38^-$ subpopulations (Figure 3.3). This indicates 7G3 can effectively inhibit IL-3 signalling in these cells. This provides additional support for a CD123 targeting monoclonal as having biological and biochemical effects on AML LSCs.

Using the approaches developed above for the biochemical and functional analysis of defined FACS-purified populations of AML cells, we then examined the therapeutic potential of targeting CD123. In the paper below, we have shown that AML-LSCs can be targeted with the CD123-specific 7G3 mAb resulting in impaired human AML cell engraftment, reduced disease burden and improved long-term survival in NOD/SCID mice. Our studies identify at least three mechanisms by which 7G3 targetted AML LSCs. Firstly, 7G3 was able to inhibit LSC homing to the BM niche. Secondly, using NOD/SCID mice depleted of T, B and NK cells, we demonstrate that 7G3 recruits the residual innate immunity in NOD/SCID recipients to allow targeting of LSCs expressing CD123. Thirdly, we show that 7G3 is able to block IL-3-mediated intracellular signaling and cellular responses in $CD34^+CD38^-$ AML cells (enriched for LSCs) *in vitro* thereby potentially blocking deregulated IL-3 autocrine loops in leukaemia. Collectively, these results were important as they provided the first demonstration that targeting CD123 that is over-expressed on the surface of LSCs may have therapeutic utility for the treatment of AML.

They were also important in providing the first demonstration of a targeted therapy against LSCs had therapeutic potential. On the basis of these results, 7G3 has been developed as a drug in Phase I clinical trials for patients with relapsed and refractory AML (http://clinicaltrials.gov/ct2/show/NCT00401739?term=CSL-360&rank=1).

Thus far, Phase I clinical data using a recombinant chimeric IgG1 mAb derived from 7G3 is very encouraging (Roberts *et al*. 2010). The drug, termed CSL360, is safe and tolerable in humans and biological effects have been demonstrated at doses up to 10mg/kg. Patients received 12 weekly intravenous infusions of CSL360 and bone marrow aspirates/trephines samples were obtained after screening and after dose 3, 5 and 11. The mean plasma half-life was 117 ± 59 hrs with a low systemic clearance (0.21 ± 0.16 L/h) and volume of distribution (0.39 ± 0.22 L/kg). Intriguingly a sustained complete remission was achieved after 3 doses and sustained for > 9 weeks in a advanced refractory AML patient that had relapsed after allogeneic stem cell transplant. A total of 17 doses were administered to this patient before withdrawal to treat comorbidities. Further clinical studies are ongoing but these studies suggest the mAb has activity *in vivo* in patients.

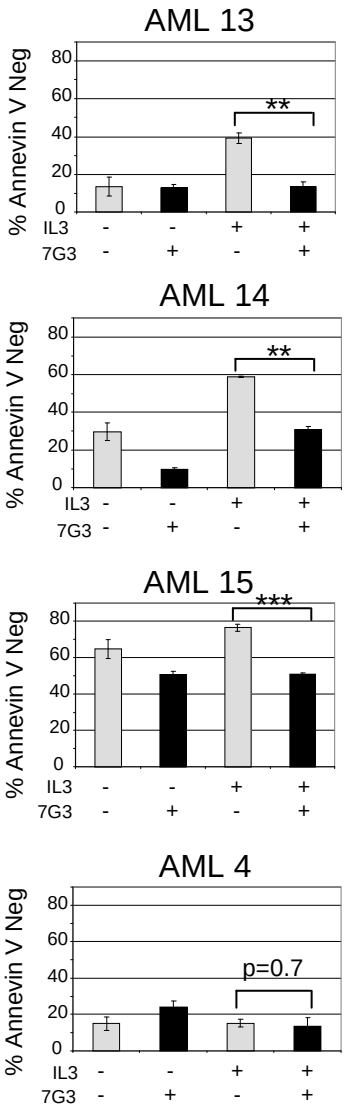

Figure 3.2: Survival analysis of $CD34^+CD38^-CD123^+$ AML cells in response to IL-3 and blocking mAb 7G3. Primary BM or PB AML cells from patients at diagnosis were sorted for $CD34^+CD38^-$ sub-populations after thawing and culturing in 20% FBS/IMDM/DNAse overnight. Approximately $2x10^5$ to $1x10^6$ cells were obtained per patient isolation procedure. These were plated at $2x10^5$/ml in 96 well round-bottomed plates in 0.5% FBS/IMDM +/- 7G3 100μg/ml or IgG2a (clone BM4) 30min prior to addition of human IL-3 100nM. The cells were assayed for survival by 1:100 Annexin V-FLUOS and Cell Count fluorospheres (100μl added to 300μl cell suspension) at 72 hrs. ** denotes $p<0.01$, *** $p<0.0001$

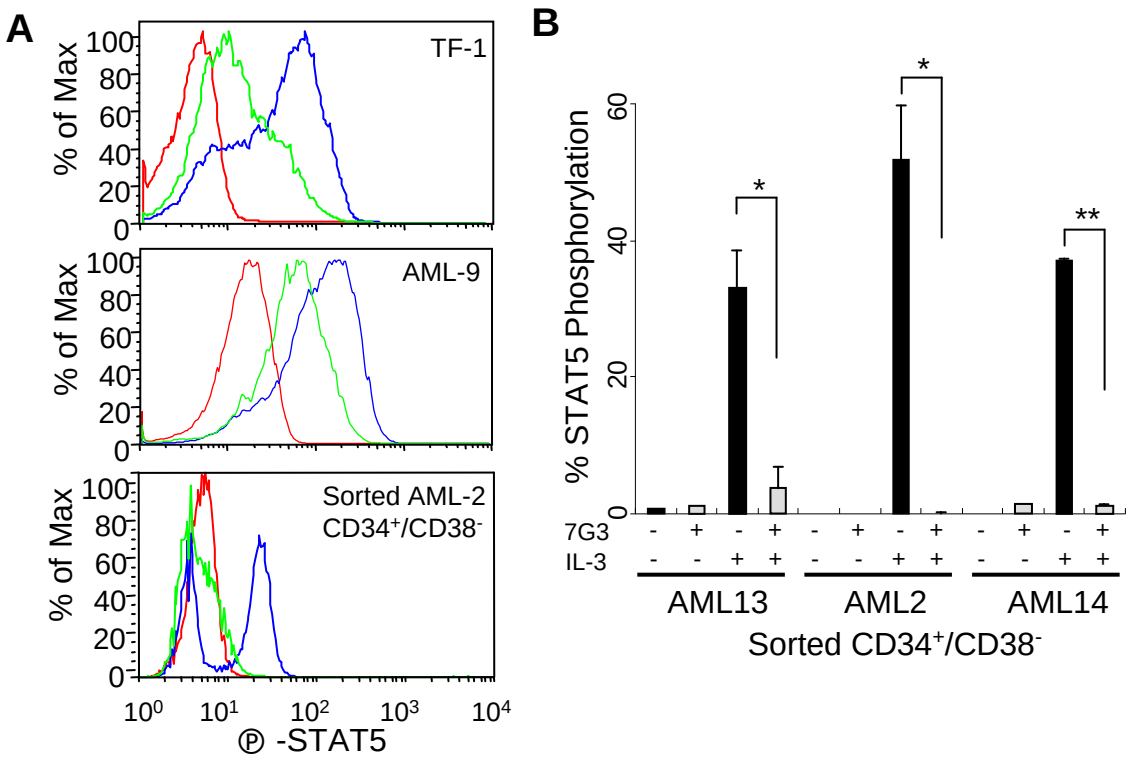

Figure 3.3: Analysis of intracellular Stat5 tyrosine phosphorylation in AML stem/progenitor cells. (A) TF-1, bulk and sorted primary AML cells were stimulated with 20ng/ml IL-3 plus 20µg/ml IgG2a or 7G3 for 1h and analysed by FACS for phospho-STAT5 activation shown by representative histograms. Colour coding: red, no IL-3; blue, IL-3 with IgG2a; green, IL-3 with 7G3. (B) Sorted $CD34^+CD38^-$ subpopulations were incubated with 150µg/ml 7G3 or IgG2a for 30 min on ice before simulation 1nM IL-3 for 15 min. Cells were fixed with BD Cytofix Buffer (BD Biosciences), methanol permeabilized, and stained with anti-phopshoSTAT5 (BD Biosciences) or isotype control. Cells were then analysed using a FACSCalbur flow cytometer (BD Biosciences). Mean ± SEM in duplicates, *=p<0.05,**=p<0.01.

3.6 (Publication)

Monoclonal antibody-mediated targeting of CD123 (IL-3 receptor α chain) eliminates human acute myeloid leukemic stem cells

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Article

Monoclonal Antibody-Mediated Targeting of CD123, IL-3 Receptor α Chain, Eliminates Human Acute Myeloid Leukemic Stem Cells

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SUMMARY

Leukemia stem cells (LSCs) initiate and sustain the acute myeloid leukemia (AML) clonal hierarchy and possess biological properties rendering them resistant to conventional chemotherapy. The poor survival of AML patients raises expectations that LSC-targeted therapies might achieve durable remissions. We report that an anti-interleukin-3 (IL-3) receptor α chain (CD123)-neutralizing antibody (7G3) targeted AML-LSCs, impairing homing to bone marrow (BM) and activating innate immunity of nonobese diabetic/severe-combined immunodeficient (NOD/SCID) mice. 7G3 treatment profoundly reduced AML-LSC engraftment and improved mouse survival. Mice with preestablished disease showed reduced AML burden in the BM and periphery and impaired secondary transplantation upon treatment, establishing that AML-LSCs were directly targeted. 7G3 inhibited IL-3-mediated intracellular signaling of isolated AML $CD34^+$ $CD38^-$ cells in vitro and reduced their survival. These results provide clear validation for therapeutic monoclonal antibody (mAb) targeting of AML-LSCs and for translation of in vivo preclinical research findings toward a clinical application.

INTRODUCTION

The 5 year survival rate of patients under 60 years of age with AML is less than 30%, with progressively worse prognosis for more elderly patients (Estey and Dohner, 2006). The cellular and molecular basis for this dismal picture is unclear; however, a large body of work is emerging in experimental systems that predicts LSCs may lie at the heart of posttreatment relapse and chemoresistance. AML is organized as a cellular hierarchy sustained by LSCs at their apex (Bonnet and Dick, 1997; Guan and Hogge, 2000; Guzman et al., 2001; Hope et al., 2004; Lapidot et al., 1994; Wang and Dick, 2005). LSCs are the only AML cells capable of self-renewal while still generating rapidly proliferating progenitors and terminal leukemic blasts. The rare occurrence, generally dormant nature, and abnormal apoptotic response via the NF-κB pathway of LSCs are all properties that may render them resistant to conventional chemotherapeutics that target proliferating cells (Bonnet and Dick, 1997; Guan and Hogge, 2000; Guzman et al., 2001; Hope et al., 2004; Ishikawa et al., 2007; Lapidot et al., 1994; Wang and Dick, 2005). In addition, minimal residual disease occurrence and poor survival have been attributed to the ability of LSCs to engraft NOD/SCID mice (Pearce et al., 2006) and high $CD34^+CD38^-$ frequency at time of diagnosis in AML patients (van Rhenen et al., 2005). Consequently, it is imperative that new treatments are developed to complement established chemotherapy by specifically eliminating AML-LSCs for the long-term management of the disease (Abutalib and Tallman, 2006; Aribi et al., 2006; Morgan and Reuter, 2006; Stone, 2007).

As with normal hematopoietic stem cells (HSCs), very little is known of the molecular regulation that governs the self-renewal, differentiation, and survival of AML-LSCs, although both of these stem cell types share the properties of slow division, self-renewal ability, and expression of some surface markers including the $CD34^+CD38^-$ immunophenotype (Bhatia et al., 1997; Bonnet and Dick, 1997; Lapidot et al., 1994). CD123, which is widely reported to be overexpressed on AML blasts, $CD34^+$ leukemic progenitors, and AML-LSCs in comparison with normal HSCs (Florian et al., 2006; Graf et al., 2004; Hauswirth et al., 2007; Jordan et al., 2000; Munoz et al., 2001; Riccioni et al., 2004; Sperr et al., 2004; Testa et al., 2002; Yalcintepe et al., 2006), represents a promising cell-surface target for the development of therapeutics that specifically target AML-LSCs but not HSCs. CD123 is the α subunit of the IL-3 receptor (IL-3R), the major binding protein for IL-3, which together with CD131 (β_c) forms the functional heterodimeric high-affinity IL-3R. The binding of IL-3 to CD123 is species specific and leads to activation of the receptor that promotes cell survival and proliferation (Bagley et al., 1997; Miyajima et al., 1993).

Overexpression of CD123 on AML cells confers a range of growth advantages over normal HSCs; AML cells proliferate

extensively with IL-3 treatment in vitro (Budel et al., 1989; Miyauchi et al., 1987; Pebusque et al., 1989; Vellenga et al., 1987), and some AML samples secrete cytokines including IL-3 (Elbaz and Shaltout, 2001; Guan et al., 2003; Nowak et al., 1999). Moreover, high-level CD123 expression on AML cells correlates with the level of IL-3-stimulated and spontaneous signal transducer and activator of transcription 5 (STAT5) activation, the proportion of cycling cells, a more primitive cell-surface phenotype, and resistance to apoptosis (Graf et al., 2004; Testa et al., 2002, 2004). Clinically, high CD123 expression in AML is associated with higher blast counts at diagnosis and a lower complete remission rate that results in reduced survival (Graf et al., 2004; Testa et al., 2002, 2004). Collectively, these data point to the significance of CD123 expression in leukemia cell stimulation and AML patient outcome.

The increased expression of CD123 on LSCs compared with HSCs presents an opportunity for selectively targeting AML-LSCs with a therapeutic antibody. Besides the possibility that IL-3 is required for LSC functions, an antibody to CD123 could stimulate host immune-mediated mechanisms for cell killing. An antibody with both IL-3R-neutralizing and innate immunity-activating properties could represent an ideal therapeutic candidate for clinical testing. The mAb 7G3, raised against CD123, has previously been shown to inhibit IL-3-mediated proliferation of leukemic cell lines (Sun et al., 1996). While AML-LSCs are often cited to be enriched in the $CD34^+CD38^-$ fraction, recent reports have demonstrated that other fractions, such as the $CD34^+CD38^+$ subpopulation, also have NOD/SCID repopulating capacity (McKenzie et al., 2006; Taussig et al., 2008). In this report, we show that CD123 is highly expressed on the bulk of AML cells as well as the $CD34^+CD38^-$ fraction compared to normal hematopoietic cells. Importantly, we demonstrate that 7G3 targeting of CD123 in the absence of exogenous human cytokines impairs AML-LSCs in vivo. This occurs through at least two mechanisms involving inhibition of homing of $CD34^+CD38^-$ cells and engraftment of AML-LSCs in the NOD/SCID xenograft model, as well as activation of innate immunity in NOD/SCID mice. As a prerequisite for the potential role of 7G3 in inhibiting IL-3-mediated growth advantages on AML-LSCs, we demonstrate that both the unsorted and the $CD34^+CD38^-$ subpopulations of AML cells proliferate and survive via IL-3-mediated intracellular signaling pathways and that these are inhibited by 7G3 in vitro. The recent characterization of defined populations of cancer stem cells (CSCs) in a range of human malignancies (Wang, 2007), as well as their relative resistance to conventional chemotherapy and radiotherapy (Rich and Bao, 2007), supports the broad applicability of our approach and provides rationale for the progression of AML-LSC-targeted therapeutics from preclinical evaluation to clinical trials.

RESULTS

Ex Vivo 7G3 Treatment Selectively Inhibits AML Engraftment in NOD/SCID Mice

Since AML-LSCs are central to long-term AML growth and they are difficult to assay in vitro, we used the SCID-leukemia initiating cell (SL-IC) assay to determine whether 7G3 can directly target AML-LSCs and inhibit their repopulating ability. Ex vivo 7G3 incubation markedly reduced the engraftment of 10 of 11 primary AML samples in sublethally irradiated NOD/SCID mice to a mean of 11.4% ± 1.9% of isotype-matched (IgG2a-treated) controls ($p = 0.00021$, Figure 1A, Table 1). This reduction in engraftment was sustained in five of seven samples when assessed between 8 and 10 weeks following inoculation (5.7% ± 1.7% of controls, $p = 0.004$). Ex vivo 7G3 treatment inhibited the engraftment of AML-8 harvested at both diagnosis and relapse to a similar extent. AML-5 was the only AML sample in which engraftment was not reduced by ex vivo 7G3 treatment. Although the reason for this is unknown, it is noteworthy that AML-5 is a monosomy 7 sample (noted for poor prognosis) with relatively low CD123 expression (Table 1).

We next investigated the sensitivity of normal cord blood (CB) and BM (NBM) to 7G3 using the same strategy as for AML samples to determine if there was differential targeting of normal HSCs. When measured at 4–11 weeks postinoculation, 7G3 significantly reduced the engraftment of only two of five normal samples (Figure 1B and Table 1). The inhibitory effect of 7G3 on the engraftment of normal cells (76.5% ± 8.9% engraftment relative to IgG2a controls) was significantly less ($p < 0.0001$) than against AML cells. Additionally, 7G3 treatment did not alter the differentiation profiles of the engrafted normal human hematopoietic populations (data not shown). Furthermore, to demonstrate the clinical relevance and specificity of 7G3 treatment against LSCs and not normal HSCs, we showed that a mouse anti-human HLA-A,B,C antibody indiscriminately inhibited the engraftment of two AML and three normal samples (see Figure S1 available online). Independent analysis at two different institutions (Sydney and Toronto) revealed that CD123 expression on AML $CD34^+CD38^-$ cells (relative fluorescence index [RFI] 38.2 ± 6.6) was significantly higher than on their normal counterparts (RFI 9.6 ± 1.6) (Figure 1C and Table 1). The engraftment levels of ex vivo 7G3-treated samples were inversely correlated with the intensity of CD123 expression on the $CD34^+CD38^-$ population (Figure S2; Spearman $R = -0.69$). Taken together, we can conclude that normal HSCs are considerably less sensitive to 7G3 than AML-LSCs, due, at least in part, to their relatively low levels of cell-surface CD123 expression.

The reduction in AML engraftment caused by ex vivo 7G3 treatment was also associated with improved survival. Mice transplanted with IgG2a- or 7G3-treated AML-9 cells exhibited median survival of 11.5 and 24 weeks, respectively (Figure 1D), with 40% of the 7G3 group surviving beyond the end of the experiment (25 weeks), in contrast with the control group, in which no mice survived beyond 20 weeks.

7G3 Inhibits AML Homing Capacity in NOD/SCID Mice

To gain insight into the mechanism whereby 7G3 inhibited AML-LSC engraftment, we investigated the influence that antibody binding had on AML cell trafficking, since the SL-IC assay requires AML-LSCs to traffic to the BM in order to survive and proliferate, thereby establishing a leukemic graft. Homing assays were performed on two AML samples (AML-8-rel and -9) following ex vivo 7G3 treatments. 7G3 reduced the homing efficiency of AML-9 in the BM to 12.2% ± 2.7% and in the spleen to 9.4% ± 2.4% of controls (Figure 2A), and inhibited the homing of AML-8-rel in the BM to 34.7% ± 5.6% (Figure 3A) and in the spleen to 46.9% ± 3.5% of controls. To better distinguish the

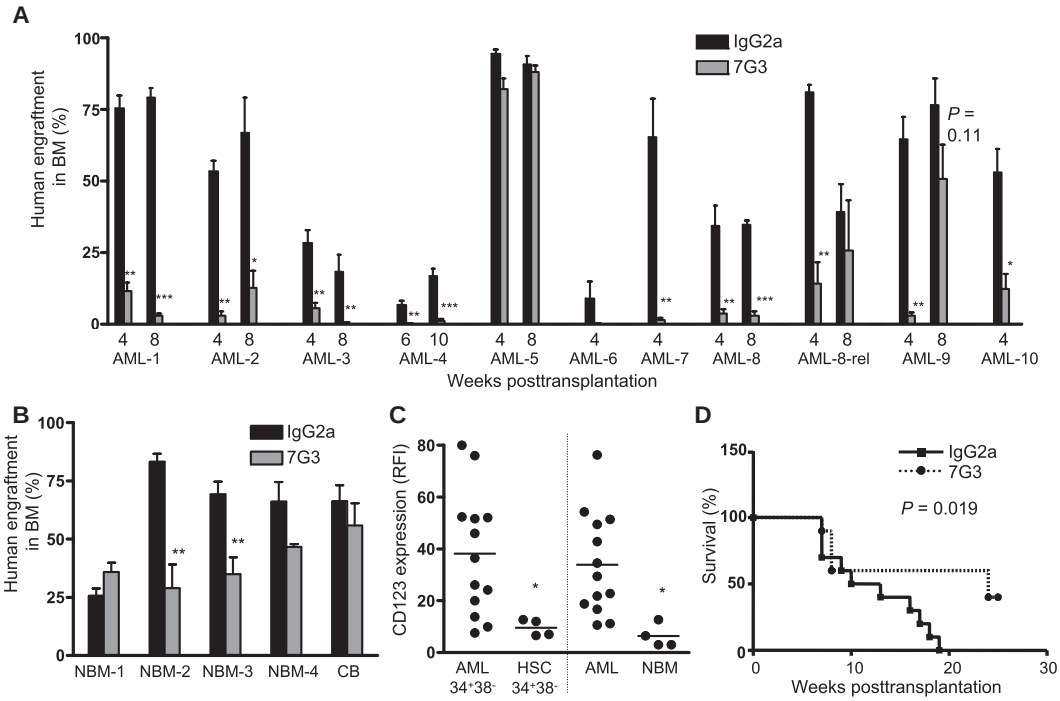

Figure 1. Ex Vivo 7G3 Treatment Selectively Inhibits the Repopulating Ability of AML Primary Cells in NOD/SCID Mice

(A) Percentage of human AML cells in the BM of mice transplanted with 7G3 or IgG2a control-treated AML cells at indicated time points. n = 3–10 per treated group.

(B) Levels of human engraftment in the BM of mice transplanted with 7G3 or IgG2a control-treated CB and NBM cells. Bars of CB represent the results from three separate experiments. n = 4–6 mice per group in each experiment.

(C) CD123 expression on total and $CD34^+CD38^-$ fractions of AML and normal cells. Each point represents an individual sample. Bars represent the mean.

(D) Kaplan-Meier survival curves of mice transplanted with IgG2a or 7G3 ex vivo-treated AML-9 cells. Survival curves were compared by log rank test. n = 10 per group. Error bars represent mean ± SEM; *$p < 0.05$, **$p < 0.01$, and ***$p \leq 0.0001$ between selected groups.

effects of 7G3 on AML homing, lodgment, and proliferation, ex vivo-treated AML-8-rel cells were transplanted intravenously (i.v.) via the tail vein or directly into the right femur (RF). The intrafemoral (IF) approach circumvents the AML-LSC trafficking/homing processes associated with the circulation (Mazurier et al., 2003). While 7G3 remained effective in significantly reducing the engraftment in both the injected femur and the noninjected bones, IF inoculation did attenuate the inhibitory effects of 7G3 on engraftment in comparison with i.v. inoculation (Figure 2B).

In order to more directly demonstrate 7G3 inhibition of AML-LSCs, we investigated the impact of 7G3 treatment on $CD34^+CD38^-$ cells since AML-LSCs are significantly enriched in this fraction (Bonnet and Dick, 1997). The number of $CD34^+CD38^-$ cells from AML-8-rel and AML-9 homing to the BM was reduced by ex vivo 7G3 treatment to 8.4% ± 0.018% and 12.0% ± 4.3% of control, respectively (Figure 2C). Similarly, the number of AML-9 $CD34^+CD38^-$ cells homing to the spleen was reduced to 3.8% ± 1.5% of control. To further confirm this finding, the homing assay was repeated with sorted $CD34^+CD38^-$ cells from AML-9 following ex vivo antibody treatment. The homing efficiency of human cells in the 7G3-treated group was reduced to 7.8% ± 1.7% of IgG2a controls in the BM and 11.2% ± 0.84% in the spleen (Figure 2D). Consistent with the observation that 7G3 diminished AML-LSC homing capacity, ex vivo 7G3 treatment reduced the number of $CD34^+CD38^-$ cells in the BM xenografts of three AML samples (Figure 2E). By contrast, the number of $CD34^+CD38^-$ cells present in xenografts established from four independent normal hematopoietic samples following ex vivo 7G3 treatment was 81.9% ± 11.6% of IgG2a controls (p = 0.19, data not shown). Collectively, we can conclude that 7G3 inhibits not only homing but also lodgment and proliferation of AML-LSCs in the BM microenvironment.

7G3-Mediated Inhibition of AML-LSC Homing and Engraftment Is Fc Dependent

In order to determine whether the inhibitory effects of 7G3 are Fc mediated, the homing efficiency of AML cells was examined following treatment with F(ab′)2 fragments of various CD123-targeting antibodies. Incubation of AML-8-rel with two MAbs clones, 6H6 and 9F5, that bind CD123 but are weakly neutralizing reduced the homing efficiency in the BM to a similar extent as 7G3 (Figure 3A). In contrast, when AML-8-rel cells were treated ex vivo with 7G3 or 6H6 F(ab′)2 fragments, the inhibitory effects of each antibody on AML homing were attenuated.

In addition, the Fc requirement for inhibition of NOD/SCID repopulation was also examined. While ex vivo incubation of AML-9 and AML-10 cells with 7G3 or 9F5 significantly reduced their ability to repopulate mouse BM, the corresponding F(ab′)2

Table 1. Patient Characteristics, CD123 Expression, and Effects of mAb 7G3 on Engraftment of AML and Normal Hematopoietic Cells

Cells Transplanted	Age/ Sex	AML Subtype	Cytogenetics	Flt3 Mutational Status	WCC	Current Clinical Status	Sample Type	Overall Survival (Days)	$CD34^+$ $CD38^-$ (%)	CD123 (RFI)	Effect of 7G3 (Engraftment as % Control)
AML											
1	69/F[a]	M0[b]	Normal	Mutant	231[a]	Deceased	Apheresis	288[c]	2.9[d]	52.1[e]	15.2[f]
2	70/F	M1	Normal	Mutant	270	Deceased	Apheresis	196	2.2	26.1	5.3
3	64/F	M5b	Normal	Wild-type	80	Deceased	BM	1378	0.048	9.9[g]	19.5
4	75/M	M5a	Trisomy 8	Mutant	300	Deceased	Apheresis	27	3.5	36.5	4.7
5	19/F	M2	Maturation/ monosomy 7	NA	108	Deceased	NA	NA	6.2	13.8	97.1
6	53/M	M4Eo	Inv16	Mutant	300	Deceased	BM	504	4.9	24.2	1.5
7	80/M	M5	NA	NA	122	NA	Apheresis	45	1.9	80	2
8	47/F	M4	NA	NA	33	Deceased	Apheresis	436	8.28	46	11.1
8—Relapse	47/F	M4	NA	NA	33	Deceased	Apheresis	436	6.6	52.4	17.3
9	55/F	M5a	46XX	NA	161	NA	Apheresis	4	35.1	76.1	1.4
10	80/F	M2	46XX	NA	130	NA	Apheresis	NA	38.5	7.6	23.2
11	78/M	M2	Normal	Wild-type	166	Deceased	BM	26	0.2	51.7[g]	NE
12	67/M	M5b	Normal	Mutant	212	Deceased	BM	2	0	20[g]	NE
Normal											
NBM-1	26/M	–	–	–	–	–	BM	–	0.4	12	139.9
NBM-2	35/M	–	–	–	–	–	BM	–	2.3	6.7	34.8
NBM-3	32/M	–	–	–	–	–	BM	–	0.4	7	50.4
NBM-4	NA	–	–	–	–	–	BM	–	NA	NA	70.3
CB-1	NA	–	–	–	–	–	CB	–	NA	12.7	79.1

NBM-3 was a $CD34^+$ sorted normal BM sample. BM, bone marrow. CB, cord blood. NA, not available. NE, no engraftment in controls. WCC, peripheral blood white cell count ($\times 10^9$/L).
[a] At diagnosis (this column).
[b] FAB criteria (this column).
[c] From date of initial diagnosis (this column).
[d] Percent of total population (this column).
[e] RFI of $CD34^+CD38^-$ population (this column).
[f] Mean engraftment in the ex vivo 7G3-treated group as a percentage of the IgG2a-treated group, based on Figures 1A and 1B (this column).
[g] Sample had very low proportion of $CD34^+$ cells.

antibody fragments were ineffective (Figure 3B), despite 7G3 F(ab′)2 retaining its IL-3Rα-neutralizing activity (data not shown). The requirement for Fc regions to inhibit homing and repopulation, combined with the reduced efficacy of 7G3 when trafficking in the circulation was circumvented by IF transplantation, strongly supports a role for the innate immune system in mediating at least a portion of the inhibitory effects of 7G3.

$CD122^+$ Cells Contribute to 7G3-Mediated Inhibition of AML Homing and Repopulation in NOD/SCID Mice

While NOD/SCID mice are devoid of functional T and B cells, and are defective in complement fixation, they retain residual levels of innate effector activity (principally due to NK cells and macrophages) that can affect stem cell engraftment. To determine whether residual NOD/SCID innate immunity contributed to the inhibitory effects of 7G3 on LSCs, mice were injected with anti-CD122 mAb prior to transplantation with ex vivo 7G3-treated AML-8-rel cells. In the IgG2a control-treated groups, leukemic engraftment in the $CD122^+$ cell-depleted mice was increased to 113.3% ± 2.8% of nondepleted mice (Figure 3C), reflective of our earlier data showing increased detection of HSCs in such recipients (McKenzie et al., 2005). We found that depletion of $CD122^+$ cells significantly attenuated the ability of 7G3 to reduce leukemia engraftment from 82.7% ± 9.4% to 39.8% ± 14.3% inhibition relative to control antibody (Figure 3C), although a significant difference still remained between 7G3-treated and IgG2a-treated groups. Similarly, when we used NOD/SCID interleukin-2 receptor γ chain null mice (NOD/SCID/IL-2Rγ^{null}), which have lower residual NK cell activity than NOD/SCID mice (Ito et al., 2002), we observed similar attenuation, but not complete ablation, of the inhibitory effects of ex vivo 7G3 treatment on AML-1 engraftment in the BM (33.3% ± 12.6% of control compared with 1.1% ± 0.9% of control for NOD/SCID mice, Figure S3).

Additionally, anti-CD122 antibody treatment also partially attenuated the ability of 7G3 to block AML cell homing to the BM observed in both AML-8-rel and AML-9. As shown in Figure 3D, the homing efficiency of AML-9 cells treated with 7G3 was 8.4% ± 1.4% of control, and this was attenuated to 18.2% ± 3.1% with depletion of $CD122^+$ cells. The number of

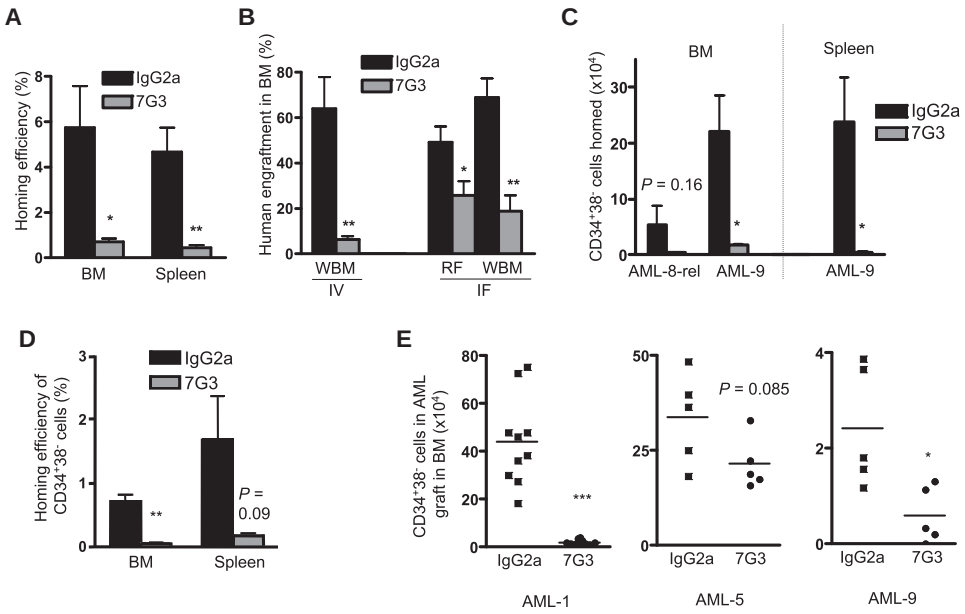

Figure 2. Inhibition of AML-LSC Homing Contributes to the Inhibitory Efficacy of 7G3

(A) Homing efficiency of AML-9 cells to the BM and spleen of mice following ex vivo 7G3 treatment from two separate experiments. n = 3–6 per group.

(B) Engraftment of ex vivo antibody-treated AML-8-rel cells in the injected femur (RF) and whole BM (WBM) after i.v. (IV) or intrafemoral (IF) transplantation. n = 4–5 mice per group.

(C) Absolute number of $CD34^+CD38^-$ AML cells homed in the BM and spleen of NOD/SCID mice injected with ex vivo 7G3-treated leukemic cells. n = 2–3 or 5 mice per group for AML-8 and AML-9, respectively.

(D) Homing efficiency of sorted $CD34^+CD38^-$ AML-9 cells after ex vivo treatment into both BM and spleen of mice. n = 3 mice per group.

(E) The number of $CD34^+CD38^-$ cells in the AML graft of mouse BM transplanted with AML-1, -5, and -9 after ex vivo IgG2a or 7G3 treatment. Each symbol represents a single mouse; horizontal bars indicate the mean. Error bars represent mean ± SEM; *$p < 0.05$, **$p < 0.01$, and ***$p \leq 0.0001$ between IgG2a and 7G3 groups.

$CD34^+CD38^-$ cells that homed to the BM of mice was also reduced to 5.3% ± 1.1% of control (Figure 3E), and this number was only marginally increased by the addition of anti-CD122 antibody (8.2% ± 1.9% of control, Figure 3E).

Collectively, our results indicate that the ability of 7G3 to inhibit engraftment and homing of AML cells in NOD/SCID mice is mediated by at least two cooperative pathways: immune effector activity caused by NK and/or other $CD122^+$-dependent cells, and specific inhibitory effects of 7G3 on AML-LSC homing and engraftment.

7G3 Reduces AML Burden in NOD/SCID Mice

Several in vivo treatment strategies were adopted to determine whether direct injection of 7G3 into NOD/SCID mice affected AML engraftment: (1) administering 7G3 to the mice 6 hr before cell transplantation almost completely ablated AML-1 engraftment in mouse BM to 1.3% ± 0.9% of IgG2a control at 5 weeks posttransplantation (Figure 4A); (2) initiating 7G3 treatment at 24 hr posttransplantation, to allow for LSC homing, also reduced the engraftment of two of three AML samples at 5 weeks posttransplantation (Figure 4B), indicating that early administration of 7G3, when the leukemic burden is low, can efficiently impair the engraftment of AML cells in NOD/SCID mice; (3) commencing 7G3 or IgG2a administration 28 days posttransplantation, in an established disease model, and continuing treatment until time of sacrifice, a significant reduction in the BM burden of AML was seen in two of five samples, likely reflective of the heterogeneity of AML seen clinically. AML-2 responded to 7G3 with reductions in BM engraftment at 9 and 14 weeks posttransplantation (Figure 4C), while treatment of mice with only four doses of 7G3 over 8 days significantly reduced the engraftment of AML-1 (Figure 4D). Moreover, while some AML samples did not have a significant reduction in leukemic burden in the BM with initiation of 7G3 treatment at either 4 or 28 days posttransplantation, a significant reduction in AML burden in the liver and spleen, but not the peripheral blood, was observed (Figures 4E–4G).

To further assess the clinical potential of a CD123-targeting mAb, 7G3 treatments were commenced in mice at 35 days posttransplantation with NBM cells and caused no significant reduction in BM infiltration when administered for 8 days or continuously for 5 weeks (Figure 4D and data not shown). Moreover, 7G3 caused no significant impairment of multilineage engraftment of normal cells (data not shown). Together, these data suggest that 7G3 is biologically active in vivo and can repress the growth of AML with lesser effects on normal human hematopoietic cells.

Since murine NOD/SCID cells do not bind 7G3, we carried out preclinical toxicity studies in a more relevant large animal model, the cynomolgus monkey. A chimeric variant of mAb 7G3 was engineered that maintains CD123 binding specificity and neutralization activity reformatted with a human IgG_1 Fc region. This model permits evaluation of any effect on resting hematopoiesis where there is a source of endogenous IL-3 and a normal immune system. The mAb was administered by i.v. infusion once

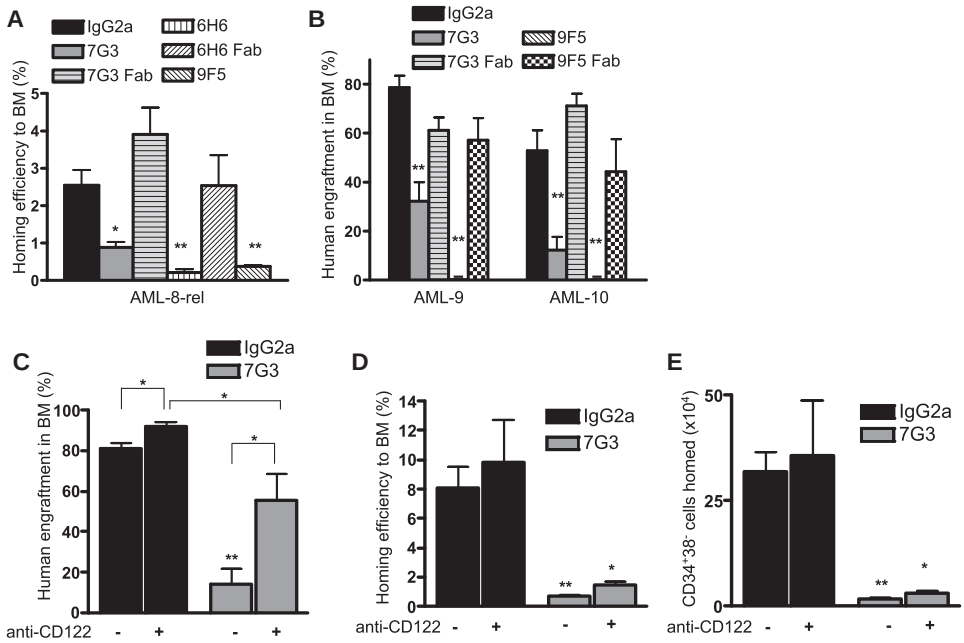

Figure 3. Fc Region of the Antibody and Innate Immunity Mediate 7G3 Antileukemic Effects

(A) Homing efficiency of AML-8-rel cells to the BM following ex vivo treatment with IgG2a, 7G3, 7G3 F(ab′)2, 6H6, 6H6 F(ab′)2, or 9F5. n = 3 per group.

(B) Engraftment of AML-9 and -10 in the BM of mice following ex vivo IgG2a, 7G3, 7G3 F(ab′)2, 9F5, or 9F5 F(ab′)2 treatment. n = 5 per group.

(C and D) 7G3-mediated inhibition of AML-9 engraftment (C) and homing efficiency (D) was attenuated in mice depleted of $CD122^+$ cells (+). n = 3 per group.

(E) Numbers of $CD34^+CD38^-$ AML-9 cells homed to the BM of irradiated NOD/SCID mice with (+) or without (–) $CD122^+$ cell depletion. n = 3 per group. Data are representative of results obtained with two AML samples. Error bars represent mean ± SEM; *$p < 0.05$, **$p < 0.01$ between selected groups.

weekly for 4 consecutive weeks at 0, 10, 30, and 100 mg/kg to a total of 32 cynomolgus monkeys (16 males and 16 females). CD123 binding by the chimeric variant was confirmed to be equivalent to the original parent 7G3 mouse mAb, and binding of both MAbs to cynomolgus CD123 was also demonstrated. There were no antibody-related effects on clinical observations nor on a comprehensive list of hematological parameters measured over 70 days after the first antibody treatment (data not shown). Overall, these data indicate that a CD123-targeting antibody does not exert adverse effects on normal hematopoiesis and are consistent with our NOD/SCID mouse experiments demonstrating that 7G3 treatment can specifically inhibit AML engraftment.

In Vivo Treatment with 7G3 Targets AML-LSCs

In order to determine if key properties of LSC such as self-renewal are targeted, serial transplantation was performed following in vivo 7G3 treatment. While 10 weeks of 7G3 treatment did not overtly decrease the engraftment of AML-10 in the BM or spleen of primary engrafted mice (Figure 5A), the AML cells harvested from 7G3-treated mice had significantly impaired homing ability to the BM and spleens of secondary recipient mice compared with IgG2a-treated controls (Figure 5B). The repopulation ability was also significantly impaired: while eight of nine secondary recipient mice transplanted with untreated control cells were engrafted, only three of eight mice inoculated with cells from 7G3-treated mice showed evidence of engraftment in the BM (Figure 5C). In the secondary mice, 7G3 also significantly reduced the proportion of $CD34^+CD38^-$ primitive cells in the BM (Figure 5D). Similar results were obtained in an independent experiment with AML-9 cells (Figure S4). In addition, when antibody treatment was combined with a suboptimal dose of cytarabine (Ara-C) to assess the potential for synergistic effects against another independent AML sample (AML-10), 7G3 again caused a marked reduction in the proportion of secondary mice engrafted (Figure S5). Collectively over three experiments, 26 of 27 (96%) secondary mice showed evidence of engraftment by cells harvested from IgG2a-treated mice, while only 12 of 23 (52%) were engrafted by cells from 7G3-treated mice. These results demonstrate that in vivo 7G3 administration specifically targets AML-LSCs in NOD/SCID mice, resulting in decreased homing and engraftment in secondary recipients.

7G3 Inhibits Spontaneous and IL-3-Induced Proliferation of Primitive AML Cells In Vitro

Due to the lack of cross-reactivity between the human and mouse IL-3 and CD123 systems, the ability of 7G3 to eliminate LSCs through targeting IL-3 signaling pathway is unable to be directly tested. To determine whether blocking IL-3 signaling can be one of the 7G3 inhibitory functions on AML-LSCs, we incubated different subtypes of primary AML cells with 7G3 or IgG2a in the medium containing IL-3. 7G3 inhibited exogenously added IL-3-induced proliferation in 32 of 35 primary AML samples (Figure 6A). Interestingly, 7G3 inhibited the growth of cells in nine of the samples to 50%–75% of control in the absence of exogenous IL-3, suggesting that these samples may possess an autocrine/paracrine IL-3 pathway or alternate growth mechanisms that can be blocked by 7G3. This profound

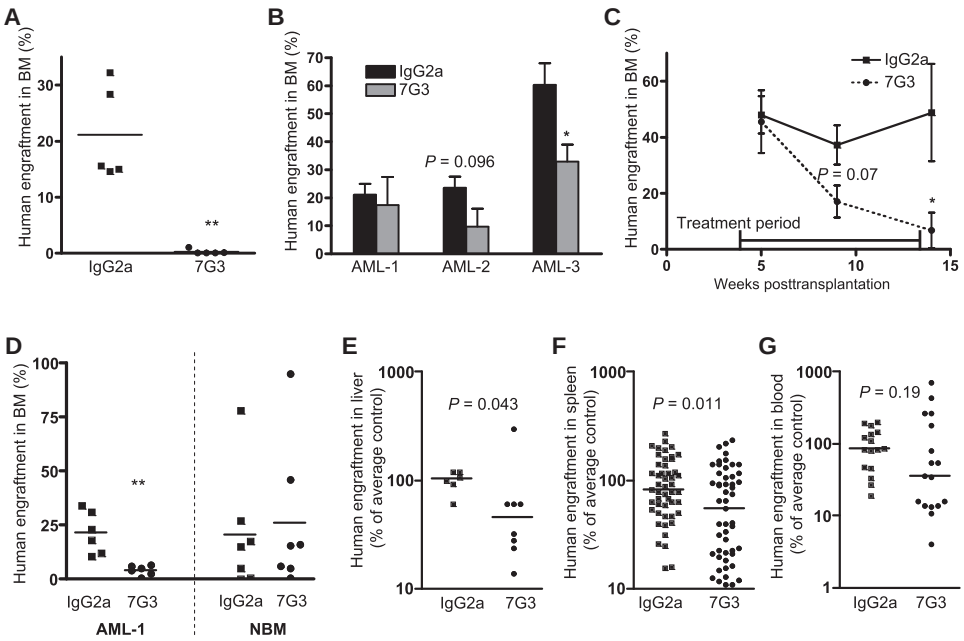

Figure 4. The Schedule of 7G3 Administration Influences Its Antileukemic Efficacy in NOD/SCID Mice

(A) Engraftment levels of AML-1 cells in the BM of mice treated with a single dose of IgG2a or 7G3 (300 μg) 6 hr prior to transplantation.

(B) Percentage of AML cells in the BM of mice (n = 5–6 per group) when the treatment was commenced 24 hr posttransplantation for four doses.

(C) Engraftment levels of AML-2 in the BM of mice when treatment was initiated at day 28 posttransplantation for 9 weeks' duration. n = 3–5 per group for each time point.

(D) Percentage of human AML-1 or NBM cells in the BM of mice after four doses of IgG2a or 7G3 starting on day 28 (AML-1) or 35 (NBM) posttransplantation.

(E–G) Assessment of leukemia infiltration in the liver (E), spleen (F), and peripheral blood (G) for the experiments in which initiating 7G3 treatments at 4 or 28 days did not cause a significant reduction in leukemic burden in the BM. Data are collected from six experiments for the different organs with n = 6–53 mice per group. Each symbol represents data from an individual mouse as a percent of average control for each experiment. Horizontal bars indicate the median in (E)–(G) and mean in (A) and (D). Otherwise results were expressed as mean ± SEM; *p < 0.05, **p < 0.01 between IgG2a and 7G3 groups.

inhibition by 7G3 was IL-3 specific since 7G3 had no effect on GM-CSF-induced cell proliferation (Figure 6B). In order to more directly link the 7G3-mediated reduction in proliferation to primitive AML cells, we demonstrated that 7G3 was able to significantly reduce IL-3-mediated survival of $CD34^+CD38^-CD123^+$ cells in three of four different patient samples (Figure 6C). In addition, 7G3 significantly reduced the survival of $CD34^+CD38^-$ cells from two samples (AML-14 and AML-15) in the absence of exogenously added IL-3. These data verify that 7G3 inhibits IL-3-induced and spontaneous proliferation of primary leukemic cells, and $CD34^+CD38^-CD123^+$ cell survival through binding to CD123.

7G3 Blocks IL-3-Mediated Signaling in AML Cells

We next tested whether 7G3 inhibited leukemic cell growth by blocking the activation of the IL-3R and its downstream pathway. The IL-3R β_c chain (CD131) was found to be coexpressed with CD123 on $CD34^+$ primary AML cells measured by both flow cytometry and PCR analyses (data not shown). Furthermore, IL-3-induced CD131 activation in primary AML cells and TF-1 assessed by tyrosine phosphorylation was inhibited by 7G3 in a concentration-dependent manner (Figure 6D and Figure S6, respectively). Inhibition of downstream STAT5 phosphorylation was also observed in TF1, bulk, and $CD34^+CD38^-$ AML cells (Figure 6E), as well as inhibition of both STAT5 and Akt phosphorylation in TF-1 cells (Figure S6), while the weakly neutralizing clones, 9F5 and 6H6 (Sun et al., 1996), were ineffective at inhibiting IL-3-mediated proliferation (data not shown) or signaling (Figure S6), demonstrating that different CD123 epitopes are functionally distinct. Collectively, these in vitro studies establish that 7G3 has the potential to also target LSCs by blocking IL-3-mediated signaling. Thus, in a clinical context, CD123 targeting has the potential to deliver antileukemic effects via activation of host immunity and inhibition of the IL-3 pathway.

DISCUSSION

In this report, we show that AML-LSCs can be targeted with the CD123-specific 7G3 mAb, resulting in impaired human AML cell engraftment and proliferation in NOD/SCID mice and improved long-term survival. The mechanism of LSC impairment by 7G3 treatment in the NOD/SCID model appeared complex and multifactorial, involving inhibition of LSC homing to the BM niche, and stimulation of residual innate immunity in NOD/SCID recipients. Although the consequences of blocking huIL-3 signaling with 7G3 in LSCs cannot be fully assessed in NOD/SCID mice, the in vitro data we generated showed marked impairment of the signaling, survival, and proliferation of primitive $CD34^+CD38^-$ AML cells. Since this subpopulation is highly enriched for LSCs, this result strongly suggests that impairment of IL-3 signaling will also be part of the multifactorial mechanism of action of 7G3 in a human context. Collectively, our results

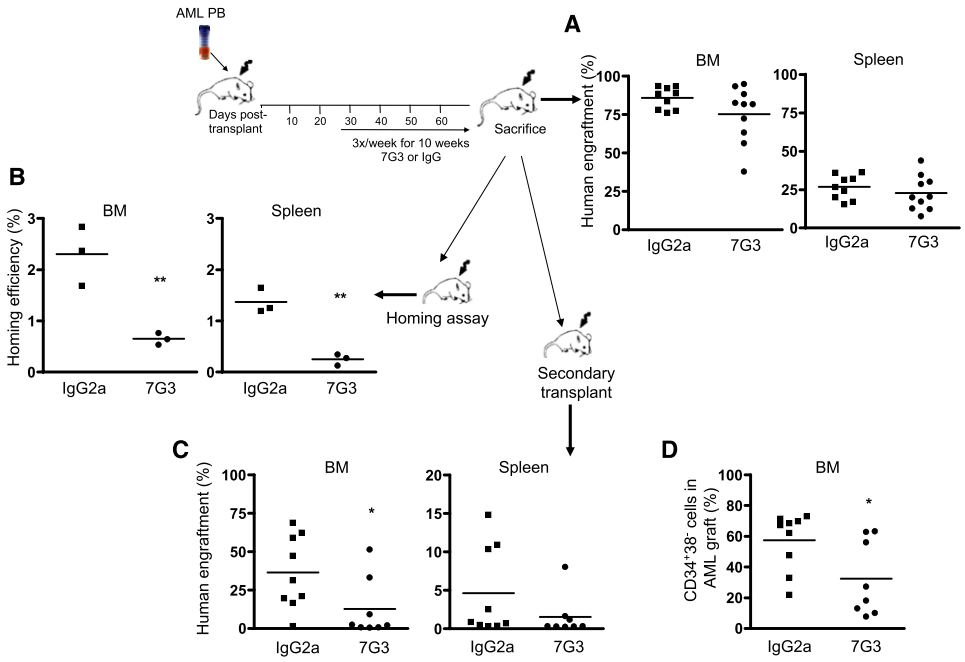

Figure 5. 7G3 Inhibits Self-Renewal Ability of AML-LSCs

(A) Engraftment levels of AML-10 cells in BM and spleen after 10 weeks of 7G3 or IgG2a treatment. The schedule of antibody treatment is shown in the schematic overview.

(B–D) (B) Homing efficiency, (C) levels of engraftment in the BM and spleen, and (D) the percentage of $CD34^+CD38^-$ cells in the BM of secondary recipient mice. Mice in (C) and (D) were analyzed at 12 weeks posttransplantation. Each symbol represents a single mouse, and horizontal bars indicate the mean value. *$p < 0.05$, **$p < 0.01$ between the two groups.

demonstrate that CD123 is an important marker for the targeting of LSCs and downstream progenitors that are capable of rapid proliferation. Our studies also show that, while the NOD/SCID mouse strain is immune deficient due to depleted T, B, and NK cells, it still proves to be an effective animal model for the preclinical testing of antibody-mediated immunotherapy.

Targeting LSCs by means of the 7G3 antibody against CD123 is an attractive approach, since (1) this receptor has been widely shown to be selectively overexpressed in LSCs; (2) the IL-3R classically stimulates multiple biological functions; and (3) 7G3 has the dual benefit of being a blocking antibody as well as mediating ADCC by effector cells providing additional and specific efficacy against leukemic cells, which a small molecule inhibitor of downstream signaling (e.g., JAK/STAT) may not be able to provide. Initial in vitro characterization showed that 7G3 robustly impaired IL-3 binding to its receptor in a broad panel of AML samples, thereby preventing IL-3-dependent CD131 tyrosine phosphorylation and downstream signaling, which are required to promote both cell survival and proliferation (Guthridge et al., 2000, 2006). Furthermore, inhibition of AML proliferation by 7G3 in the absence of exogenous IL-3 in 9 of 32 samples suggests that there is autocrine or paracrine secretion of IL-3 in some AML samples at physiologically significant levels. These experimental data are consistent with other reports demonstrating the expression of IL-3 mRNA and protein in primary AML samples (Guan et al., 2003; Nowak et al., 1999), as well as elevated serum IL-3 levels associated with leukemic burden in AML patients (Elbaz and Shaltout, 2001). By contrast, normal $CD34^+CD38^-$ cell proliferative potential is not affected by IL-3 (De Bruyn et al., 2000), and lineage-negative NBM cells did not have detectable IL-3 mRNA expression (Guan et al., 2003), suggesting that normal HSC function is relatively independent of IL-3 and is consistent with IL-3 being dispensable for normal hematopoiesis in IL-3-deficient mice (Nishinakamura et al., 1996). In AML, the level of CD123 expression and responsiveness to cytokines including IL-3 have been associated with poor prognosis (Graf et al., 2004; Testa et al., 2002, 2004; Tsuzuki et al., 1997). Thus, 7G3 inhibition of the CD123 signaling pathway in the context of AML patients, many of whom are likely to express high levels of circulating IL-3 (Elbaz and Shaltout, 2001), may provide significant additional benefit beyond the mechanisms we have already uncovered with the NOD/SCID model.

Residual murine NK cells, macrophages, or other host immune cells clearly contribute to the action of 7G3. Depletion of innate immunity in NOD/SCID mice with anti-CD122 mAb significantly, but not completely, attenuated the inhibitory effects of 7G3 on the homing and repopulating abilities of AML-LSCs. Additional evidence supporting this mechanism of 7G3-mediated inhibition of LSC function includes evidence that the Fc portion of 7G3 is critical for its activity, as well as the reduced potency of 7G3 in a NOD/SCID strain without NK cell activity. These findings support the further modification of 7G3 to enhance ADCC activity.

Our experiments provide two key findings that support the development of MAbs targeting CD123 as a novel therapy for AML. First, combined with those from other groups, our data showed that CD123 was highly expressed on the surface of $CD34^+CD38^-$ populations enriched for AML-LSCs compared

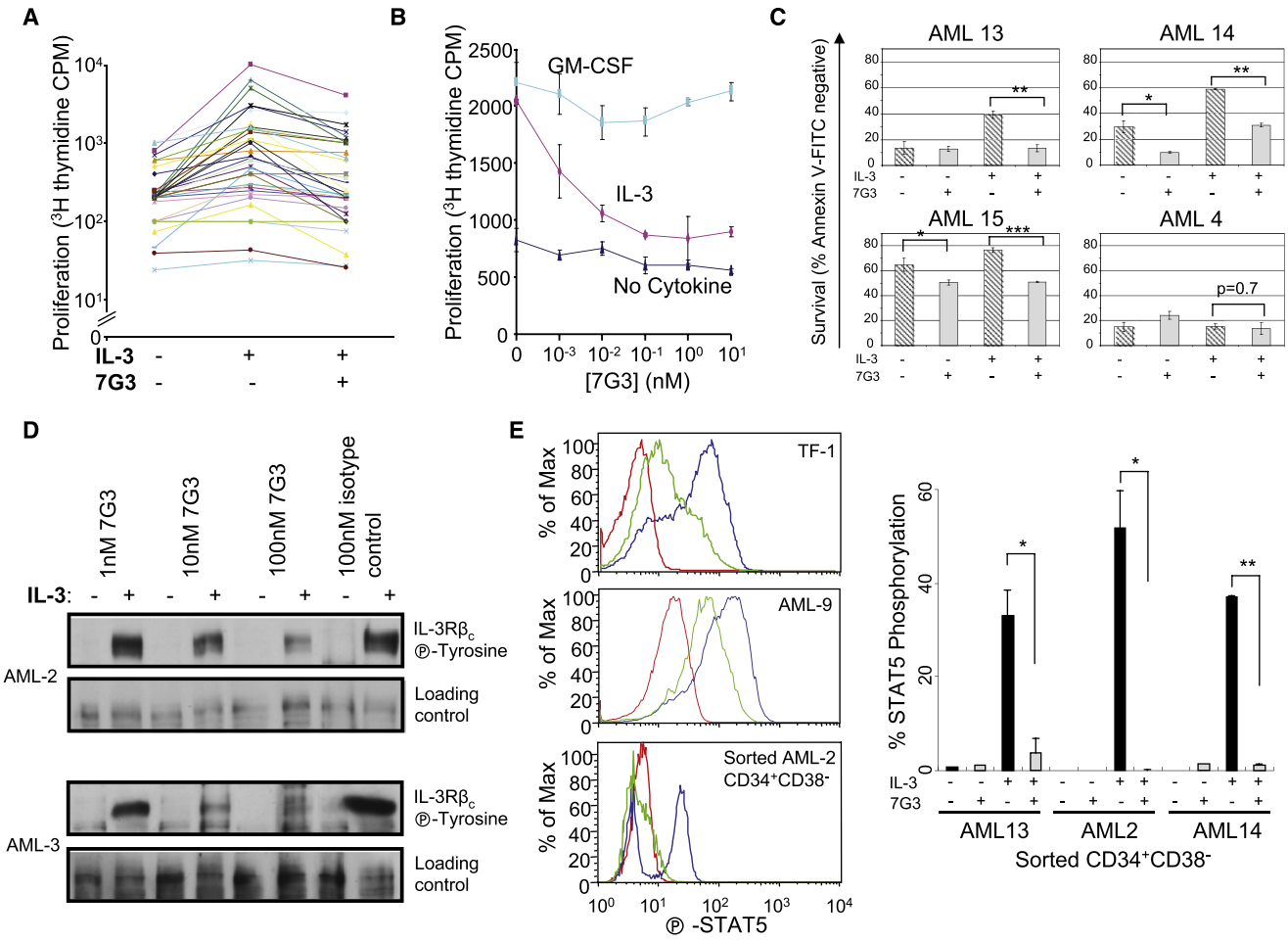

Figure 6. mAb 7G3 Inhibits Proliferation of Primary AML Cells

(A) Inhibition of primary AML cell proliferation by 7G3. Each line represents an individual AML sample exposed to the three different conditions. n = 35.

(B) Concentration-dependent effects of 7G3 on the proliferation of a primary AML sample induced by GM-CSF (0.1 ng/ml) or IL-3 (1 ng/ml). Data are representative of results obtained with 21 different AML samples.

(C) 7G3 inhibits IL-3-mediated survival of isolated $CD34^+CD38^-CD123^+$ primary AML cells. The percentage of surviving cells is shown.

(D) Western blot showing that 7G3 inhibits IL-3-induced CD131 tyrosine phosphorylation in a dose-dependent manner. n = 2 AML samples.

(E) 7G3 inhibits IL-3-induced phosphorylation of STAT5 in TF-1, primary AML, and sorted $CD34^+CD38^-$ AML cells shown by representative histograms of intracellular FACS (red, no IL-3; blue, IL-3 with IgG2a; green, IL-3 with 7G3); bar graphs represent the cumulative data in sorted $CD34^+CD38^-$ cells. Mean ± SEM in triplicates (B) or duplicates (C and E); *$p < 0.05$, **$p < 0.01$, ***$p \leq 0.0001$ between indicated groups.

to their normal hematopoietic counterparts from both newborn CB and adult BM. Reduction of AML engraftment by ex vivo 7G3 treatment with less effect on normal HSCs, in comparison with the nonspecific ablation of both normal and AML sample engraftment by the antibody against HLA-A,B,C epitope, is consistent with the CD123 expression data. Similarly, in vivo 7G3 treatment appears to preferentially reduce AML engraftment relative to NBM xenograft. Second, the marked impairment of engraftment in secondary recipients demonstrates that 7G3 treatment targets the AML-LSCs in vivo, impairing LSC homing and reducing the repopulation of secondary recipients similar to the data from ex vivo treatment. Overall, this establishes 7G3 as a compelling LSC therapeutic in this preclinical model. Interestingly, the IF injection method established that at least a part of the action of 7G3 on LSC homing occurred during lodgment in microenvironmental niches and not during circulation through the blood, or during extravasation across endothelial membranes, since this method directly bypasses the latter processes by delivering cells to the femoral cavity.

The clinical potential for a CD123-targeting mAb is supported by three lines of evidence. First, our study has shown that ex vivo or in vivo 7G3 treatments selectively target AML cells compared with their normal counterparts. Second, toxicity testing in primates has shown that a chimeric IgG1 variant of 7G3 had no significant effects on any measured hematological parameters over 70 days. If normal hematopoiesis or HSCs had been affected, alteration of some of the parameters would be expected within this time frame. Third, the same chimeric variant mAb is being investigated in a phase I clinical trial as weekly treatment of patients with relapsed or refractory or high-risk AML. To date, with a total of >180 infusions administered to 26 patients comprising five dose-level cohorts up to 10 mg/kg, no signal of

treatment-related toxicity has been detected from hematology, biochemistry, or vital signs. Other than two mild infusion reactions, only one serious adverse event, an infection, was considered possibly related to treatment with the mAb. The incidence of adverse events did not increase with escalating dose, no grade 3–4 adverse events have been considered treatment related, and unrelated adverse events have been consistent with complications and risks of AML (A.W. Roberts et al., 2008, ASH Annual Meeting, abstract).

Although 7G3 effectively targeted AML-LSCs, it was most successful under conditions where the leukemic burden was low. When 7G3 treatment began at 4 weeks posttransplantation, BM engraftment was significantly impaired in only two of five primary AMLs. However, in this model of established AML, 7G3 distinctly reduced the AML burden in peripheral hematopoietic tissues (spleen, liver) in the majority of the samples we tested, perhaps due to a greater access of innate immune cells eliminating 7G3-coated AML cells (Fujii et al., 2007). While sample-to-sample variability was encountered at high leukemic burden, the increased effectiveness of 7G3 at low leukemic burden suggests a potential application of anti-CD123 treatment during remission following treatment with conventional chemotherapeutic agents.

The concept of antibody targeting of malignancy is well established. For example, several MAbs directed at hematological malignancies have been evaluated in clinical trials, including rituximab (which targets CD20) and epratuzumab (CD22) in B cell malignancies, alemtuzumab (CD52) in chronic lymphocytic leukemia, daclizumab (CD25) in T cell malignancies, and gemtuzumab ozogamicin (CD33) in AML. However, these mAb therapies are unlikely to target CSCs, and, while impressive cytoreduction and clinical responses have been observed, none are curative. Therefore, the multifaceted properties of 7G3 shown in this preclinical model of AML support a broader proposal for CSC-targeted cancer drug development (Wang, 2007) in which potential therapies that target key traits of CSCs are identified and tested using primary patient samples in relevant in vivo xenotransplantation models. The ongoing clinical evaluation of a chimeric CD123 mAb in advanced AML (http://clinicaltrials.gov/ct2/show/NCT00401739?term=CSL360&rank=1) will be the first of its kind to test whether the significant activity of an LSC-targeted mAb therapy in the xenograft models shown in this study translates into a clinical benefit for patients. Ultimately, this clinical testing will also provide more definitive proof of a role for IL-3 in the pathology of AML.

EXPERIMENTAL PROCEDURES

AML Patient Samples, Normal Hematopoietic Cells, and Cell Lines

Patient samples were collected after informed consent according to institutional guidelines, and studies were approved by the Royal Adelaide Hospital Human Ethics Committee, Melbourne Health Human Research Ethics Committee, Research Ethics Board of the University Health Network, and the South Eastern Sydney and Illawarra Area Health Service Human Research Ethics Committee. Diagnosis was made using cytomorphology, cytogenetics, and leukocyte antigen expression and evaluated according to the French-American-British (FAB) classification. Mononuclear cells were enriched by Lymphoprep (Axis-Shield PLC, Dundee, Scotland) or Ficoll (GE Healthcare, Uppsala, Sweden) density gradient separation and frozen in liquid nitrogen. Human CB and NBM cells were obtained from full-term deliveries or consenting patients receiving hip replacement surgery, or commercially from Cambrex Corporation (East Rutherford, NJ) and Lonza (Basel, Switzerland), respectively, and processed as previously described (Mazurier et al., 2003).

Ex Vivo Antibody Treatment

Thawed AML or normal hematopoietic cells were incubated with IgG2a, 7G3, 7G3 F(ab′)2, 9F5, 9F5 F(ab′)2, 6H6, or 6H6 F(ab′)2 (10 μg/ml) for 2 hr in X-VIVO 10 medium (Cambrex Corporation or Lonza) supplemented with 15%–20% bovine serum albumin-insulin-transferrin (StemCell Technologies Inc, Vancouver, Canada) at 37°C before i.v. transplantation into sublethally irradiated NOD/SCID mice for repopulating assays. Engraftment was measured at 4–10 weeks at two different time points.

Xenotransplantation of Human Cells into NOD/SCID Mice and In Vivo Antibody Treatment

Animal studies were performed under the institutional guidelines approved by the University Health Network/Princess Margaret Hospital Animal Care Committee or the Animal Care and Ethics Committee of the University of New South Wales. Transplantation of human cells into NOD/SCID mice was performed as previously described (Mazurier et al., 2003). Briefly, all mice received sublethal irradiation 24 hr before i.v. or IF transplantation with either 5–10 × 10^6 human AML cells, 3 × 10^5 lineage-depleted $CD34^+$ CB cells, 8 × 10^6 BM cells, or 1 × 10^6 sorted $CD34^+$ BM cells per mouse. Anti-CD122 antibody purified from the hybridoma cell line TM-β1 (generously provided by Professor T. Tanaka, Hyogo University of Health Sciences) (Tanaka et al., 1993) was injected intraperitoneally (i.p.) immediately after irradiation (200 μg/mouse). Engraftment levels of human AML and normal hematopoietic cells were evaluated by the percentage of $huCD45^+$ cells by flow cytometry (Lock et al., 2002). The number of $CD34^+CD38^-$ AML cells in the BM and spleen was also calculated based on the average number of cells harvested, and the engraftment levels and percent of $CD34^+CD38^-$ AML cells in each mouse. To measure effects on LSC activity, secondary transplantations were also performed by i.v. transplantation of 7–10 × 10^6 AML cells isolated from the BM (two femurs and two tibias) of IgG2a- or 7G3-treated primary mice into secondary recipient mice.

For in vivo testing, IgG2a or 7G3 (300 μg per injection) was injected i.p. into mice three times a week with schedules described in the legends to each figure. In vivo treatment of 7G3 was also tested in combination with the chemotherapeutic reagent Ara-C as described in Figure S5.

In Vivo Homing Assay

Homing assays were performed on ex vivo 7G3-treated cells, sorted $CD34^+CD38^-$ cells from primary patient samples, or cells harvested from previously engrafted mice, as previously described (Jin et al., 2006). Briefly, cells harvested from BM and spleen of mice transplanted 16 hr previously were stained with anti-human CD45-FITC, CD38-PE, and CD34-PC5 followed by flow cytometry for human cells using 5 × 10^4 to 5 × 10^6 collected events. Homing efficiency of human cells into the mouse tissues was calculated based on the number of total $huCD45^+$ cells in the tissue and the number of cells injected.

Cell Staining, Sorting, and Flow Cytometry

For flow cytometry, cells were stained as previously described (Bonnet and Dick, 1997; Lock et al., 2002) with conjugated anti-human antibodies against CD15, CD14, CD19, CD33, CD34, CD38, and CD45 (BD Biosciences, or BioLegend, CA). CD123 expression was measured with anti-CD123 clone 9F5, and RFI was determined by the ratio of the geometric mean of the 9F5-stained signal to matched isotype control. Stained cells were analyzed using FACScan or FACSCalibur flow cytometers (BD Biosciences). For sorting, cells were stained with anti-human antibodies against CD34, CD38, and CD123, and propidium iodide was also added to exclude dead cells when the cells were sorted using Moflo and BD Aria cell sorters (BD Biosciences).

Survival Analysis of $CD34^+CD38^-CD123^+$ AML Cells

Sorted cells plated at 1.5 × 10^5 cells/ml in IMDM/0.5% FCS were treated with 150 μg/ml 7G3 or IgG2a (clone BM4) for 30 min prior to addition of 1 nM IL-3. Cells were analyzed for survival at 48 and 72 hr by staining with 1:100 Annexin V-FLUOS (Roche, Basel, Switzerland) as described previously (Guthridge et al., 2006). Absolute cell number was also assessed by addition of 50 μl Flow-Count fluorospheres (Beckman Coulter).

Proliferation Assays

AML cell-growth responses to IL-3 or GM-CSF were measured by ^{3}H-thymidine assay as previously described (Lopez et al., 1988). Briefly, 2 × 10^{4} mononuclear cells per well in 96-well plates were stimulated with IL-3 (1 ng/ml) or GM-CSF (0.1 ng/ml) in the presence of 0–10 nM 7G3 or IgG2a in 200 μl IMDM + 10% HI-FCS (Hyclone, UT) for 48 hr with 0.5 μCi of ^{3}H-thymidine (MP Biomedicals Australasia, Sydney, Australia) added for the last 6 hr of culture. Cells were deposited onto glass fiber paper using a Packard Filtermate cell harvester (PerkinElmer Life and Analytical Sciences, Melbourne, Australia) and counted using a Top Count (PerkinElmer). All cytokines were supplied by R&D Systems (MN).

Cytokine Signaling Assays

Phosphorylation of signaling proteins was detected by immunoprecipitation and immunoblots. TF-1 and primary AML cells were washed and rendered quiescent overnight before incubation with IgG2a, 9F5, 6H6, or 7G3 (0–100 nM) for 20 min on ice. Cells were then stimulated with 1 nM IL-3 for 10 min at 37°C. Cells were lysed in NP-40 lysis buffer, and CD131 was immunoprecipitated using 1C1 and 8E4 antibodies conjugated to Sepharose beads (Guthridge et al., 2004). Immunoprecipitates were subjected to SDS-PAGE and immunoblotting as previously described (Guthridge et al., 2004). Antibodies used were the following: antiphosphotyrosine mAb 4G10 (Upstate Biotechnology Inc, NY), anti-phospho-Akt Ser473 (Cell Signaling Technology Inc, MA), and, anti-phosphorylated STAT5 mAb (Zymed Laboratories Inc, CA). All antibodies were used according to manufacturers' instructions. Blots were stripped and reprobed with antibody to βc (1C1) as a loading control.

For intracellular FACS, quiescent TF-1, bulk, and sorted primary AML cells were stimulated with 20 ng/ml IL-3 plus 20 μg/ml IgG2a or 7G3 for 1 hr. Sorted subpopulations were incubated with 150 μg/ml 7G3 or IgG2a for 30 min on ice before stimulation with 1 nM IL-3 for 15 min. Cells were fixed with BD Cytofix Buffer (BD Biosciences), methanol permeabilized, and stained with anti-phosphoSTAT5 (BD Biosciences) or isotype control. Cells were then analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Statistical Analysis

Data are presented as the mean ± SEM. The significance of differences between groups was determined using the unpaired, two-sided Student's t test, or the nonparametric Mann-Whitney U test. Survival curves were compared using the log rank test.

SUPPLEMENTAL DATA

Supplemental Data include six figures and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00207-0.

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Monoclonal antibody-mediated targeting of CD123 (IL-3 receptor α chain) eliminates human acute myeloid leukemic stem cells

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SUPPLEMENTARY MATERIAL

Figure S1. *Ex vivo* 7G3 treatment specifically inhibited repopulating ability of AML but not NBM primary cells in NOD/SCID mice compared with anti-human HLA-A,B,C antibody. Percentage of human AML cells in the BM of NOD/SCID mice transplanted with 7G3 (gray) or IgG2a control (black) or anti-HLA-A,B,C (white) antibody -treated AML or NBM cells at 6-11 weeks post transplantation. For each treatment group, 3 - 6 mice were used. Values represent mean ± SEM *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ between control IgG2a and 7G3 or HLA-A,B,C groups.

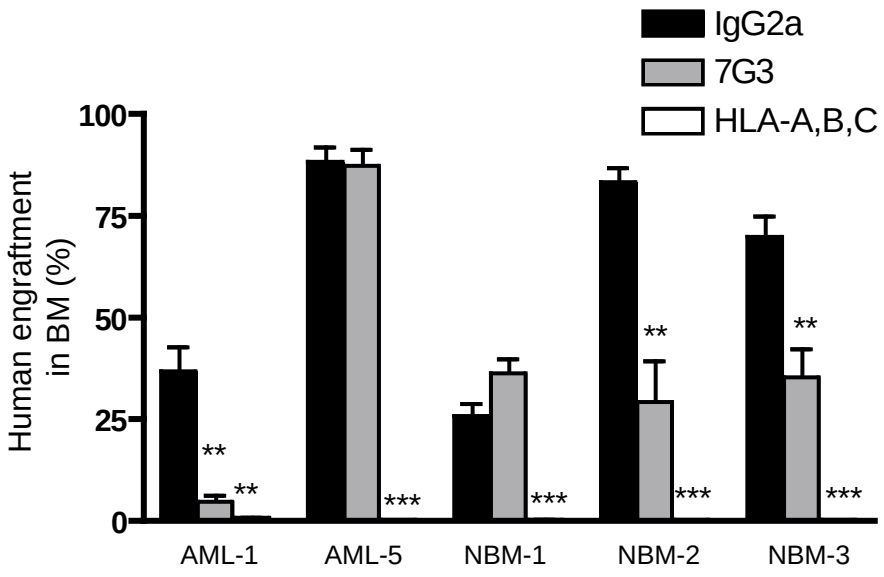


Figure S2. The intensity of CD123 expression on $CD34^{+}/CD38^{-}$ cells directly correlates with the ability of 7G3 to inhibit engraftment in NOD/SCID mice. Spearman correlation plot of CD123 cell surface expression (log RFI) versus engraftment of the 7G3 *ex vivo* treated group relative to IgG2a controls (log% of IgG2a control) at 4-6 weeks post transplantation. Data are taken from Table 1.

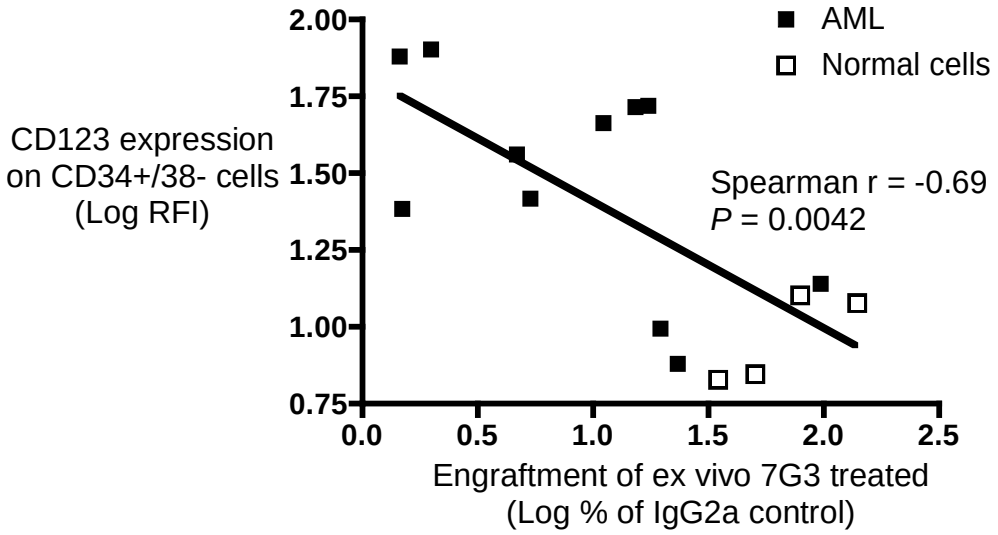


Figure S3. 7G3 inhibits self-renewal ability of AML-LSC. (A) Engraftment levels of AML-9 cells in BM and spleen after 10 weeks of 7G3 or control IgG2a treatment. Antibody treatment was initiated at Day 28 post transplantation, 300 µg per mouse thrice weekly, as shown in the schematic overview. (B) Levels of engraftment in the BM of secondary recipient mice. Secondary mice were analyzed at 8 weeks post transplantation. Each symbol represents a single mouse, horizontal bars indicate the mean value. **, $P < 0.01$ between control IgG2a and 7G3 groups.

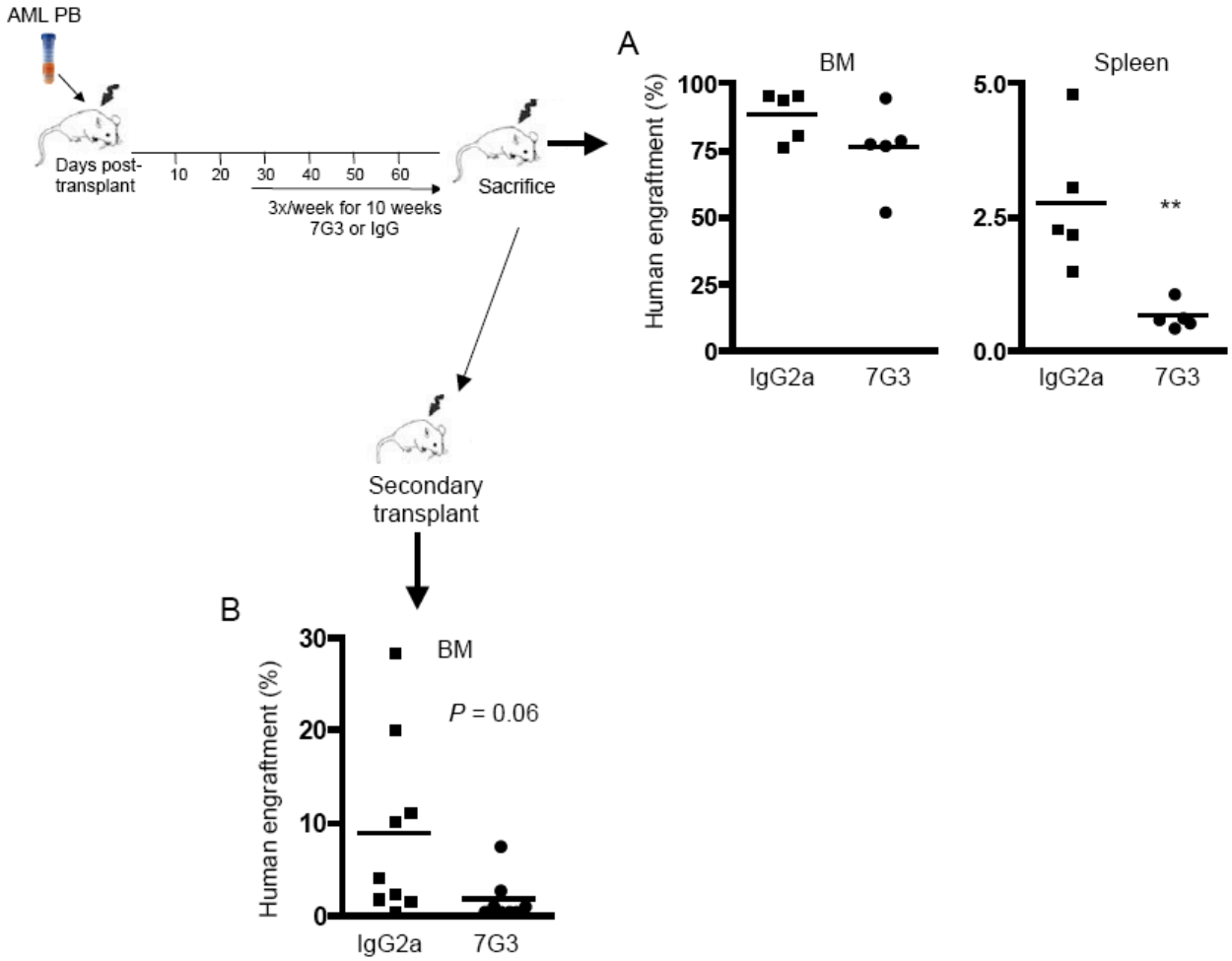


Figure S4. 7G3 inhibits self-renewal ability of AML-LSC. (A) Engraftment levels of AML-10 cells in BM and spleen after 4 weeks of 7G3 or control IgG2a treatment in combination with Ara-C. Antibody treatment was initiated at Day 35 post transplantation for 3 consecutive days at 500 μg followed by 5 days of Ara-C treatment. Antibody treatment was then continued at the same dose thrice weekly for a total of 4 weeks, as shown in the schematic overview. (B-D) Homing efficiency (B), levels of engraftment in the BM and spleen (C), and the percentage of $CD34^{+}CD38^{-}$ cells in the BM (D) of secondary recipient mice. Mice in C and D were analyzed at 8 weeks post transplantation. Each symbol represents a single mouse, horizontal bars indicate the mean value. *, $P < 0.05$; **, $P < 0.01$ between control IgG2a + Ara-C and 7G3 + Ara-C groups.

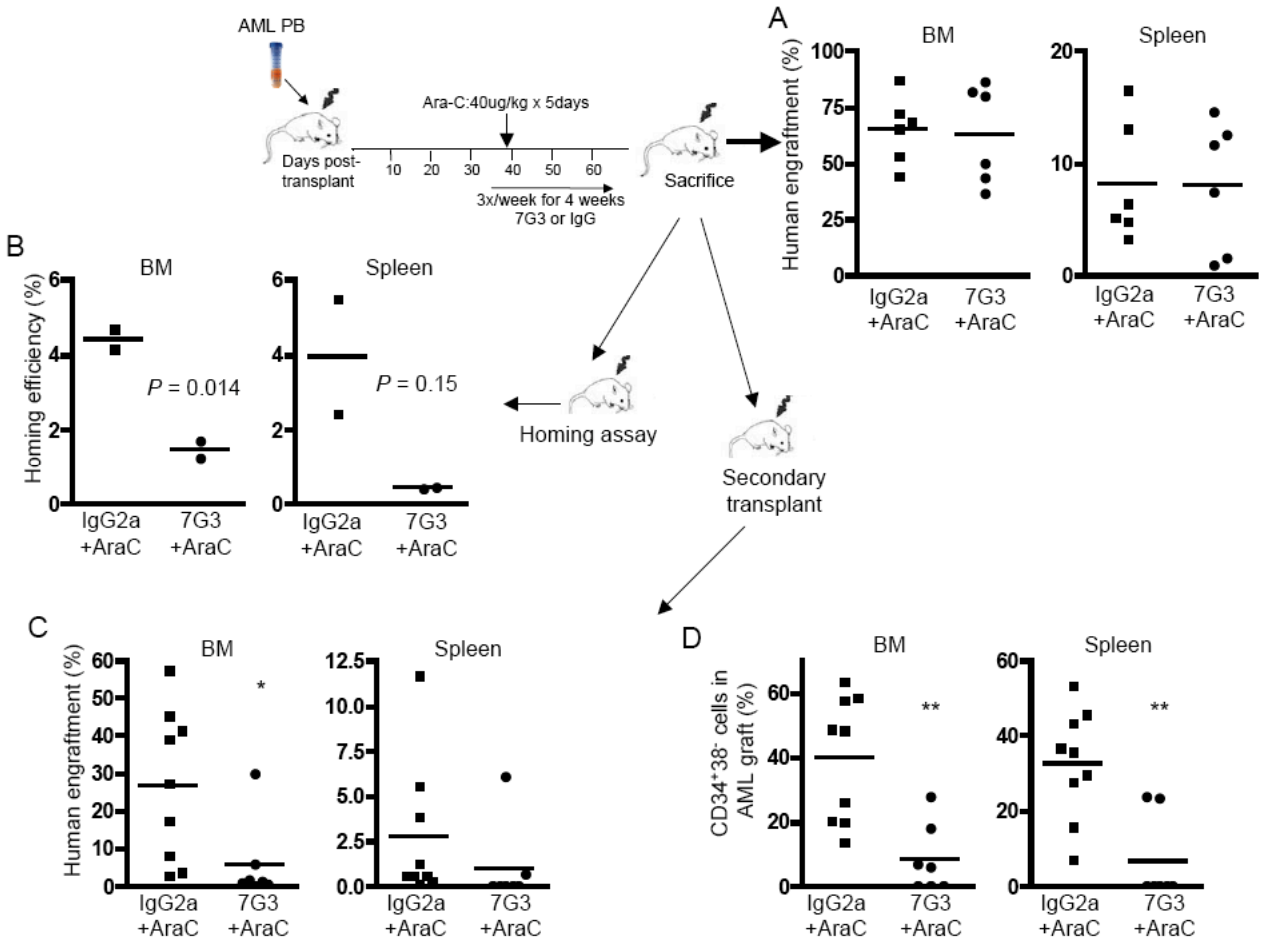
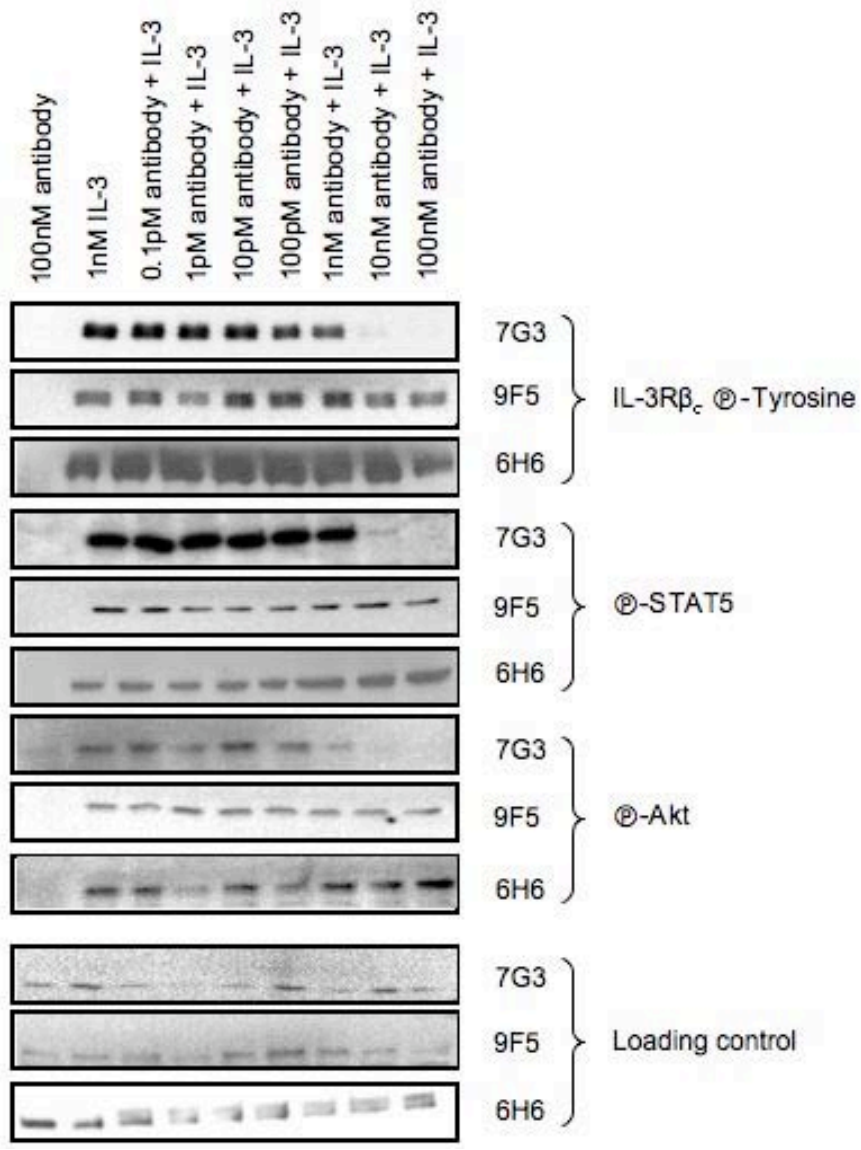


Figure S5. MAb 7G3, but not 6H6 or 9F5, inhibits IL-3 stimulated phosphorylation of CD131, STAT5 and Akt in the IL-3-dependent TF-1 cell line. TF-1 cells were incubated with the indicated concentrations of 7G3, 9F5 or 6H6 for 20 min on ice. Without washing, cells were stimulated with IL-3 (1 nM for 10 min at 37℃). Immediately following stimulation cells were lysed and β_c was immunoprecipitated using 1C1 and 8E4 antibodies conjugated to Sepharose. Immunoprecipitates were run on SDS-PAGE and immunoblotted with antibodies to phosphorylated tyrosine residues (4G10), phosphorylated STAT5 or phosphorylated Akt. Blots were stripped and re-probed with antibody to β_c (1C1) as a loading control.



Chapter 4

Protein kinase activity of phosphoinositide 3-kinase promotes cell survival

4.1 Summary

Acute myeloid leukaemia (AML) is heterogeneous disease with poor prognosis that consists of a clonal hierarchy of myeloid progenitor cells that accumulate in the bone marrow and whose growth is propagated and maintained by leukaemia stem cells (LSCs). In the previous chapter we demonstrated the feasibility of targeting LSCs to eradicate AML *in vivo* by using monoclonal antibody therapy directed against the interleukin-3 (IL-3) cytokine receptor subunit, CD123. Emerging studies have shown that LSCs possess robust cell survival properties and low proliferation rates that may contribute to their intrinsic resistance to anti-metabolite chemotherapy such as cytarabine(Hope *et al.* 2004). However little is known about the molecular mechanisms that govern LSC survival and persistence over many months in the human body prior to relapse.

The IL-3 receptor consists of a ligand-specific α subunit and a common signal-transducing beta subunit, βc (Figure 1.9) and is over-expressed and biologically active in stimulating the survival and proliferation of leukaemic stem/progenitor cell populations isolated *in vitro* (Jin *et al.* 2009). Our previous studies in non-transformed cells had demonstrated that Ser585 of βc is phosphorylated in response to cytokine in non-transformed cells and specifically promotes cell survival (Guthridge et al, 2006; Guthridge et al, 2000). In normal mononuclear cells from healthy donors, Ser585 undergoes phosphorylation at low picomolar concentrations of cytokine in the absence of detectable βc tyrosine phosphorylation. However our initial studies indicated that in the majority of AML patient cases examined Ser585 was constitutively phosphorylated (Guthridge et al, 2006). This suggested that constitutive Ser585 signalling could lead to deregulated cell survival in AML cells and constitute at least part of their transformed phenotype. We therefore wished to examine the kinases responsible for Ser585 phosphorylation with a view toward targeting those kinases in AML.

We sought to profile cytoplasmic extracts for their ability to phosphorylate Ser585 in search of novel kinase activity that could be tractable for targeting in leukaemia. We have isolated a kinase activity that is able to phosphorylate Ser585 in the cytoplasmic domain of

the interleukin 3 (IL-3) and granulocyte macrophage colony stimulating factor (GM-CSF) receptors and shown it to be phosphoinositide 3-kinase (PI3K).

The dual specificity protein/lipid kinase, PI3K, promotes growth factor-mediated cell survival and is frequently deregulated in cancer. However, in contrast to canonical lipid-kinase functions, the role of PI3K *protein* kinase activity in regulating cell survival is unknown. We show that the phosphorylation of the IL-3 and GM-CSF receptor on Ser585 by PI3K protein kinase activity is essential for the regulation of cell survival. Importantly, the activation of the protein kinase activity of PI3K, but not its lipid kinase activity, required physiological concentrations of cytokine in the picomolar range and was essential for promoting Ser585 phosphorylation and cell survival in the absence of phosphotyrosine pathways and proliferation (the survival-only response). Furthermore, pharmacological/genetic activation or blockade of PI3K lipid signalling had no significant effect on the ability of picomolar concentrations of cytokine to promote cell survival. Pharmacological blockade of p110α with 3 independent p110α-selective inhibitors blocked cytokine-regulated Ser585 phosphorylation under conditions where only the protein kinase activity of PI3K was activated. Additionally, the p110α-selective inhibitor, PIK75, not only down-regulated constitutive Ser585 phosphorylation in a panel of primary human AML samples but also blocked the autonomous survival of leukemic blasts. Our findings demonstrate a new role for the protein kinase activity of PI3K in phosphorylating the cytoplasmic tails of cytokine receptors to selectively regulate cell survival and highlight the importance of targetting such pathways in cancer.

4.2 Significance

While much is known regarding the targets and biological functions of PI3K lipid signalling, little is known of the substrates and functional roles of its protein kinase activity (Cantley, 2000). This is the first demonstration of a new role for the protein kinase activity of PI3K in phosphorylating the cytoplasmic tails of cytokine receptors to selectively regulate cell survival. This work underscore the central role of PI3K p110α rather than β,δ

or γ isoforms in contributing to cell survival in AML. It suggests that both lipid and protein kinase substrates are important signalling mediators in transformed cells. Lipid and protein kinase activity of PI3K appears to be regulated by cytokine in a concentration dependent manner with protein kinase activity active at low picomolar concentrations in the absence of lipid signalling and Akt activation. Further work in elucidating other protein kinase substrates of PI3K in cancer is likely to be fruitful and may be linked to cell survival. This chapter also underscores the therapeutic potential of PI3K p110α-selective inhibitors in killing AML cells that have deregulated and constitutive PI3K signalling.

4.3 Introduction

A key mechanism by which growth factors and cytokines promote cell survival is via the phosphatidyl inositol 3-OH kinase (PI3K) pathway and constitutive PI3K signaling is known to promote autonomous cell survival and transformation (Cantley, 2002). The recruitment and activation of Class 1A isoforms of PI3K by cytokine and growth factor receptors leads to the phosphorylation of phosphatidyl inositol phosphates (PIPs) and the subsequent docking of pleckstrin homology (PH) domain proteins such as Akt that activate downstream signaling cascades and biological responses (Cantley, 2002). However, in addition to their lipid kinase activity, all members of the Class 1 PI3K family also possess intrinsic protein kinase activity (Dhand *et al.* 1994a; Dhand *et al.* 1994b; Foukas and Shepherd, 2004a; Hunter, 1995). While much is known regarding the targets and biological functions of PI3K lipid signalling, little is known of the substrates and functional roles of its protein kinase activity.

We and others have shown that the phosphorylation of specific serine residues in the cytoplasmic tails of growth factor and cytokine receptors is critical for initiating intracellular signaling pathways that selectively control cell survival (Guthridge *et al.* 2006; Guthridge *et al.* 2004; Guthridge *et al.* 2000; Lonic *et al.* 2008; Peruzzi *et al.* 1999). In non-transformed cells, physiological concentrations of granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin 3 (IL-3) in the picomolar (pM) range are able to promote Ser585 phosphorylation in the cytoplasmic domain of the βc receptor subunit to regulate cell survival in the absence of other biological responses such as proliferation (the “survival-only” response) (Guthridge *et al.* 2006). Importantly, this "survival-only" pathway is deregulated in leukaemia with constitutive Ser585 phosphorylation clearly detectable in >85% of primary AML samples (Powell *et al.* 2009).

We now show that primary leukemic progenitors isolated from acute myeloid leukaemia (AML) and chronic myeloid leukaemia (CML) patients demonstrate autonomous cell survival that is refractory to tyrosine kinase inhibitors (TKIs). In order to identify the kinase(s) that promote cellular transformation via the constitutive serine phosphorylation

of cytokine and growth factor receptors leading to autonomous cell survival and TKI resistance, we have employed an approach that allows the biochemical isolation and pharmacological profiling of "survival kinases". Using primary human AML patient samples, we have isolated a kinase that phosphorylates Ser585 in the cytoplasmic tail of the GM-CSF/IL-3 βc receptor. We have identified this Ser585 kinase as the p110 catalytic subunit of PI3K and show that physiological picomolar concentrations of cytokine activate the protein kinase activity of PI3K leading to Ser585 phosphorylation and cell survival. While Ser585-survival signalling is constitutive in AML, inhibition of PI3K with p110α-selective inhibitors not only down-regulated Ser585 phosphorylation, but also induced rapid apoptosis in primary AML blasts. Our findings identify a new role for the protein kinase activity of PI3K in promoting growth factor-mediated cell survival and provide a novel functional link between the deregulated PI3K protein kinase activity and phosphotyrosine-independent survival programs in leukaemia.

RESULTS

4.4 Isolation of a Ser585 survival kinase.

GM-CSF and IL-3 receptor signaling regulate both proliferation and survival of normal myeloid cells and play an important role in myeloid leukaemia (Guthridge *et al.* 1998). However, while GM-CSF promotes cell proliferation in both primary leukemic progenitors from either an AML or CML patient in a tyrosine kinase-dependent manner, cell survival was autonomous, growth factor-independent and resistant to tyrosine kinase inhibition (Fig. 4.1A and B). Consistent with our previous findings (Powell *et al.* 2009), Ser585 phosphorylation of the GM-CSF/IL-3 βc receptor was constitutive in primary leukemic blasts (Fig. 4.2A) or CML leukaemic cell-line (Fig. 4.2B) and was not affected by TKIs. These results indicate that cell survival pathways in leukaemia, such as those regulated by Ser585, are constitutively activated in leukaemia and are refractory to TKI treatment.

In order to identify the kinases responsible for phosphorylating Ser585 and promoting cell survival we performed chromatographic fractionation of an AML patient sample exhibiting constitutive Ser585 phosphorylation (Fig. 4.3A). Eluted fractions were tested for Ser585-kinase activity *in vitro* using a βc peptide encompassing Ser585 and a single peak of activity was observed that was distinct to the kinase activity detected by the protein kinase A (PKA) substrate, Kemptide (Fig. 4.3A). Pharmacological profiling of the Ser585-kinase ctivity revealed that only the PI3K inhibitor, LY294002, significantly reduced Ser585 phosphorylation (Fig. 4.3B). Further analysis using a panel of four PI3K inhibitors indicated that each was able to inhibit the Ser585-kinase activity in a dose-dependent manner (Fig. 4.3C). Although little is known of the protein substrates of PI3K, our results suggested the intriguing possibility that the serine kinase activity of PI3K could phosphorylate Ser585.

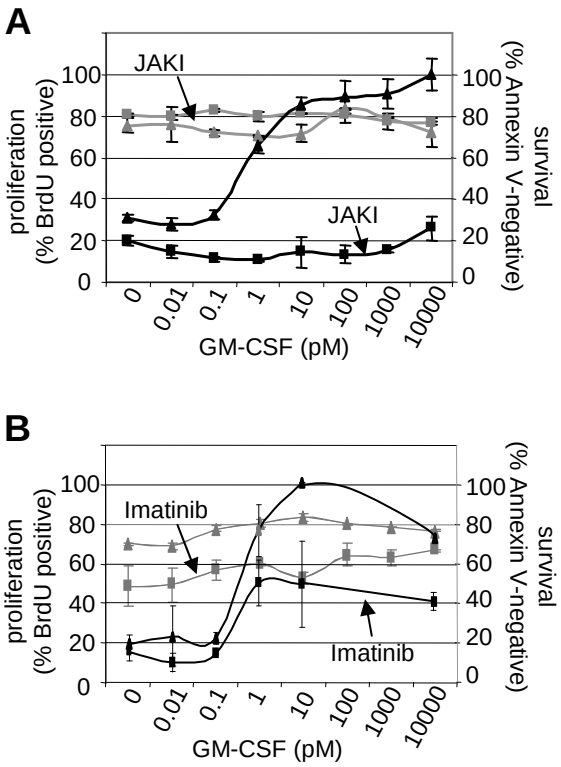


Figure 4.1: Cell survival is autonomous in primary human AML and CML and is refractory to tyrosine kinase inhibitors (TKIs). Primary human AML MNCs (A) or CML CD34+ progenitors (B) were cultured in DMSO, 10μM JAK inhibitor I (JAKI) (A) or 2μM imatinib (B) and GM-CSF. Cell survival (annexin V)(black) or proliferation (BrdU)(grey) were measured by flow cytometry.

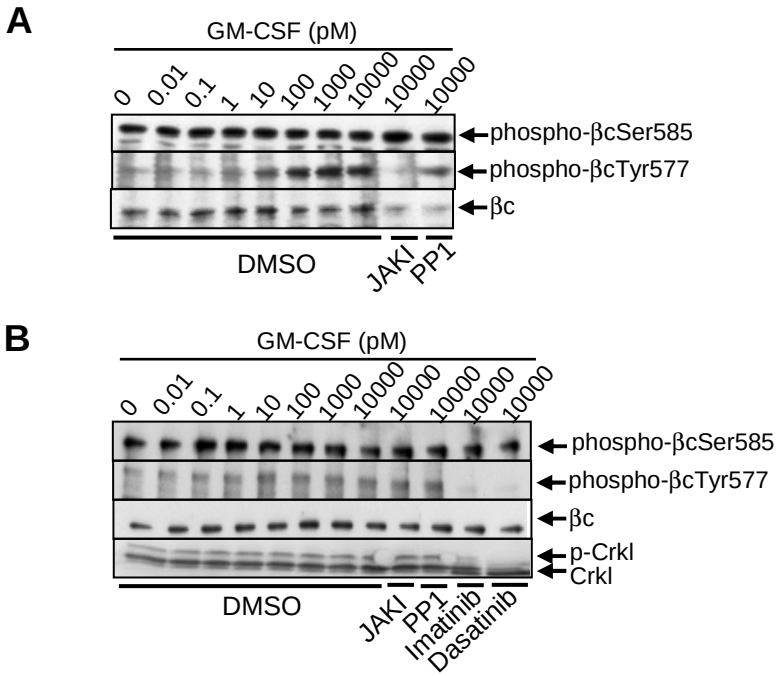


Figure 4.2: Ser585 phosphorylation is constitutive in primary human AML and CML. (A) Primary AML blasts were preincubated in JAKI (10μM), src kinase inhibitor, PPI (10μM) or vehicle (DMSO) for 20min following which the cells were stimulated with GM-CSF for 5min. βc was then immunoprecipitated and subjected to immunoblot analysis with anti-phospho-βcSer585 pAb, anti-phospho-βcTyr577 pAb or anti-βc (1C1) mAb. (B) CML cell-line (KG-1) cells were preincubated in JAKI (10μM), src kinase inhibitor, PPI (10μM), 2μM Imatinib, 2μM Dasatinib or vehicle (DMSO) for 20min following which the cells were stimulated with GM-CSF for 5min and processed as in (A).

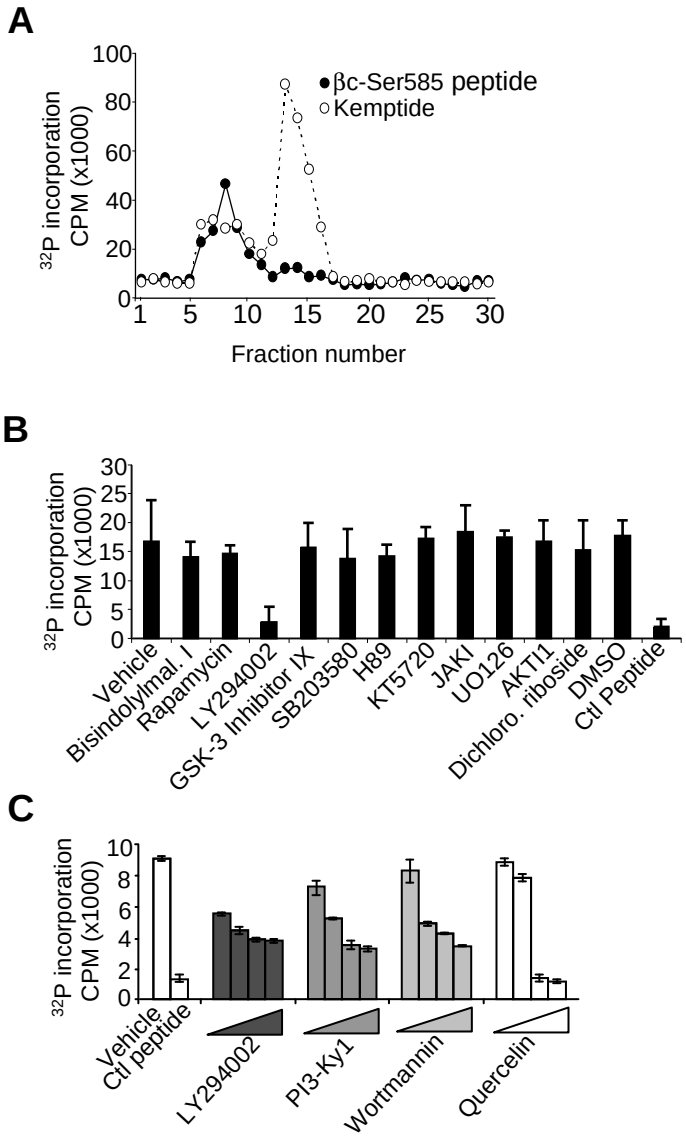


Figure 4.3: Purification and pharmacological profiling of a Ser585 kinase from AML. AML MNCs (from patients AML1 & AML2; both samples gave same profile) were subjected to hypotonic lysis and then FPLC on a Superdex 200PC column (A). Aliquots of eluted fractions were analysed for kinase activity *in vitro* using either a βcSer585 peptide substrate (filled circles) or Kemptide (open circles). Fraction 8 from (A) was examined for kinase activity using the βcSer585 peptide in the presence of 10μM inhibitors (B). Kinase reactions in the presence of increasing concentrations of LY294002 (1, 10, 50 and 100 μM), PI3-Kγ1 (0.01, 0.1, 1 and 10 μM), wortmannin (0.01, 0.1, 1 and 10 μM) and quercetin (1, 10, 100 and 1000 nM)(C). Error bars indicate +/- standard deviation .

4.5 The p110 catalytic subunit of PI3K can directly phosphorylate Ser585.

To address this possibility, we immuno-purified PI3K from TF-1 hemopoietic cells and examined its ability to phosphorylate Ser585 *in vitro*. PI3K immuno-purified using an anti-p85 antibody was able to phosphorylate a Ser585 peptide but not a control peptide in an LY294002-dependent manner (Fig. 4.4A). Consistent with the known divalent cation and redox requirements for PI3K(Dhand *et al.* 1994a; Dhand *et al.* 1994b), robust Ser585 phosphorylation only occurred under conditions where both Mn^{++} and DTT were present (Fig. 4.4B). Using isoform-specific antibodies, we immuno-purified individual Class I p110 catalytic subunits from TF-1 cells and examined their ability to phosphorylate Ser585 *in vitro*. TF-1 cells express predominantly p110α with detectable amounts of p110γ as determined by a PI3K lipid kinase activity assay (Fig. 4.4C). Consistent with this activity profile, our results show that immuno-purified p110α and p110γ were able to phosphorylate Ser585 in an LY294002-dependent manner (Fig. 4.4D).

To determine whether immuno-purified PI3K could phosphorylate βc within the context of a full-length protein, we performed *in vitro* kinase assays using the purified recombinant intra-cytoplasmic portion of βc (βic) (Guthridge *et al.* 2000). After first showing that immuno-purified PI3K was able to phosphorylate the p85 subunit as previously described (Dhand *et al.* 1994a; Dhand *et al.* 1994b)as well as purified recombinant βic (Fig. 4.4E), we then specifically examined the ability of a p110α mutant (K941-944A) that was defective in its lipid kinase activity but retained full protein kinase activity to phosphorylate βic (Naga Prasad *et al.* 2005). Although the K941-944A mutant was defective in phosphorylating PIPs (Fig 4.5A), it was clearly able to phosphorylate not only p85, but also βic (Fig. 4.5B). Furthermore, purified recombinant p110α, p110β and p110γ were able to phosphorylate βic in an LY294002-sensitive manner (Gif. 4.5C). Importantly, we also showed that purified recombinant p110α and p110γ can directly phosphorylate Ser585 in the context of the full-length purified recombinant βic protein by immunoblot analysis using a phospho-specific anti-phospho-Ser585 pAb (Fig. 4.5D). Although PKA

was also able to phosphorylate Ser585, it was less selective and phosphorylated multiple residues as evidenced by the mobility shift in βic (Fig. 4.5D, asterisks). Importantly, purified recombinant p110α was able to directly phosphorylate a Ser585 peptide and this phosphorylation was blocked by the p110α-selective inhibitor, PIK75 (Table 4.1)(Kim *et al.* 2009)(Fig. 4.5E). Taken together, these results indicate that the protein kinase activity of p110α can directly phosphorylate Ser585.

Table 4.1 PI3K selective p110 isoform inhibitors

Inhibitor	Selectivity	IC_{50} for p110α (μM)	IC_{50} for p110β (μM)	IC_{50} for p110δ (μM)	IC_{50} for p110γ (μM)	References
PIK-75	α	0.0078	0.345	0.907	-	Knight et al, 2006; Hayakawa et al 2007
PI-103	α	0.008	0.300	1.0	-	Knight et al., 2006
YM024	α, δ	0.3	2.65	0.33	9.07	Condliffe et al., 2005
TGX-221	β	5	0.05	0.1	3.5	Kim et al, 2009
IC87114	δ	>100	75	0.5	29	Billottet et al, 2006
AS25424	γ	1.07	>20	>20	0.035	Chaussade et al., 2007

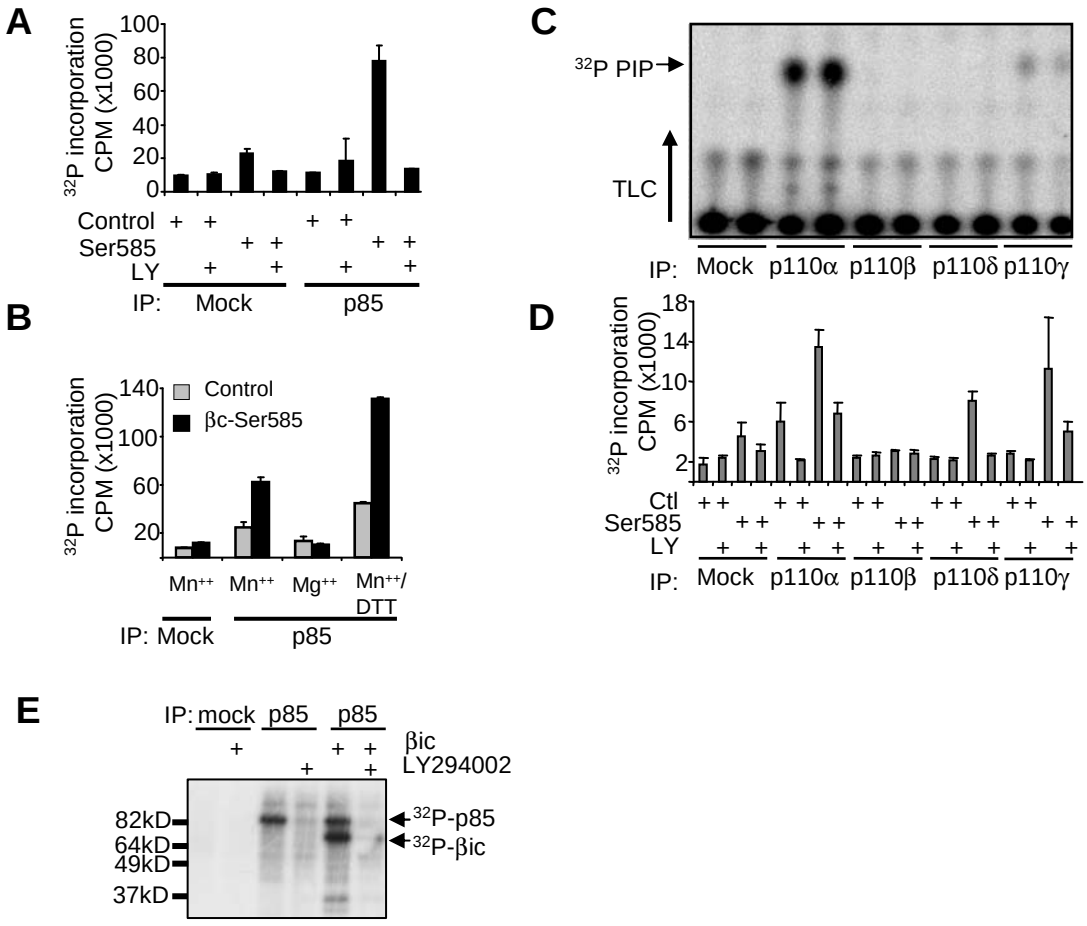


Figure 4.4: The protein kinase activity of PI 3-kinase can directly phosphorylate Ser585. PI3K was immunopurified from TF-1 cells by immunoprecipitation with anti-p85 pAb (no anti-p85 was used for the mock) following which *in vitro* kinase assays were performed using a βc-phosphoSer585 peptide (non-phosphorylatable control peptide) or a βcSer585 peptide and +/- 10μM LY294002 (A). Kinase activity was examined in either mock or p85 immunoprecipitates using either a βc-phosphoSer585 control peptide or a βcSer585 peptide in Mn^{++} kinase buffer(B). Immunoprecipitation of specific isoforms of p110 from TF-1 cells was performed and either lipid kinase assays (C) or βcSer585 peptide kinase assays were performed +/- 10μM LY294002 (D). Anti-p85 pAb immunoprecipitates from TF-1 cells were added to protein kinase reaction as above with 0.5μg purified recombinant intra-cytoplasmic domain of βc substrate (βic, see Chapter 2). Reactions were performed for 30 min at 30ºC before SDS-PAGE and autoradiography. Mock immunoprecipitates were performed in which no p85 pAb was included. LY294002 (10μM) was added to the kinase reactions where indicated. ^{32}P-labelled p85 and βic are indicated. Error bars indicate standard deviation of 2 replicates.

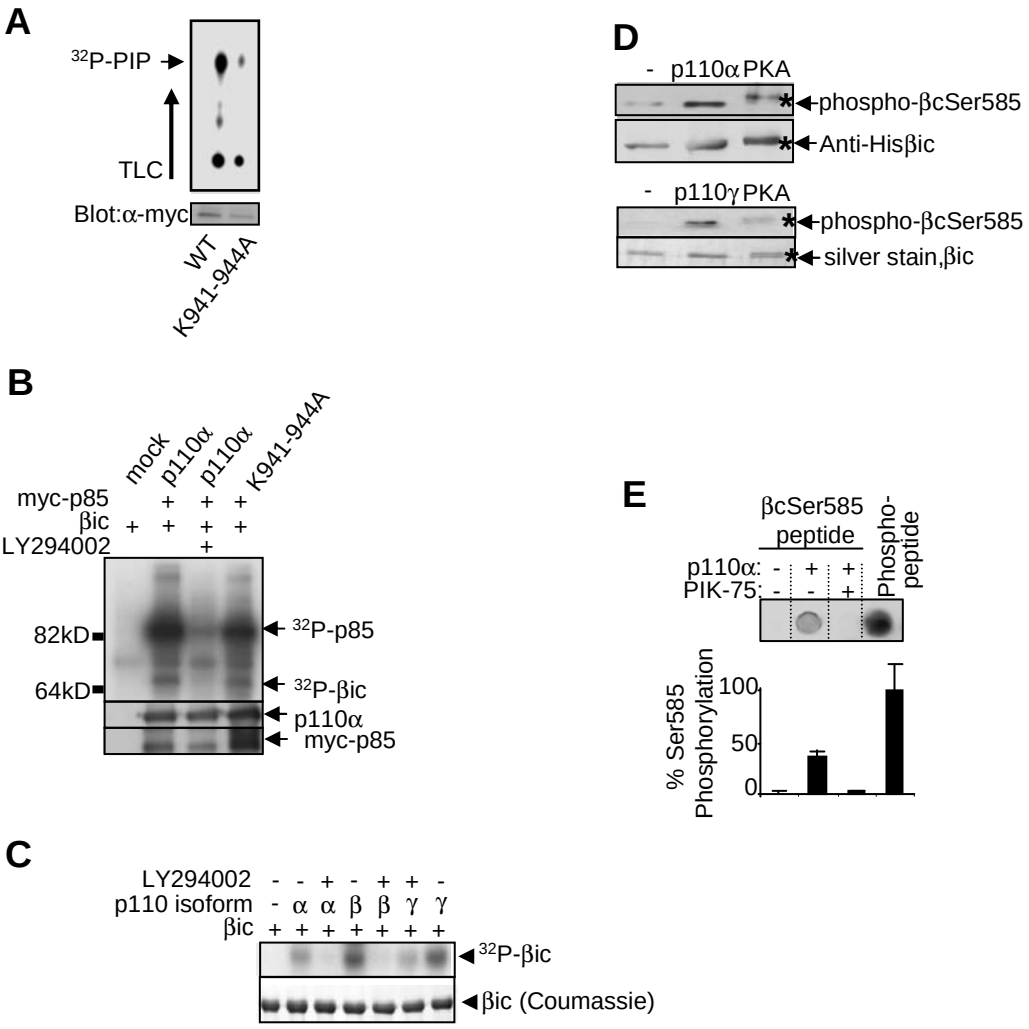


Figure 4.5: Protein kinase-only mutant of PI 3-kinase can directly phosphorylate βc on Ser585. (A). Constructs for the expression of Flag-tagged wild-type p110α (WT), a p110α-K941-944A mutant (K941-944A) and/or myc-tagged p85α were transfected into HEK293T cells. After 48h, PI3K was immunoprecipitated with the 9E10 anti-myc mAb and lipid kinase assays were performed and subject to thin layer chromatography (TLC). The direction of TLC as well as the migration of ^{32}P-PIP are indicated. (B) HEK293T cells were transfected with Flag-tagged wt p110α (p110α), a Flag-tagged lipid-kinase defective form of p110α (K941-944A) and/or myc-tagged p85. myc-p85 was immunoprecipitated and subjected to *in vitro* kinase assays using purified recombinant intracytoplasmic domain of βc (βic) following which reactions were subjected to SDS-PAGE and autoradiography or immunoblot analysis for p110α and myc-p85. (C) Purified recombinant p110α, p110β or p110γ (0.5μg) were incubated with 0.5mg βic and 0.25mCi[γ-^{32}P]ATP in kinase buffer and products examined by SDS-PAGE and autoradiography. Coumassie staining of the gel indicates loading. (D) Purified PKA, purified recombinant p110α or p110γ were examined for their ability to phosphorylate Ser585 in purified recombinant βic *in vitro* and reactions were subjected to immunoblot analysis using anti-phospho-βcSer585 pAb. Asterisks indicate hyperphosphorylated βic as evidenced by a mobility shift. (E). Purified recombinant p110α was incubated with either 50mM βc-phosphoSer585 control peptide or 50mM βcSer585 peptide +/- 50nM PIK75 p110α-selective inhibitor (F). Reactions were spotted onto nitrocellulose and blotted with the anti-phospho-βcSer585 pAb. 50mM phospho-βcSer585 peptide was included as a positive control for staining with the anti-phospho-βcSer585 pAb. The histogram shows laser densitometry quantification of signals. Error bars represent standard deviation.

4.6 The protein kinase activity but not the lipid kinase activity of PI3K regulates cell survival.

While the ability of PI3K to promote cell survival has almost exclusively been attributed to its lipid kinase activity, the potential biological roles of PI3K protein kinase activity have been largely overlooked. Our previous studies have shown that very low concentrations of cytokine in the picomolar range can promote Ser585 phosphorylation and cell survival in the absence of phosphotyrosine pathways and proliferation (Guthridge *et al.* 2006) while our current studies indicate that PI3K protein kinase activity can phosphorylate Ser585. If PI3K was able to phosphorylate Ser585 in cells, then picomolar concentrations of cytokine that induce Ser585 phosphorylation should also activate PI3K protein kinase activity. We therefore examined the regulation of both the protein kinase and lipid kinase activities of PI3K in response to increasing concentrations of cytokine. In order to examine the regulation of PI3K protein kinase activity, we analysed p85-Ser608 phosphorylation which has been shown to be a direct substrate of p110 (Dhand *et al.* 1994a; Foukas *et al.* 2004). Low picomolar concentrations of GM-CSF that were able to promote Ser585 phosphorylation (0.1-1pM) were also able to activate the protein kinase activity of PI3K as evidenced by increased p85-Ser608 phosphorylation (Fig. 4.6A). However, such low picomolar concentrations did not detectably activate PI3K lipid signaling as evidenced by the lack of both Akt and GSK-3 phosphorylation (Fig. 4.6B), p85 tyrosine phosphorylation (Fig 4.6A) or activation of PI3K lipid kinase activity (Fig. 4.6C). Thus, PI3K demonstrates two distinct modes of signaling with PI3K protein kinase signaling being regulated by low picomolar cytokine concentrations and PI3K lipid kinase signaling being regulated by higher nanomolar concentrations.

We then examined whether PI3K lipid kinase activity was essential for regulating cell survival by examining the role of the key downstream lipid signaling target of PI3K, Akt. Our results show that 1pM GM-CSF was able to promote the survival of primary mouse BM progenitor cells derived from either Akt1-/- mice or derived from wild-type (wt) mice and treated with an Akt1 inhibitor (AKTI-1) (Fig. 4.6D). Conversely, experiments performed in collaboration with Dr Paul Ekert (WEHI, Parkville) showed that inducible-expression of a constitutively active form of Akt (myr-Akt-1) was not sufficient to support

the long-term viability of factor-dependent myeloid cells in the absence of cytokine (Fig. 4.7A, Fig. 4.7B).

Because picomolar concentrations of cytokine activated the protein kinase but not lipid kinase activity of PI3K, we examined the ability of low cytokine concentrations to promote cell survival under conditions where the protein kinase activity of PI3K was blocked by the p110α-selective PI3K inhibitor, PIK75, but downstream PI3K lipid signalling was enforced by expression of myr-Akt-1. Blockade of PI3K protein kinase activity induced by 1pM GM-CSF using PIK75 resulted in cell death despite constitutive signaling by myr-Akt-1 indicating that the protein kinase activity of PI3K was required for cell survival (Fig. 4.7C). We also performed an inverse experiment and examined the effect of selectively blocking PI3K lipid signaling while allowing PI3K protein kinase signalling. For these experiments we over-expressed the PH domain of Akt1 fused to GFP (PH-Akt-1) in order to block the binding of endogenous PH-domain proteins (such as Akt) to PIPs in the plasma membrane thereby abrogating PI3K lipid signaling but permitting PI3K protein kinase signalling. Using this approach, we examined the regulation of cell survival in response to either 1pM cytokine (that was able to promote PI3K protein kinase activity) or 1000pM cytokine (that was able to promote PI3K lipid kinase activity)(Fig 4.6A-C). While expression of PH-Akt-1 was able to reduce cell viability under conditions where PI3K lipid kinase signaling is activated by high cytokine concentrations (Fig. 4.7D, 1000pM), it had no significant effect on cell survival under conditions where only the protein kinase activity of PI3K is induced by low cytokine concentrations (Fig. 4.7D, 1pM). Together the data shown in Figure 4.6 and 4.7 indicates that lower concentrations of cytokine (1pM) are able to promote a survival-only response that requires the protein kinase activity of PI3K but not its lipid kinase activity.

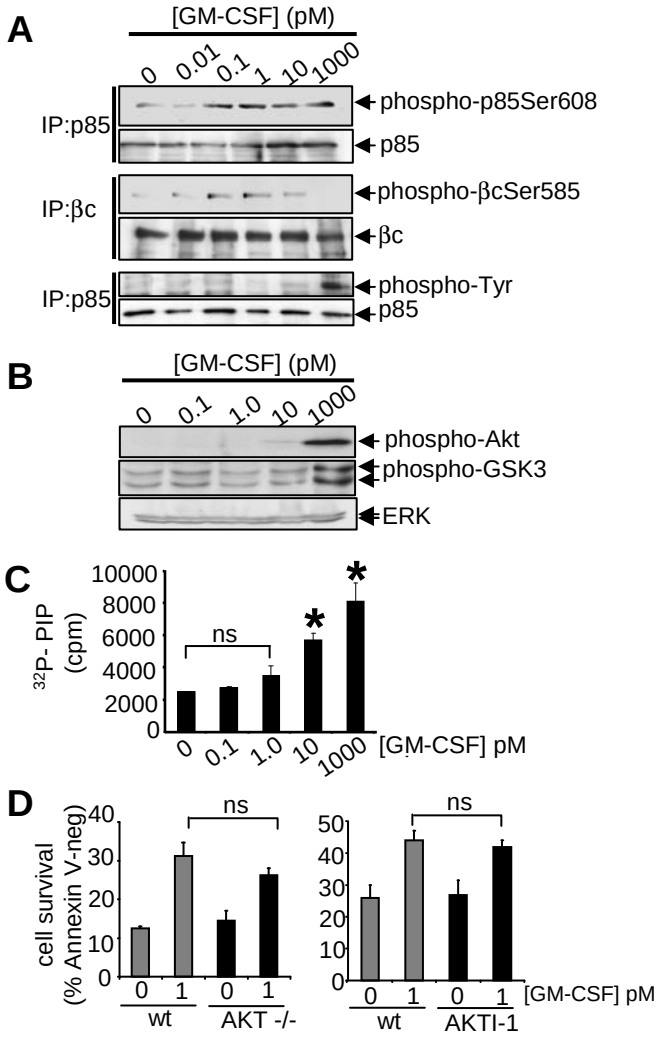


Figure 4.6: PI3K protein kinase activity but not lipid kinase activity promotes the survival-only response. Factor-deprived TF-1 cells were stimulated for 20min with GM-CSF, the cells lysed and p85 or βc immunoprecipitates were subjected to immunoblot analysis (A). Lysates from (A) were blotted with an anti-phospho-Ser473 Akt pAb, anti-phospho-Ser21/9-GSK3α/β pAb or total ERK pAb (B). Lysates from (A) were subjected to 4G10 immunoprecipitation and lipid kinase assays were performed using [γ-^{32}P]ATP and phosphatidyl inositol (PI) as substrates (C). Error bars represent SEM from 3 independent experiments shown, * indicating $p<0.05$. Primary mouse Lin- haemopoietic progenitor cells from fetal livers of wt and AKT1-/- mice were plated in GM-CSF and cell survival was determined after 72h (D, left panel). The survival of Lin- cells isolated from wt mice was also assessed in the presence of 10μM Akt inhibitor (AKTI-1)(D, right panel).

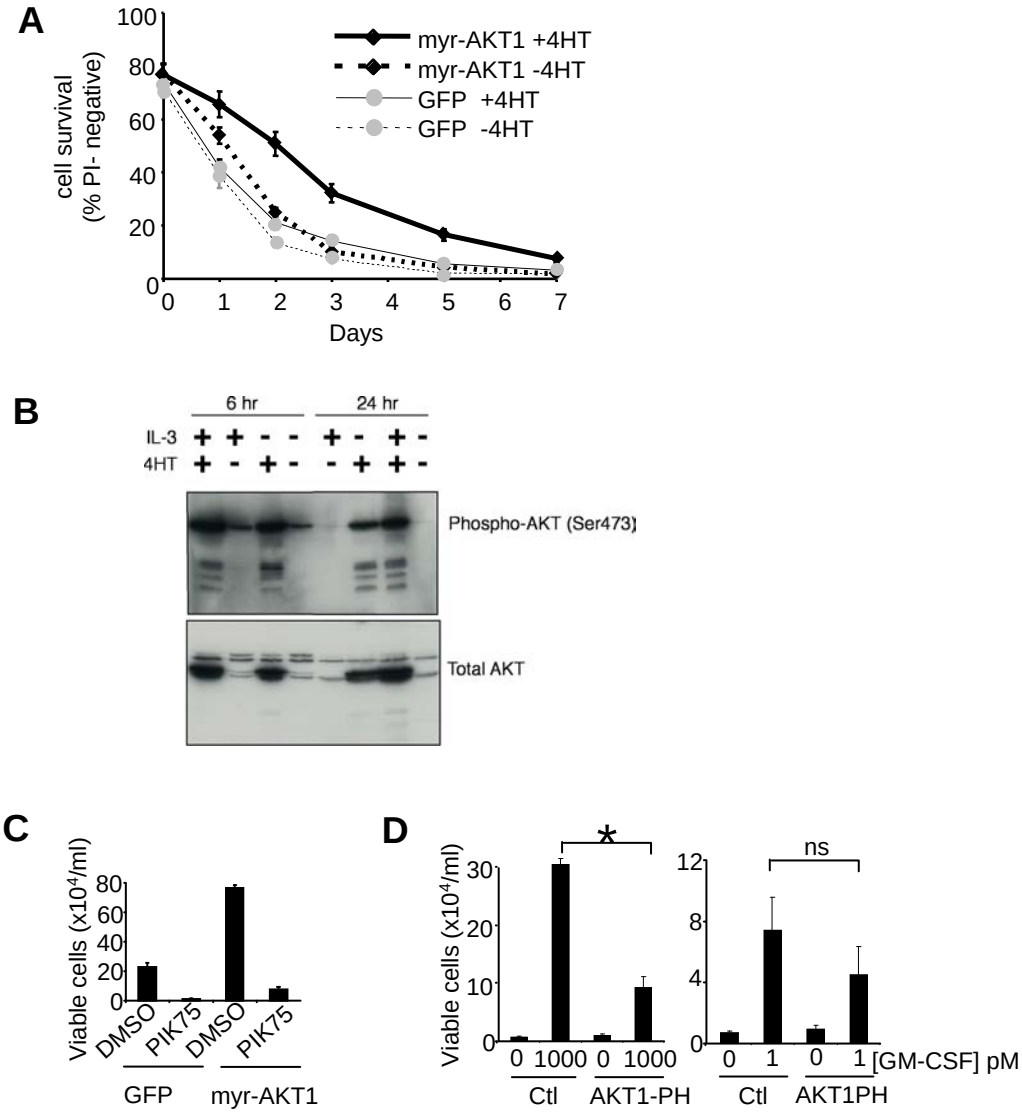


Figure 4.7: Lipid kinase activity insufficent for survival-only response. FDM cells expressing either GFP (GFP) or a tamoxifen-inducible constitutively active form of myristoylated Akt1 (myr-Akt1) were plated in the absence of cytokine and +/- 4-hydroxy tamoxifen (4HT) and cell survival was assessed by propidium iodine exclusion and flow cytometry (A). The same pools of GFP+ FDM cells treating with 1µM 4-hydroxytamoxifen (4-OHT) for 6 or 24h followed by immunoblotting lysates with the 12CA mAb. Indicated is the total Akt (12CA blots) and phosphorylated Akt (phospho473-Akt) (B). TF-1 cells were co-transfected with myr-Akt1 and GFP or GFP alone and plated in 1pM GM-CSF and either 100nM PIK-75 or DMSO. The number of GFP+ viable cells was counted at 48h using Flowcount fluorospheres and flow cytometry (C). TF-1 cells were transfected with plasmids encoding either GFP or GFP-Akt1-PH and the number of GFP+ viable cells was counted at 48h using Flowcount fluorospheres and flow cytometry (D). Error bars represent standard deviation, * indicating $p<0.05$. The experiments in (A) and (B) were performed in the laboratory of Dr Paul Ekert, Walter and Eliza Hall, Parkville.

4.7 Selective inhibition of p110α down-regulates Ser585 phosphorylation

We next examined the impact of targeting PI3K on Ser585 phosphorylation and cell survival. As shown in Figure 4.8A, TF-1 factor-dependent cells rapidly lose viability in the absence of GM-CSF (0pM) and are able to proliferate in response to higher concentrations of cytokine (1000pM). Importantly, lower concentrations of cytokine (1pM) that were able to promote PI3K protein kinase signaling but not lipid signaling (Fig. 4.6) were also able to maintain the viability of TF-1 cells for up to 2 weeks in the absence of detectable proliferation ("survival-only" response)(Fig. 4.8A). To test whether Ser585 of endogenous βc was a substrate for p110 under these “survival-only” conditions, cells were pretreated with both pan-specific (LY294002 and Wortmannin) and isoform-selective PI3K inhibitors (Table 4.1) and then stimulated with 1pM cytokine. Our results show that both LY294002 and Wortmannin down-regulated Ser585 phosphorylation induced by 1pM GM-CSF (Fig. 4.9A,E). Furthermore, 2 different p110α-selective inhibitors (PIK-75, PI-103 see Table 4.1) as well as a p110α/δ-selective inhibitor (YM024) were able to down-regulate Ser585 phosphorylation (Fig. 4.9A,B,DE). In contrast, p110β-selective (TGX-221) and p110γ-selective (AS252424) inhibitors had no detectable effect on Ser585 phosphorylation while p110δ-selective (IC87114) and the PKA inhibitor, H89, had modest effects (Fig. 4.9E). Consistent with its ability to block Ser585 phosphorylation, the p110α-selective inhibitor, PIK-75, was also able to block the survival-only response of TF-1 cells and murine lin- BM progenitor cells in the presence of 1pM GM-CSF (Fig. 4.10A,B). In addition YM024, an α/δ selective inhibitor and PI-103 α-selective inhibitor blocked 1pM survival in BM progenitor cells. Overall, these results show that under survival-only conditions in which low picomolar cytokine concentrations activate the protein kinase activity of PI3K, inhibition of p110α not only blocks Ser585 phosphorylation of endogenous βc but also cell survival.

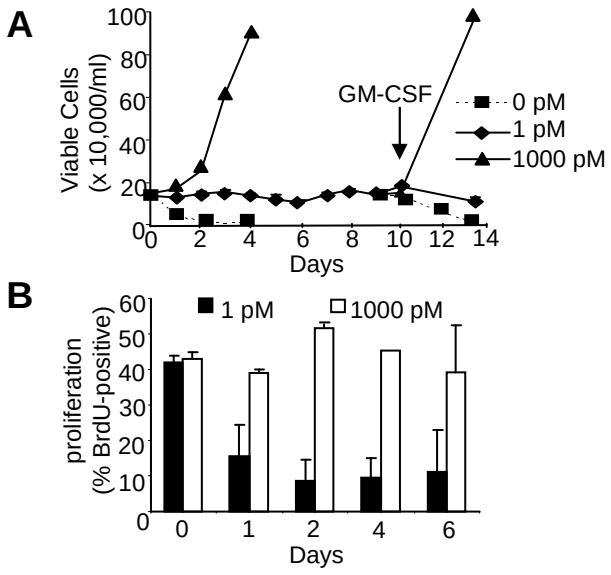


Figure 4.8: Robust survival but slow proliferation in TF-1 cells maintained at 1pM GM-CSF. TF-1 cells were plated in GM-CSF for up to 14 days (media changed every 2 days) at the concentrations shown and cell viability was determined by trypan blue exclusion (A). On day 10, an aliquot of cells growing in 1pM was washed and 1000pM GM-CSF added (arrow). TF-1 cells were plated in GM-CSF and proliferation was measured by BrdU-incorporation (B).

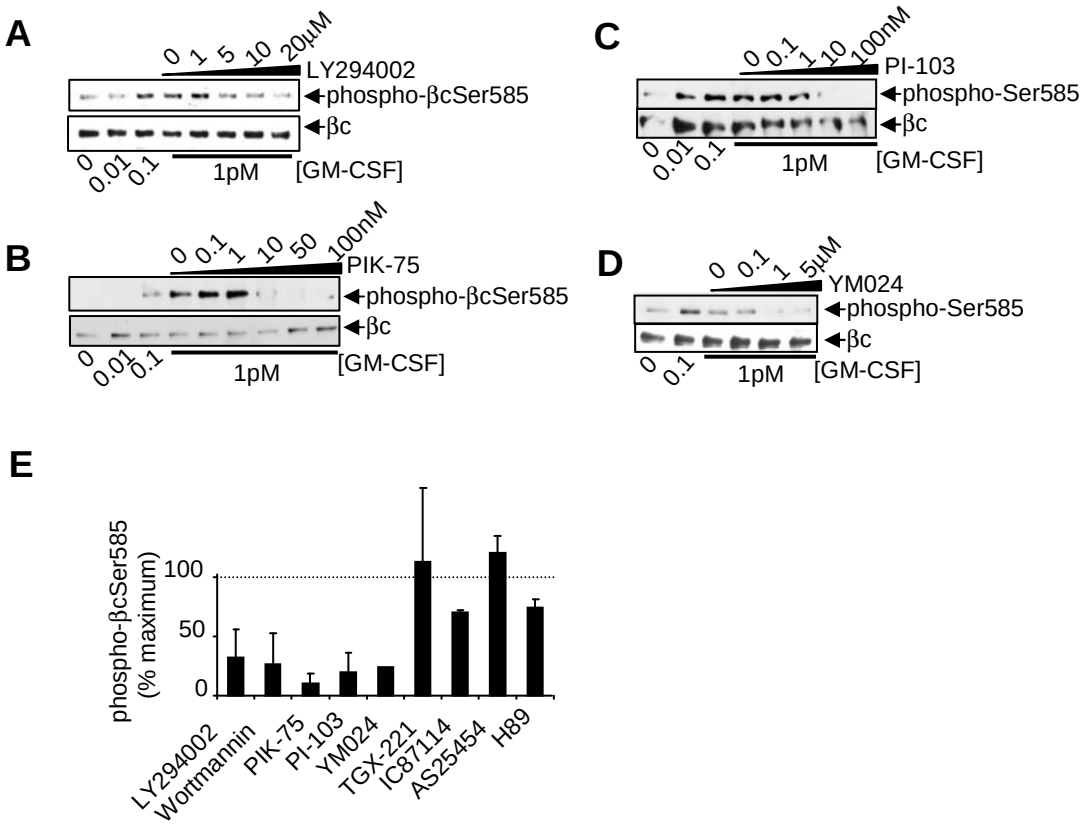


Figure 4.9: Inhibition of endogenous Ser585 phosphorylation with PI3K p110α-selective inhibitors. Factor-deprived TF-1 cells were pre-incubated for 45min with LY294002 (A), PIK75 (B), PI-103 (C), YM024 (D) at the concentrations shown and then stimulated for 5min with 0.01, 0.1 and 1pM GM-CSF as shown before lysis and immunoblot analysis of βc immunoprecipitates using anti-phospho-βcSer585 pAb and anti-βc mAb. Laser densitometry quantification of the ability of p110 isoform-selective inhibitors to block Ser585 phosphorylation in which the ratio of phospho-Ser585 to total βc in the presence of drug is expressed as a percentage of the maximum Ser585 phosphorylation in DMSO (set at 100%, dotted line)(E).

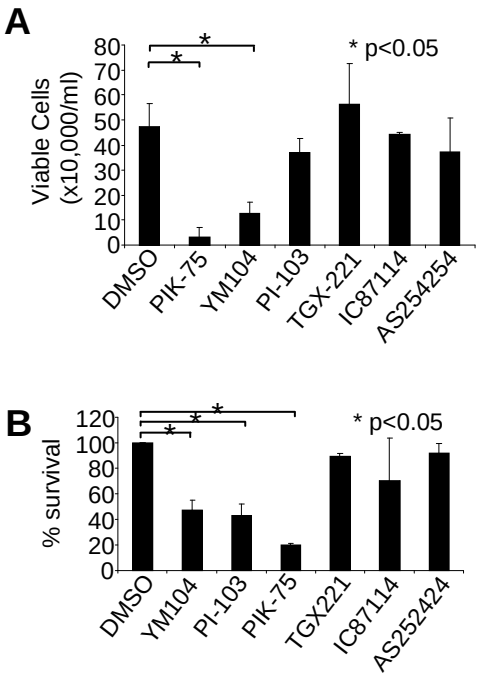


Figure 4.10: Inhibition of cell survival at 1pM GM-CSF. (A) TF-1 cells were cultured in 1pM GM-CSF in the presence of 100nM PIK-75, 5μm YM024, 100nM PI-103, 1μM TGX-221, 5μM IC87114 or 100nM AS254254 and cell viability was determined at 72h by trypan blue exclusion. This experiment was repeated 3 times, error bars indicate standard devision. (B) Lineage negative mouse bone marrow progenitors were isolated as described in Chapter 2 using MACS beads and plated for 72 h in 1pM murine GM-CSF in the presence of DMSO or PI3K inhibitors at the same doses as used in (A) Error bars indicate standard deviation. * indicating $p<0.05$.

4.8 Selective inhibition of p110α with PIK-75 down-regulates endogenous Ser585 phosphorylation in primary human AML blasts.

Because PI3K was isolated as a Ser585-kinase from human AML (Fig. 4.3), and p110α was able to phosphorylate Ser585 (Fig. 4.4 & 4.5), we wished to further assess the effect of p110α inhibition on constitutive Ser585 phosphorylation in primary AML cells. PIK75 was able to significantly down-regulate constitutive Ser585 phosphorylation in a panel of primary AML samples (for patient details see Table 4.2) (Fig. 4.11A, median decrease 54%, range 2-84%, p=0.003, Mann-Whitney U with 6/11 AMLs showing >50% decrease) as well as block in PI3K lipid kinase targets as evidenced by a decrease in Akt phosphorylation (Fig. 4.11B, median decrease 69.5%, range 33-80%, p<0.002). Selective inhibition of p110β with TGX-221 (median decrease 27%, range 6-34%, p=0.03, Mann-Whitney U), p110δ with IC87114 (median decrease 13%, range 0-38%, p=NS) or p110γ with AS25424 (median decrease 21%, range 0-53%, p=NS) were less effective at down-regulating Ser585 (Fig 4.11A). We therefore tested the ability of PIK75 to induce apoptosis in AML blasts derived from patient samples that were sensitive to PIK75-mediated down-regulation of Ser585 phosphorylation. Our results show that PIK75 was able to potently and selectively induce apoptosis in primary AML blasts (Fig. 4.11C) and block their clonogenic growth (data not shown).

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Table 4.2 Patient clinical characteristics

Patient ID*	Age/Sex	Blast Count	WCC	FAB	Karyotype	Flt3-ITD
AML1	75/M	92%	300	M5a	Trisomy 8	Yes
AML2	69/F	96%	135	M0	XX	Yes
AML3	19/M	79%	150	M4	XY	Yes
AML4	85/M	46%	179	M4	+8	Yes
AML5	48/F	99%	110	M1	11q23	No
AML6	63/F	97%	192	M4	t(2;11)	No
AML7	76/M	96%	56.8	M1	XY	ND
AML8	41/M	24%	1.6	M2	XY	No
AML9	42/F	98%	49	M1	t(9;11)	No
AML10	67/M	14%/ALIP	12.5	M2	complex	ND
AML11	71/M	90%	101	M1	-5q	No
AML12	39/F	92%	102	M1	XX	Yes
AML13	72/F	96%	210	M1	XX	Yes

ND= not determined

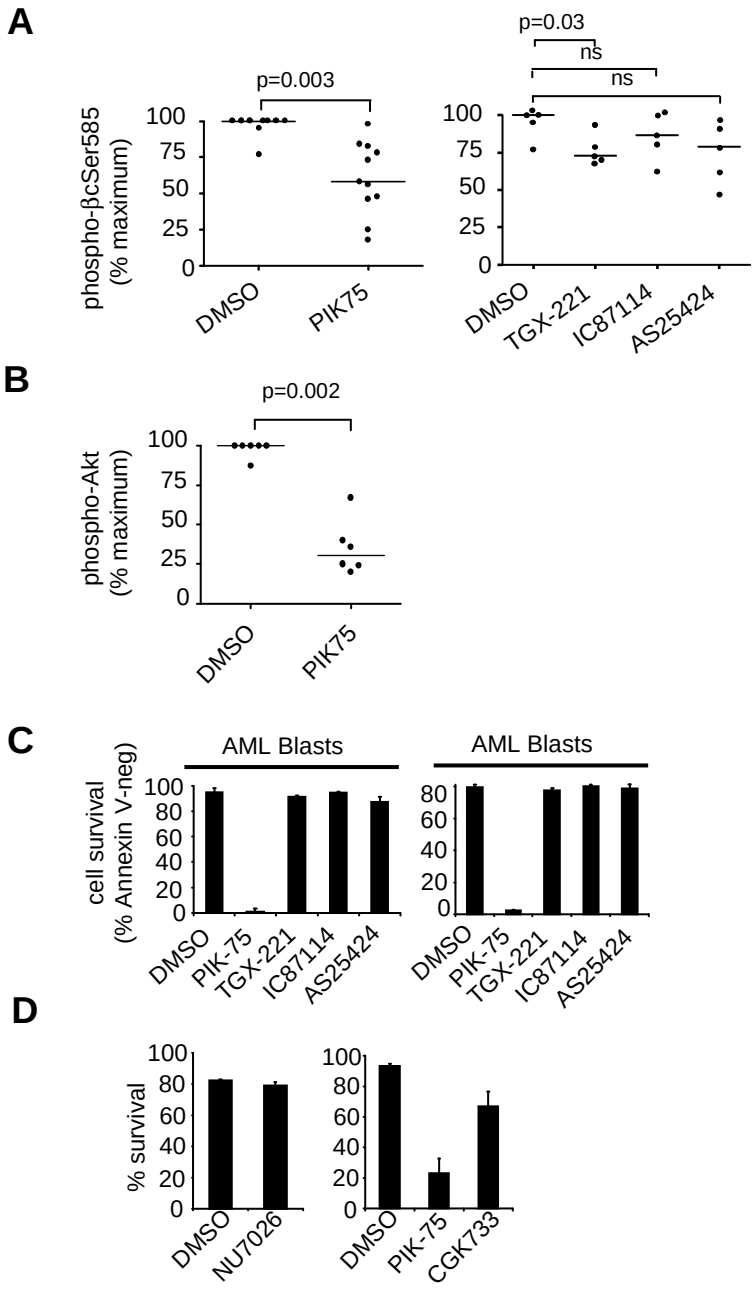

Figure 4.11: Inhibition of p110α with PIK75 selectively down-regulates Ser585 phosphorylation and induces apoptosis in primary AML MNCs. AML MNCs (from patients AML2-AML13) were incubated with either 1μM PIK-75, 1μM TGX-221, 5μM IC87114, 100nM AS254254 or DMSO for 1h following which the cells were lysed and βc immunoprecipitates were subject to immunoblot analysis with either anti-phospho-βcSer585 pAb or 1C1 anti-βc mAb (A) and anti-phospho-Akt Ser473 pAb or ERK pAb (B). Results were quantified by laser densitometry and analysed by Mann-Whitney U. The ratio of phospho-Ser585 to total βc or phospho-Akt to total ERK in the presence of drug is expressed as a percentage phosphorylation in DMSO. AML MNCs from AML patients were plated in PIK-75 (100nM), TGX-221 (1μM), IC87114 (5μM) or AS25424 (100nM) and cell survival examined at 24h by annexin V staining and flow cytometry (C). AML mononuclear cells from AML patients (AML3, AML4) were plated in DNA-PK inhibitor NU7026 (10μM), PIK-75 (100nM) or ATM kinase inhibitor CGK733 (10μM) and cell survival examined at 24h by annexin V staining and flow cytometry (D).

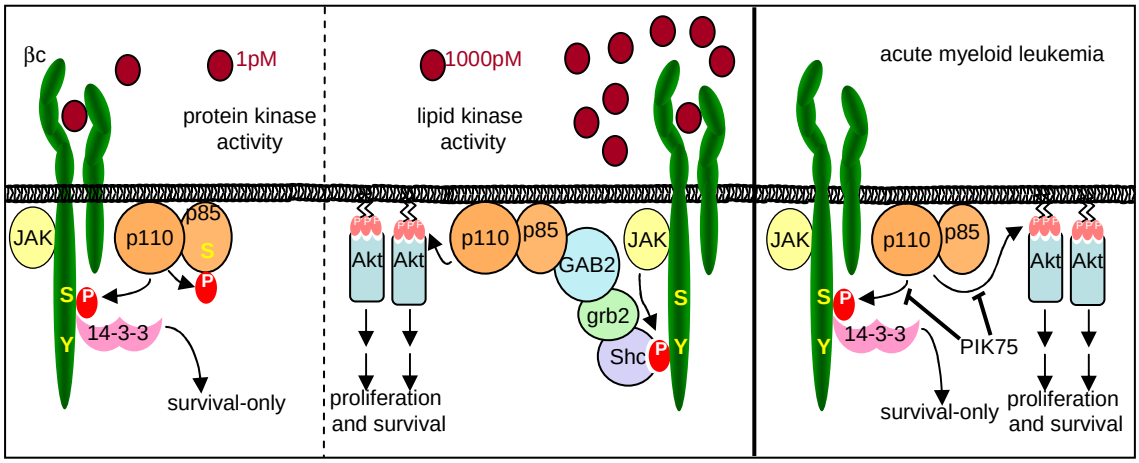

Figure 4.12: Model for the regulation of cell survival by the protein kinase or lipid kinase activities of PI3K. (Left panel) Low physiological concentrations of cytokine in the picomolar range activate the protein kinase activity of PI3K leading to Ser608 phosphorylation of p85 and Ser585 phosphorylation of the GM-CSF/IL-3 βc receptor to promote cell survival in the absence of phosphotyrosine pathways and proliferation (survival-only response). (Middle panel) Nanomolar concentrations of cytokine result in activation of the JAK2 tyrosine kinase, Tyr577 phosphorylation of βc, the recruitment of a PI3K signaling complex via Shc and the activation of canonical PI3K lipid signaling via AKt to promote cell proliferation and survival. (Right panel) Blockade of the p110α catalytic subunit of PI3K with PIK75 blocks both protein kinase targets (Ser585) and lipid kinase targets (Akt) and induces apoptosis in primary human AML cells.

4.9 Conclusion

PI3K is a key regulator of cell survival and deregulated PI3K signaling occurs frequently in cancer. While many cytokines and growth factors are able to regulate PI3K lipid signaling, little is known of their ability to regulate PI3K protein kinase signaling or whether the protein kinase activity of PI3K is important in promoting cell survival. We and others have identified key serine residues in the cytoplasmic tails of cytokine and growth factor receptors that selectively control cell survival (Guthridge *et al.* 2006; Guthridge *et al.* 2004; Guthridge *et al.* 2000; Lonic *et al.* 2008; Peruzzi *et al.* 1999; Powell *et al.* 2009). In the case of the GM-CSF and IL-3 βc receptor, constitutive Ser585 phosphorylation is associated with deregulated cell survival programs in myeloid leukaemia (Powell *et al.* 2009). Importantly, constitutive Ser585 phosphorylation in leukemic cells is refractory to tyrosine kinase inhibition and thus may provide a mechanism by which transformed cells are able to survive in the presence of TKIs (Fig. 4.1). We have now isolated a kinase activity from primary AML samples that is able to phosphorylate Ser585 *in vitro* and shown that this activity is uniquely sensitive to PI 3-kinase inhibitors (Fig. 4.3). We have further shown that purified recombinant p110α can directly phosphorylate Ser585 *in vitro* (Fig. 4.4) and that inhibition of p110α using three independent p110α-selective inhibitors down-regulated endogenous Ser585 phosphorylation (Fig. 4.9). Treatment of primary AML blasts with the p110α inhibitor, PIK75, down-regulated constitutive Ser585 phosphorylation and induced apoptosis (Fig. 4.10). These results indicate that the protein kinase activity of PI3K can phosphorylate Ser585 and that targeting the p110α isoform of PI3K with PIK75 not only reduced Ser585 phosphorylation but also induced apoptosis in primary AML blasts (Fig. 4.12, model).

While the lipid kinase activity of PI3K has been implicated in a wide array of cellular responses including cell survival (Cantley, 2002), little is known regarding the protein substrates of PI3K and their functional significance (Dhand *et al.* 1994a; Foukas and Shepherd, 2004a; Hunter, 1995). Initial reports identified several autophosphorylation sites in either the p85 regulatory subunit (p85-Ser608) (Dhand *et al.* 1994a), or the p110 catalytic subunits (p110β–Ser1070 (Czupalla *et al.* 2003), p110δ-Ser1039

(Vanhaesebroeck *et al.* 1999), and p110γ-Ser1101 (Czupalla *et al.* 2003)). Additionally, a number of other protein substrates of PI3K have been reported including p101 (Bondev *et al.* 1999), insulin receptor substrate 1 (IRS1) (Lam K *et al.* 1994; Tanti *et al.* 1994), PDE3B phosphodiesterase (Rondinone *et al.* 2000), eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) (Foukas and Shepherd, 2004b), mitogen-activated protein kinase kinase (MEK1) (Bondev *et al.* 1999) and H-Ras (Foukas and Shepherd, 2004b). However, these earlier studies did not determine the specific residues phosphorylated by PI3K nor their functional significance. To our knowledge, only one other specific phosphorylation site has been identified for the protein kinase activity of PI3K for which a functional role has been ascribed (Table 4.3). Prasad *et. al.* have shown that p110γ can phosphorylate Ser61 of non-muscle tropomyosin which is required for agonist-dependent β-adrenergic receptor internalization (Naga Prasad *et al.* 2005). From the limited substrates identified, no clear consensus motif for PI3K is apparent (Table 4.3) suggesting secondary or tertiary structural determinants may be important for target recognition.

The activation of canonical Type 1A PI3K lipid signalling requires the recruitment of p85 SH2 domains to pYXXM (where pY is phosphotyrosine) phosphotyrosine docking sites at the plasma membrane, either in the cytoplasmic tails of cell surface receptors or their associated signalling proteins (Pawson, 2004). This mode of signalling is triggered by higher concentrations of ligand in the nanomolar range that induce receptor dimerization/oligomerization and the trans-activation of tyrosine kinases (Schlessinger, 2000). However, several lines of evidence indicate that low picomolar concentrations of ligand promote Ser585 signalling and cellular survival in the absence of phosphotyrosine pathways, PI3K lipid signaling and proliferation. Firstly, while high concentrations of cytokine clearly activate the lipid kinase activity of PI3K, we were unable to observe any detectable activation of lipid kinase activity in response to 1pM cytokine (Fig. 4.6) despite the ability of these concentrations of cytokine to promote long-term cell survival (Fig. 4.8). Secondly, genetic or pharmacological blockade of the key downstream target of PI3K lipid signaling, Akt, had no effect on hemopoietic cell survival in response to 1pM cytokine (Fig. 4.6). Thirdly, while key downstream targets of PI3K lipid signaling such as Akt or GSK were clearly phosphorylated in response to high nanomolar doses of cytokine,

phosphorylation was not detected in response to 1pM cytokine (Fig. 4.6). Fourthly, although we found no evidence of PI3K lipid signaling in response to 1pM cytokine, we were clearly able to detect cytokine-regulated PI3K protein kinase activity as evidenced by the induction of p85-Ser608 and βc-Ser585 phosphorylation (Fig. 4.6). Fifthly, enforcing downstream lipid kinase signaling by targeting Akt1 to the plasma membrane while blocking the protein kinase activity of PI3K in response to 1pM cytokine with PIK75 was able to block cell survival (Fig. 4.7). Sixthly, selectively blocking the lipid kinase activity of PI3K by over-expression of an Akt1 PH domain that dominant-negatively blocks PIP docking sites in the plasma membrane but allowing PI3K protein kinase activity in the presence of 1pM cytokine permitted cell survival (Fig. 4.7). Thus, our results highlight an important distinction between the regulation of PI3K lipid kinase and protein kinase signaling. While higher concentrations of cytokine can regulate phosphotyrosine pathways, PI3K lipid signaling, the phosphorylation downstream lipid signaling targets to promote cell proliferation and survival, lower concentrations of cytokine promote the activation of PI3K protein kinase activity, Ser585 phosphorylation and cell survival in the absence of other biological responses such as proliferation (Fig. 4.12, model).

Others have also suggested that PI3K can provide multiple independent signaling outputs with p110γ regulating Akt signalling via its lipid kinase activity and regulating ERK signalling via its protein kinase activity (Bondeva *et al.* 1998). While the functional significance of this signal bifurcation remains unclear, it is intriguing that the insulin and IFNα receptors can activate the protein kinase activity of PI3K in a phosphotyrosine-independent manner (Rondinone *et al.* 2000; Uddin *et al.* 2000). In the case of the βc subunit, the mechanism by which PI3K is recruited and activated leading to Ser585 phosphorylation is not clear. Normally p110α PI3K lipid kinase activity is increased allosterically upon p85 binding to phosphotyrosine residues in βc phosphorylated by JAK2. Our previous work using a βc mutant in which all 8 cytoplasmic tyrosine residues are substituted for alanine (βcF8) still retained Ser585 phosphorylation (Guthridge et al, 2000). This suggests PI3K is not recruited in a classic phospho-tyrosine-dependent manner and must use alternative mechanisms for activation. It is possible that in addition to recruitment to phosphotyrosine docking sites, class 1A PI3Ks such as p110α can also be recruited via phosphotyrosine-independent mechanisms similar to those employed for the

recruitment of p110γ to G-protein coupled receptors (Curnock *et al.* 2002) or p110β and p110δ to ErbB3 (Jiang *et al.* 2010). For example, Perugini et al., have shown that SH3-domain containing proteins such as p85 are able to bind a conserved PXXP motif in the cytoplasmic tail of the α-subunit of the GM-CSF and IL-3 receptors (Perugini *et al.* 2010). In line with this proposal we have shown that p85 is associated with the α-subunit fo the IL-3 receptor (unpublished data) indicating that this pre-assembled p85:α-subunit complex would be available to phosphorylate βc on Ser585 following ligand stimulation.

In terms of the ability of PIK75 to induce apoptosis in AML blasts, it is important to note that PIK75 inhibits both the protein and lipid kinase activity of p110α. Thus, the potent ability of PIK75 to induce apoptosis may not only result from inhibition of PI3K protein kinase targets (such as Ser585), but also lipid kinase targets (such as Akt)(Fig. 4.12, model). Deregulated PI3K lipid signalling has been widely observed in many cancers and activating mutations in p110α are frequently observed in solid tumors. However, p110α mutations are rare in AML (Muller *et al.* 2007). Nevertheless, constitutive PI3K lipid signalling is prevalent in AML as indicated by elevated basal levels of Akt phosphorylation (Fig. 4.11) (Muller *et al.* 2007; Powell *et al.* 2009). On the other hand, the prevalence of deregulated PI3K protein kinase activity in AML is not known. While Ser585 phosphorylation is constitutive in >85% of human AMLs, PIK75 did not down-regulate Ser585 phosphorylation in all AML samples examined (Fig. 4.11) suggesting that serine kinases other than p110α may also lead to deregulated Ser585 phosphorylation.

Others have shown that targetting p110δ with IC87114 prevents the proliferation of AML blasts, but the effect on the cell survival was not determined (Sujobert *et al.* 2005). In our studies, IC87114 as well as p110β-selective (TGX-221) and p110γ-selective (AS25424) inhibitors were not effective in down-regulating either Ser585 phosphorylation or promoting apoptosis in AML blasts (Fig. 4.11) suggesting that p110α is likely to be the primary isoform promoting Ser585-survival signaling in AML. It is also important to note that while nanomolar concentrations of PIK75 were sufficient to block Ser585 phosphorylation and induce apoptosis of AML blasts, it remains possible that these activities were due to the inhibition of kinases other than p110α. However, two other

p110α–selective inhibitors, PI-103 and YM-104 were also able to abrogate Ser585-survival signaling suggesting that regardless of possible PIK75 "off-target" activities, p110α represents an important regulator of Ser585 phosphorylation. While a comprehensive screen of PIK75-sensitive kinases remains to be reported, Knight *et. al.* have shown that from a panel of 36 kinases, only DNA-PK had a similar IC_{50} to p110α (Knight *et al.* 2006). However, inhibition of DNA-PK and the related PI3K family member ATM had no effect on the survival of AML blasts (Fig. 4.11D).

There are several limitations of the data presented in this chapter. The PI3K isoform selective inhibitors used in this study may not be entirely selective. Confirmation that p110α is directly responsible for Ser585 phosphorylation requires depletion of p110α by siRNA or genetic knockout approaches *in vivo*. Future experiments should examine the effect of PI3K p110α and/or p85 knockdown by siRNA on Ser585 phosphorylation in AML cell-lines and primary material. Secondly, isoform-selective kinase inhibitors such as PIK-75 and YM024 work as ATP-analogues and not only inhibit the protein kinase activity of p110α but also its lipid kinase activity. Therefore one cannot determine whether the observed inhibition of AML blast survival is due solely inhibition of Ser585 phosphorylation. A future experiment to investigate this would be testing survival of cells depleted of wildtype p110α but re-expressing the K941-994A protein kinase-only mutant p110α and showing retained survival but not proliferation.

Our results identify a new role for PI3K in which its protein kinase activity phosphorylates growth factor receptors to initiate downstream signaling leading to survival-only responses. The ability of PI3K to switch between protein kinase and lipid kinase activities would thus allow two independent modes of signalling each functionally linked to a distinct cellular outcome. Discovery of other protein kinase substrates of PI3K that are constitutively phosphorylated in cancer may reveal useful biomarkers and therapeutic targets for PI3K-pathway drug development.

Table 4.3. PI3K protein kinase substrate phosphorylation sites

Substrate	ID/Accession	Phospho-Ser	Sequence
PI 3-kinase p85 regulatory subunit	P85A_HUMAN	Ser608[1]	LGNENTEDQY**S**LVEDDEDLPH
PI 3-kinase p110β catalytic subunit	PK3CB_HUMAN	Ser1070[2]	MAHTVRKDYR**S**
PI 3-kinase p110δ catalytic subunit	PK3CD_HUMAN	Ser1039[3]	TKVNWLAHNV**S**KDNRQ
PI 3-kinase p110γ catalytic subunit	PK3CG_HUMAN	Ser1101[2]	VLGIKQGEKH**S**A
non-muscle tropomyosin 1α	TPM1_HUMAN	Ser61[4]	KGTEDELDKY**S**EALKDAQEKL
βc subunit of the GM-CSF/IL-3 receptor	IL3B_HUMAN	Ser585	GPYLGPPHSR**S**LPDILGQPEP

Chapter 5

Expression profiling of a haemopoietic cell survival transcriptome implicates osteopontin as a functional prognostic factor in AML

5.1 Summary

Acute myeloid leukaemia (AML) comprises a number of distinct cytogenetic and transcriptional sub-groups that possess intrinsic differences in biology and clinical outcome. Targeted therapies with good efficacy and low side effect profile remain to be developed and rigorously tested in AML. The IL-3 receptor is over-expressed on AML blasts and AML $CD34^{+}CD38^{-}$ cells compared to normal haematopoietic stem cells. The common signalling subunit βc of the IL-3 receptor is constitutive phosphorylated on Ser585, a residue linked specifically linked to survival signalling in non-transformed cells. In the previous two chapters we have demonstrated two potential novel targets for AML: i) an extracellular target CD123 over-expressed on AML stem cells and ii) an intracellular target, namely PI3K as an upstream protein kinase that can phosphorylate Ser585 of βc.

In this chapter we searched for downstream transcriptional targets of the IL-3/GM-CSF Ser585 βc signalling pathway that may have prognostic and biological relevance in AML. We first performed a global microarray analysis of all Ser585 regulated genes in cell-lines bearing mutant GM-CSF receptors. We then performed validation and pathway analysis of these genes in AML samples looking for druggable and/or secreted Ser585 genes in AML that may have biological significance (Figure 5.1).

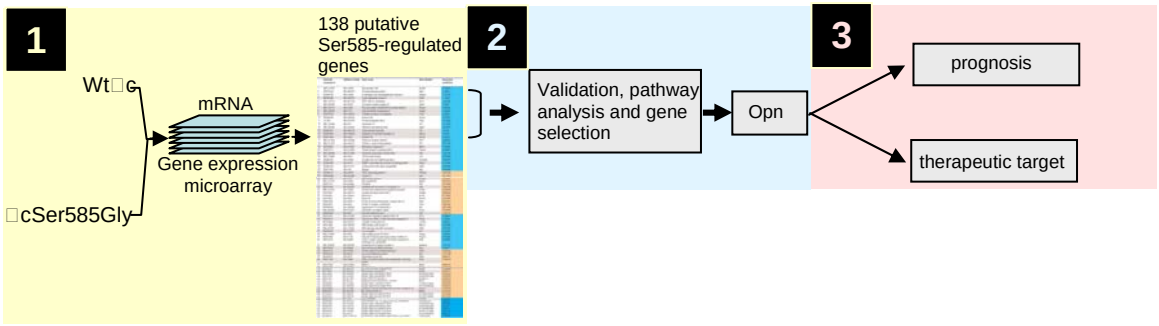

Figure 5.1: Schema for (1) global analysis of a cytokine-regulated survival –only pathway, (2) gene analysis in AML and (3) analysis of a prognostic and therapeutic target in AML.

We show that one such gene, osteopontin (*OPN*), is a functionally relevant target of the Ser585-survival pathway as demonstrated by siRNA-mediated knockdown of *OPN* expression which induces cell death in both AML blasts and $CD34^{+}CD38^{-}CD123^{+}$ leukemic progenitors. Increased expression of *OPN* at diagnosis is associated with poor prognosis with multivariate analysis indicating that it is an independent predictor of overall patient survival in normal karyotype AML (n=60,HR=2.2,p=0.01). These results delineate a novel cytokine-regulated Ser585/PI3-kinase signalling network that is deregulated in AML and identify *OPN* as a potential prognostic and therapeutic target.

Recent insights into GM-CSF signalling and receptor activation in human disease including AML are summarized in a published review in section 5.3.

5.2 Significance

This chapter, now published, was the first demonstration that the soluble extracellular matrix cytokine osteopontin is a cell survival factor in AML stem/progenitor cells and its expression in AML patients is associated with poor prognosis in normal karyotype AML. It extends our previous findings that Ser585 of the IL-3/GM-CSF receptor is constitutively phosphorylated in the majority of patients' AML and is deregulated in AML. By mapping the downstream transcriptional events regulated by Ser585 we uncovered the gene encoding a soluble extracellular molecular osteopontin (*OPN*) as a Ser585 regulated gene. *OPN* had previously been identified as a bone marrow stem cell niche regulator associated with anti-proliferative signalling that lines the endosteal layer of the bone marrow. This is the first demonstration that *OPN* expression by AML blasts may be a determinate of clinical outcome independent of cytogenetic, age, white cell count or *FLT3* mutation status and suggests *OPN* as a novel therapeutic target in AML. It also suggests further work on the role of *OPN* as a regulator of the AML stem cell niche is likely to be fruitful.

5.3 (Publication)

Expression profiling of a hemopoietic cell survival transcriptome implicates osteopontin as a functional prognostic factor in AML

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Jason A. Powell, Daniel Thomas, Emma F. Barry, Chung H. Kok, Barbara J. McClure, Anna Tsykin, L. Bik To, Anna Brown, Ian D. Lewis, Kirsten Herbert, Gregory J. Goodall, Terence P. Speed, Norio Asou, Bindya Jacob, Motomi Osato, David N. Haylock, Susan K. Nilsson, Richard J. D'Andrea, Angel F. Lopez, and Mark A. Guthridge (2009) Expression profiling of a hemopoietic cell survival transcriptome implicates osteopontin as a functional prognostic factor inAML. *Blood, v. 114 (23), pp. 4850-4870*

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It is also available online to authorised users at:

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5.4 (Publication)

The GM-CSF receptor: linking its structure to cell signaling and its role in disease

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***Blood* (2009) 114: 1289-1298**

Timothy R. Hercus, Daniel Thomas, Mark A. Guthridge, Paul G. Ekert, Jack King-Scott, Michael W. Parker, and Angel F. Lopez (2009) The granulocyte-macrophage colony-stimulating factor receptor: linking its structure to cell signaling and its role in disease.
Blood, v. 114 (7), pp. 1289-1298

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Chapter 6

Dual inhibition of CDK9 and p110α by PIK-75 blocks Mcl-1 expression and PI3K signalling and allows selective targeting of human acute myeloid leukaemic cells

6.1 Summary

Oncogenic activation of multiple intracellular cell survival pathways renders malignant cells resistant to chemotherapeutic and new-generation targeted therapies. Previously we have shown a link between PI3K protein kinase activity and Ser585 deregulation in promoting AML survival (Chapter 4). Here, we describe the potent anti-leukaemic activity of the PI3K kinase inhibitor PIK-75 and its ability to block two independent arms of the pro-survival machinery in AML. PIK-75 induces apoptosis through the simultaneous inhibition of i) PI3K p110α resulting in reduced PI3K/Akt signalling and ii) Cdk9 resulting in a transient block of RNA polymerase II-mediated transcription and the rapid loss of the Mcl-1 pro-survival protein. Our results identify a previously unrecognized alliance between PI3K p110α and Cdk9 in promoting oncogenic survival signals and show that PIK-75 represents a prototypic example of a dual PI3K p110α/Cdk9 inhibitor with anti-leukaemic activity.

6.2 Significance

Despite substantial progress in the treatment of AML, the majority of patients succumb to their disease. The ability of leukaemic stem and progenitor cells to survive genotoxic or targeted therapies (e.g. kinase inhibitors) represents a major obstacle in obtaining long-term clinical responses. We now show that simultaneous blockade of two independent survival nodes (PI3K p110α and Cdk9) using PIK-75 is sufficient for the induction of apoptosis in AML stem and progenitor cells. With PI3K and Cdk inhibitors already in clinical trials, our findings support the rational development of these drugs in AML patients. Furthermore, our findings provide proof-of-concept that dual PI3K/Cdk9 inhibitors, as exemplified by PIK-75, represent an effective anti-cancer strategy.

6.3 Introduction

Deregulation of cell survival programs is an obligate step in the tumorigenic process and renders malignant cells resistant to a variety of apoptotic triggers(Hanahan and Weinberg, 2011) . Phosphoinositol 3-kinases (PI3Ks) are potent regulators of cell survival through their ability to phosphorylate phosphoinositols in the inner leaflet of the plasma membrane that, in turn, provide docking sites for the recruitment of pleckstrin homology domain proteins such as Akt (Manning and Cantley, 2007). Class I PI3Ks are heterodimeric enzymes comprising an adapter protein bound to one of four 110kDa catalytic subunits (p110α, p110β, p110δ and p110γ). Constitutive activation of the p110α isoform of PI3K has been implicated in diverse tumors, encouraging attempts to develop of signal transduction therapies able to specifically block the p110α isoform in animal models of cancer (Liu *et al.* 2009). However, studies with PI3K inhibitors suggest that cancer cells have the inherent capacity to adaptively utilize alternative signalling pathways (Liu *et al.* 2011) . Thus, whether targeting a single cell survival pathway such as the PI3K pathway alone will be sufficient as an anti-cancer therapy or whether combined inhibition of other cell survival pathways will be a necessary adjunct remains to be determined.

In addition to PI3K, Bcl-2 and closely related pro-survival family members Bcl-xL, Bcl-w, Mcl-1 and A1 are also central regulators of cell survival in diverse cell types (Cory and Adams, 2002). Over-expression of individual members of the Bcl-2 pro-survival family is commonly observed in human cancer, and their oncogenic potential has been clearly demonstrated for both solid tumors and leukaemias (Cory and Adams, 2002). Over-expression of Bcl-2 family member Myeloid cell leukaemia 1 (Mcl-1) has been observed in relapsed AML and elevated Mcl-1 has been shown to antagonize cell death induced by a number of drugs that are either in the clinic or in clinical development (Aichberger *et al.* 2005; Schwickart *et al.* 2010; Xiang *et al.* 2010; Zhang *et al.* 2002; Zhou *et al.* 1997). Although small molecule inhibitors of Bcl-2, Bcl-xL and Bcl-w, such as ABT-737 are currently in clinical development (Oltersdorf *et al.* 2005), tumor cells may evade sensitivity to ABT-737-induced apoptosis through Mcl-1 expression (van Delft *et al.* 2006). Thus, the development of drugs capable of targeting Mcl-1 is a highly desirable therapeutic goal.

Here we show that blockade of two independent cell survival pathways, one regulated by PI3K:Akt and the other regulated by Cdk9:Mcl-1, results in the rapid and dramatic induction of apoptosis in both AML blasts as well as purified CD34+CD38-CD123+ leukaemic stem and progenitor cells (LSPCs). We discovered this previously unrecognized synergy between PI3K:Akt and Cdk9:Mcl-1 signalling using a kinase inhibitor, PIK-75, which was initially reported to block the p110α catalytic subunit of PI3K activity (Knight *et al.* 2006). Our data shows that PIK-75 blocks signalling via both PI3K:Akt and Cdk9:Mcl-1, resulting in the rapid and selective induction of apoptosis in primary human AML cells *in vitro* and AML growth *in vivo*. Our studies not only establish a biological rationale for the simultaneous blockade of PI3K p110α and Cdk9 in cancer therapy, but we also validate a new class of anti-cancer drug distinguished by its ability to target two critical signalling nodes underpinning oncogenic cellular survival mechanisms in AML.

RESULTS

6.4 PIK-75, a PI3K p110α isoform-selective inhibitor potently inhibits survival of AML cells

We and others have previously shown that PI3K-regulated survival programs are constitutively active in AML (Gallay *et al.* 2009; Guthridge *et al.* 2006; Powell *et al.* 2009, Chapter 5). We therefore compared the ability of PI3K p110 isoform-selective inhibitors to induce cell death in the MV4;11 AML cell line, which expresses a gain-of-function mutant form of the FLT3 tyrosine kinase receptor in which there is an internal tandem duplication (FLT3-ITD). The imidazolidine derivative PIK-75, which has previously been shown to inhibit the p110α isoform of PI3K (Knight *et al.* 2006), demonstrated potent killing of MV4;11 cells, whereas selective inhibitors of p110β- (TGX-221), p110δ- (IC87114) and p110γ (AS252424) were less effective (Figure 6.1A). Similar results were observed in the KG1 and U937 AML cell lines (data not shown). We and others have shown that leukaemic stem and progenitor cells (LSPCs), that are responsible for establishing and maintaining AML growth in mouse xenograft models are enriched in (although not always restricted to) the $CD34^+CD38^-CD123^+$ fraction (Dick, 2008; Jin *et al.* 2009, Chapter 3; Jordan *et al.* 2000; Martelli *et al.* 2010). Furthermore, $CD34^+CD38^-CD123^+$ AML cells have been shown to be significantly more resistant to ara-C-induced apoptosis both *in vitro* and in the bone marrows of xenografted mice (Guzman *et al.* 2001; Saito *et al.* 2010), further suggesting that this phenotype is enriched for drug-resistant leukaemic stem and progenitor cells. We therefore tested whether PIK-75 was able to reduce the survival of primary human $CD34^+CD38^-CD123^+$ cells defined as the population most enriched for chemoresistant leukaemic stem and progenitor cells (see Chapter 3, Figure 3A; patient details present in Table 6.1). The profile of killing using a panel of PI3K p110 isoform-selective inhibitors was consistent with our observations in AML cell lines, with only PIK-75 able to induce substantial apoptosis of primary $CD34^+CD38^-CD123^+$ cells (Figure 6.1B). Consistent with an apoptosis-based mechanism of cell death, PIK-75 killing was caspase-dependent (Figure 6.1C).

Table 6.1. AML patient samples

Patient ID	Age/ Sex	Marrow blasts (%)	WCC x10^9/L	FAB	Karyotype	FLT3-ITD
AML1	85/M	46	179	M4	47,XY, +8	Yes
AML2	19/M	79	150	M4	Normal	Yes
AML3	48/F	99	110	M1	46,XX, 11q23	No
AML4	63/F	97	192	M4	46,XX, t(2;11)	No
AML5	69/F	96	135	M0	Normal	Yes
AML6	42/F	98	49	M1	46,XX, t(9;11) (p21;q23)	No
AML7	71/M	90	101	M1	46,XY, -5q	No
AML8	32/M	96	7.4	M5	46,XX, t(9;11) (p21;q23)	No

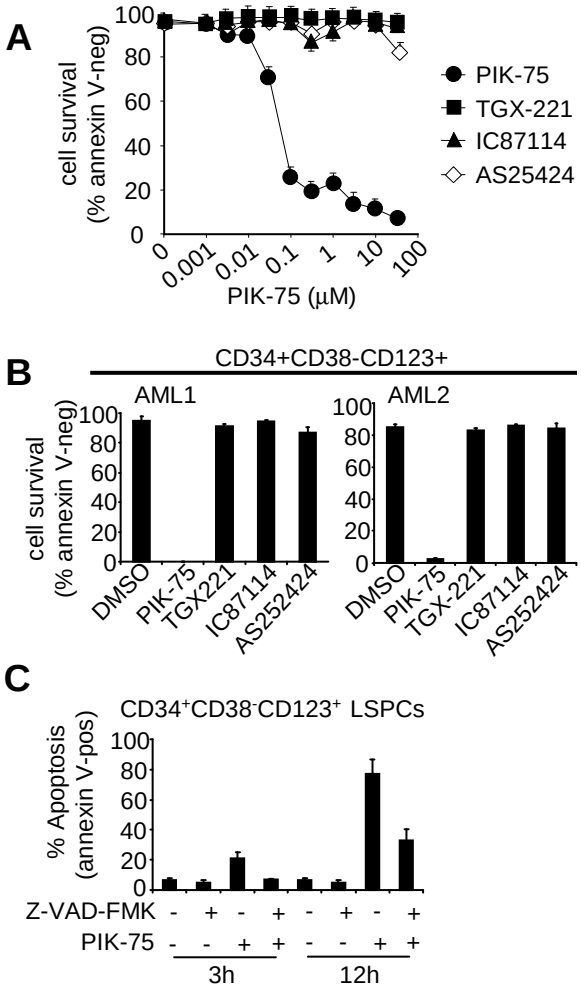

Figure 6.1: PIK-75, potently induces apoptosis in leukemic cell lines. (A) MV4-11 cells were cultured in the presence of increasing concentrations of p110α-selective (PIK-75), p110β-selective (TGX-221), p110δ-selective (IC87114) or p110γ-selective (AS252424) PI3K inhibitors and cell survival examined at 24h. (B) FACS-purified primary human AML CD34+CD38-CD123+ LSPCs were plated in DMSO (vehicle), PIK-75 (100nM), TGX-221 (1μM), IC87114 (5μM) or AS252424 (100nM) and cell survival examined at 24h. (C) FACS-purified primary human CD34+CD38-CD123+ cells were plated in PIK-75 (100nM) for 3 or 12h in the presence DMSO (vehicle) or Z-VAD-FMK (50μM) following which cell survival was examined by annexin V staining.

6.5 The pro-apoptotic activity of PIK-75 is distinct from other inhibitors targeting the PI3K signalling pathway

To determine if the activity of PIK-75 was specifically related to its ability to target the PI3K p110α isoform, additional pan-specific PI3K inhibitors (LY294002 and Wortmannin), dual PI3K/mTOR inhibitors (BEZ235 and PI-103) and a p110α-selective inhibitor (YM-024)(Knight *et al.* 2006) were examined for their ability to induce cell death in the MV4;11 cell line (Figure 6.2A) as well as in primary human AML cells (Figure 6.2B). Remarkably, the activity of PIK-75 was substantially greater than that seen with all other PI3K inhibitors (Figure 6.2A,B). Blockade of PI3K downstream targets such as Akt or mTOR in primary AML cells also failed to recapitulate the cell killing activity observed for PIK-75 (Figure 6.2C). Thus, while PIK-75 was originally identified as a PI3K p110α inhibitor (Knight *et al.* 2006), our results demonstrated that its ability to induce apoptosis was clearly distinct from all other inhibitors of the PI3K pathway tested (Figure 6.2A-C), indicating that its mechanism of action was unlikely to be due to the inhibition of p110α alone.

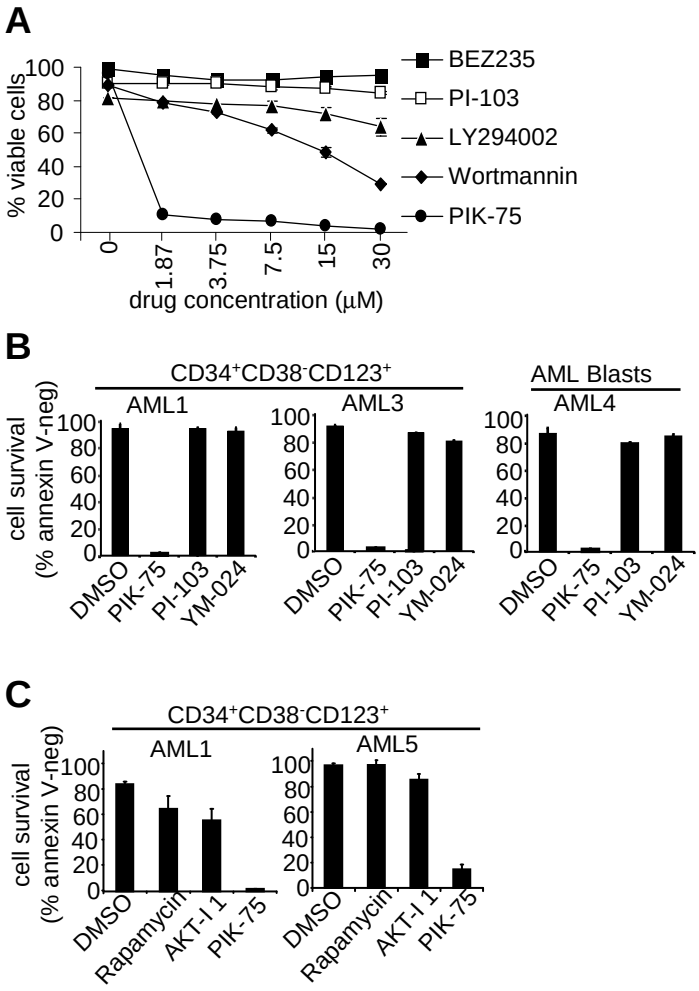

Figure 6.2: PIK-75 is more potent at inducing apoptosis than other inhibitors of the PI3K pathway. (A) MV4-11 cells were cultured in the presence of BEZ235, PI-103, LY294002, Wortmannin or PIK-75 for 24h and cell survival determined by flow cytometric enumeration of PI-negative cells. (B) FACS-purified primary human CD34+CD38-CD123+ LSPCs or AML blasts were plated in PIK-75 (100nM), PI-103 (100nM) or YM-024 (5μM) and cell survival examined at 24h. (C) FACS-purified primary human CD34+CD38-CD123+ LSPCs were cultured in the presence of rapamycin (10μM), AKT-I 1 (10μM) or PIK-75 (100nM) and cell survival measured at 24h.

6.6 PIK-75 targets Mcl-1 to induce apoptosis in a Bak dependent manner

We therefore sought to further understand the mechanism by which PIK-75 induced apoptosis in AML cells and whether additional targets may be involved. In collaboration with Dr Paul Ekert (Walter and Eliza Hall Institute) examined the ability of PIK-75 to promote cell death using factor-dependent myeloid (FDM) lines derived from mice in which specific pro-apoptotic members of the Bcl-2 family had been knocked out (Ekert *et al.* 2004). While no significant differences in PIK-75 sensitivity were observed between wild type (wt) FDM cells and FDM knockout cells derived from either *Bid*$^{-/-}$, *Bad*$^{-/-}$*Bim*$^{-/-}$ or *Noxa*$^{-/-}$*Puma*$^{-/-}$ mice, FDM cells from *Bak*$^{-/-}$ mice were completely resistant to PIK-75 (Figure 6.3). These findings indicated an important role for Bak in PIK-75-mediated apoptosis.

The pro-apoptotic activity of Bak is known to be held in check by Mcl-1 and Bcl-xL (Willis *et al.* 2005) raising the possibility that PIK-75 might target the pro-survival function of Mcl-1 and/or Bcl-xL leading to Bak-dependent apoptosis. We therefore examined whether PIK-75 affected the expression levels of Mcl-1 or Bcl-xL. As expected, both PIK-75 and LY294002 blocked Akt phosphorylation in MV4;11 cells (Figure 6.4A). However, PIK-75 treatment but not LY294002 resulted in a dramatic down-regulation of Mcl-1 protein levels while no significant reduction of Bcl-xL was observed (Figure 6.4B). Flavopiridol, which has been shown by others to reduce Mcl-1 expression (Gojo *et al.* 2002), was also able to down-regulate Mcl-1 levels in a similar manner to PIK-75 (Figure 6.5B). Furthermore, while two independent FLT3 tyrosine kinase inhibitors (AG1296 and CEP701) were able to down-regulate Akt phosphorylation, they only had modest effects on Mcl-1 levels (Figure 6.4A). Additionally, using a panel of pharmacological inhibitors to target a broad spectrum of intracellular signalling pathways, only PIK-75 was able to down-regulate *both* Mcl-1 protein and Akt phosphorylation in MV4;11 cells (Figure 6.4C). These results demonstrate that in addition to its ability to inhibit PI3K signalling, PIK-75 possessed the unique ability to down-regulate Mcl-1 protein in a PI3K- and FLT3-independent manner.

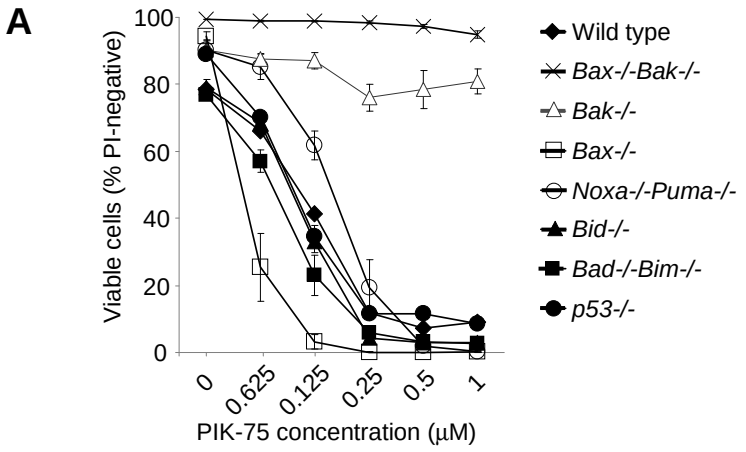

Figure 6.3: PIK-75 targets Mcl-1 to induce Bak-dependent apoptosis. (A) FDM lines generated from the indicated knockout mice were cultured for 24h in the presence of PIK-75 and cell survival measured by flow cytometric enumeration of PI-negative cells. This data was performed in the laboratory of Dr Paul Ekert, Walter and Eliza Hall, Parkville as part of a collaboration for this project.

To assess the relative importance of Mcl-1 down-regulation for PIK-75-mediated cell death, we performed siRNA-mediated Mcl-1 knockdown in AML samples and examined cell survival. Our results showed that siRNA-mediated knockdown of Mcl-1 alone in 6 primary human AML samples was not sufficient to induce significant apoptosis (data not shown) despite a >80% reduction in Mcl-1 protein levels (Figure 6.5A). However, while LY294002 alone partially reduced AML cell survival, combining LY294002 and Mcl-1 siRNA-mediated knockdown resulted in a further significant reduction in CD34+CD38-CD123+ cell survival (Figure 6.5B). These results suggest that PIK-75 inhibits two signalling pathways; one involving PI3K resulting in a block in Akt phosphorylation and the other involving a PI3K-independent pathway regulating Mcl-1 expression.

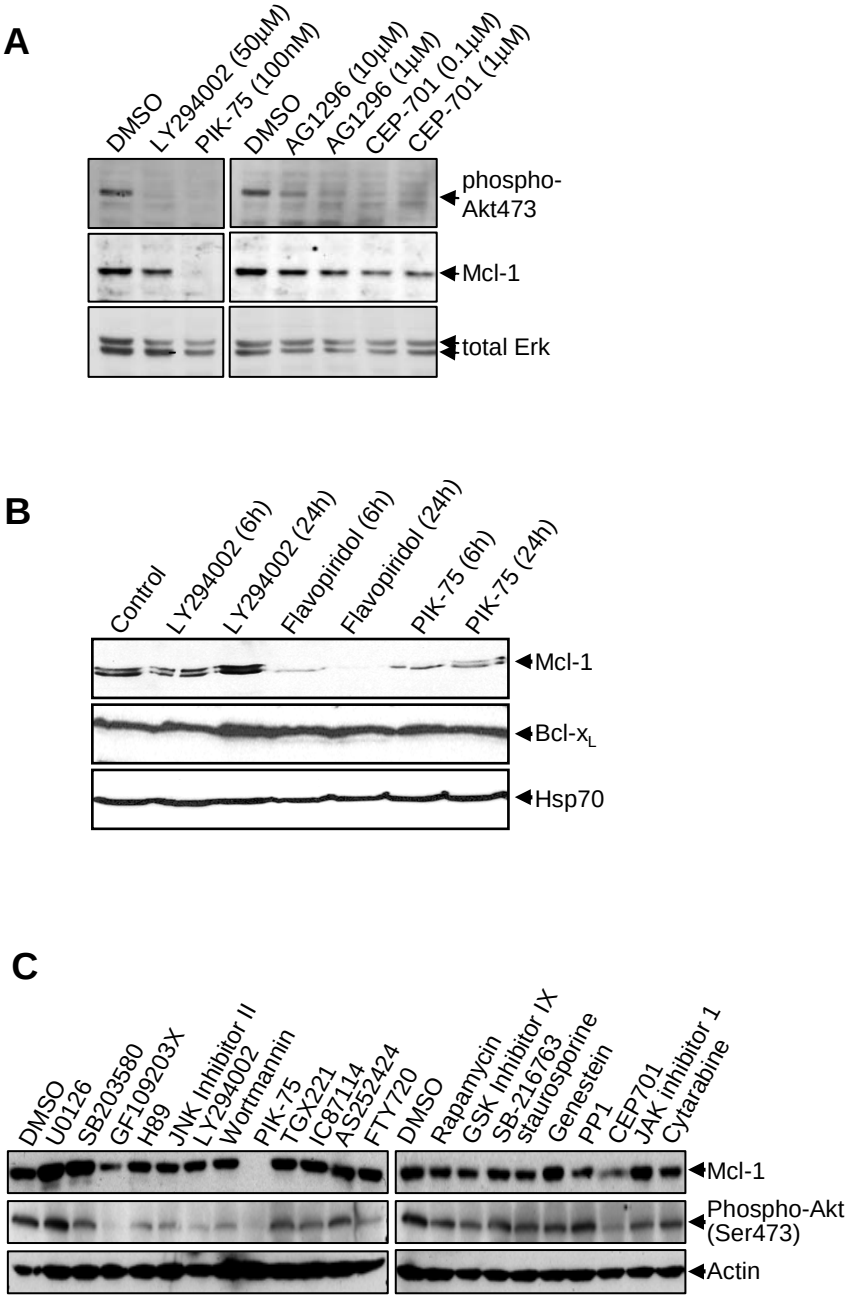

Figure 6.4: PIK-75 targets Mcl-1 and PI3K but not Bcl-x$_L$. (A) MV4;11 cells were cultured for 8h in the presence of DMSO, LY294002, PIK-75, AG1296 or CEP701 and cell lysates were immunoblotted with the indicated antibodies. (B) FDM cells were treated for either 6 or 24h with 50mM LY294002, 1mM Flavopiridol or 1mM PIK-75 following which cell lysates were immunoblotted.(C) MV4-11 cells were treated with U0126 (1μM), SB203580 (10μM), GF109203X (5μM), H89 (5μM), JNK Inhibitor II (1μM), LY294002 (10μM), wortmannin (1μM), PIK-75 (0.1μM), TGX221 (1μM), IC87114 (1μM), AS252424 (1μM), FTY720 (3μM), Rapamycin (1μM), GSK Inhibitor IX (10μM), SB-216763 (5μM), Staurosporine (1μM), Genistein (10μM), PP1 (2 μM), CEP701 (1 μM), JAK inhibitor (5μM), or cytarabine (100ng/ml) for 8h following which cell lysates were immunoblotted. The data in panel B were performed by Dr Andrew Wei, Australian Centre for Blood Diseases, Monash University.

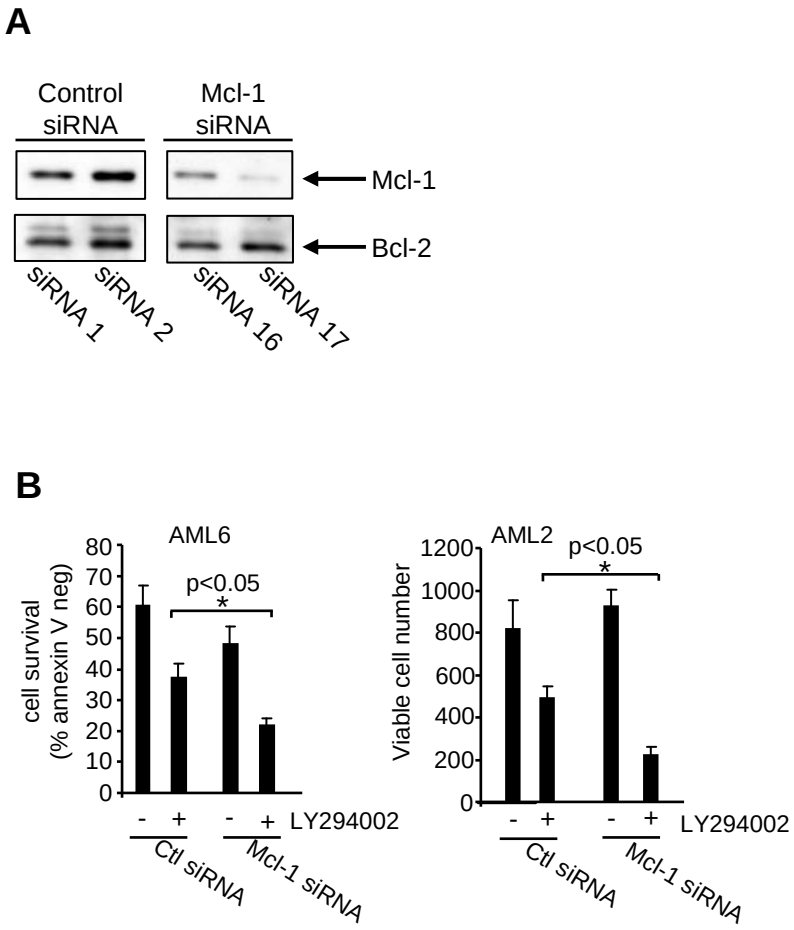

Figure 6.5: Targeting Mcl-1 with siRNA shows additional cell killing to PI3K blockade in AML stem/progenitor cells. (A) HEK293T cells were transfected with either 50nM control or Mcl-1 specific siRNA (Dharmacon, J-004501-16; target sequence GAUUAUCUCUCGGUACCUU and J-004501-17; target sequence CGAAGGAAGUAUCGAAUUU). After 48h, cell lysates were immunoblotted for Mcl-1 or Bcl-2. (B) FACS-purified primary human AML CD34+CD38-CD123+ LSPCs were transfected with 100nM siRNA targeting Mcl-1 or a non-targetting control siRNA +/-LY294002 (20μM) and cell survival was determined at 72h.

6.7 PIK-75 results in the rapid down-regulation of Mcl-1 protein through a block in Mcl-1 gene transcription.

Mcl-1 protein is known to have a short half-life owing to its ubiquitination and targeting for degradation via the proteosome (Schwickart *et al.* 2010). The loss of Mcl-1 protein following PIK-75 treatment of MV4;11 cells was rapid and followed closely by the onset of apoptosis as measured by caspase 3 and PARP cleavage (Figure 6.6A). To investigate the mechanism by which PIK-75 down-regulated Mcl-1 protein levels we first examined the effect of PIK-75 on protein stability. Neither PIK-75, nor LY294002 had a significant impact on Mcl-1 protein half-life (data not shown) indicating that the rapid depletion of Mcl-1 protein observed in the presence of PIK-75 was not due to a decrease in protein half-life. We therefore examined the effect of PIK-75 on Mcl-1 gene transcription using a luciferase reporter gene driven by the Mcl-1 promoter (Chao *et al.* 1998). PIK-75 significantly reduced luciferase reporter activity whereas PI3K inhibition with Wortmannin had no significant effect (Figure 6.6B) suggesting that PIK-75 was able to block Mcl-1 gene transcription in a PI3K-independent manner.

Initiation and elongation of Mcl-1 gene transcription is mediated by the transcriptional kinases, Cdk7 and Cdk9, which phosphorylate Ser2 and Ser5 within the C-terminal domain (CTD) of RNA polymerase II (RNAPII) (Chen *et al.* 2005; Chen *et al.* 2009; Ramanathan *et al.* 2001). To further investigate the mechanism by which PIK-75 inhibited Mcl-1 gene transcription, we performed additional Mcl-1 gene reporter assays in the presence of a panel of PI3K inhibitors as well as two independent Cdk7/9 inhibitors, Flavopiridol and SNS-032 (Chen *et al.* 2005; Chen *et al.* 2009). In these experiments, the luciferase reporter gene driven by the Mcl-1 promoter was significantly inhibited by PIK-75, Flavopiridol and SNS-032, but not by the PI3K inhibitors LY294002, PI-103, IC87114, TGX-221 or AS252424 (Figure 6.6C). Importantly, nuclear run-on experiments demonstrated that PIK-75 directly inhibited transcription of the endogenous Mcl-1 gene in a similar manner to SNS-032 and Flavopiridol, whereas LY294002 had no significant effect (Figure 6.6D). In contrast, PIK-75 did not significantly alter 18S rRNA gene

transcription (Figure 6.6D). These findings indicated that PIK-75 was able to rapidly deplete Mcl-1 protein levels through blockade of Mcl-1 gene transcription.

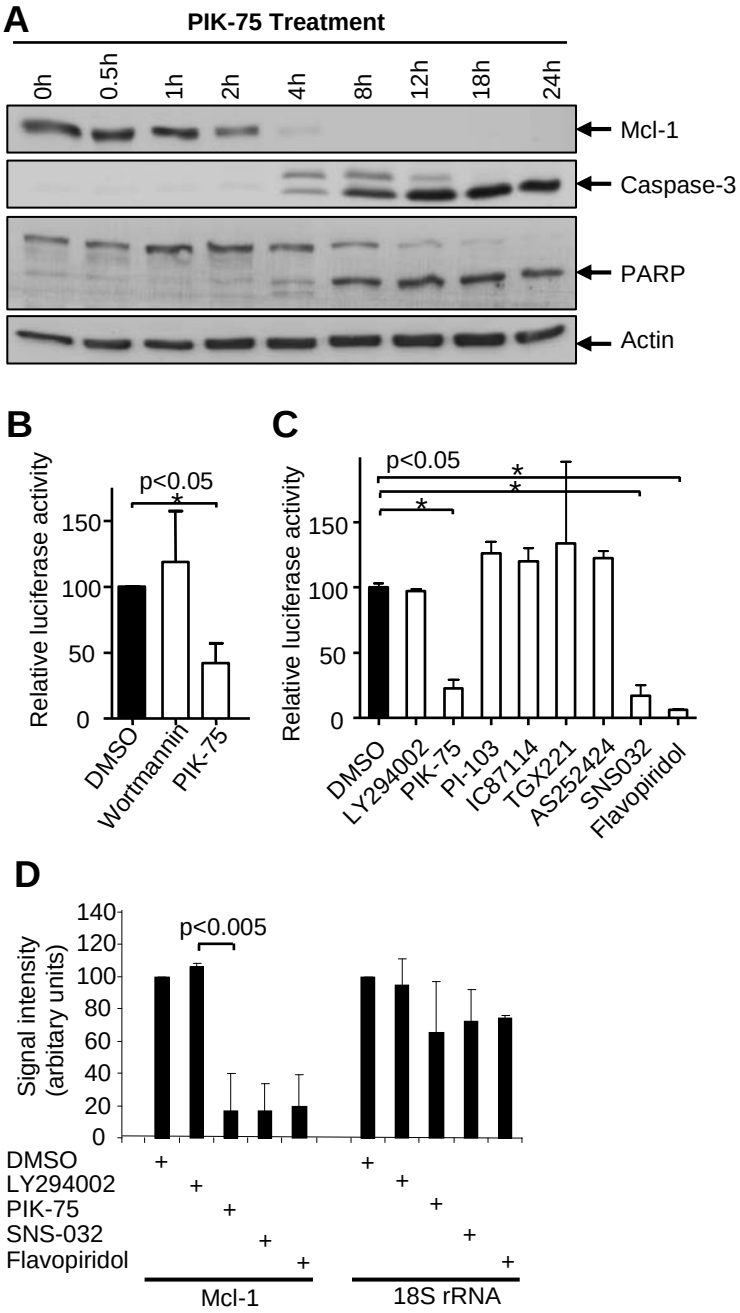

Figure 6.6: PIK-75 down-regulates Mcl-1 through inhibition of Cdk9 kinase activity and a block in Mcl-1 gene transcription. (A) MV4-11 cells were treated with 100nM PIK-75 for time periods as shown following which cell lysates were immunoblotted with the indicated antibodies.(B) HEK293T cells were co-transfected with constructs expressing the Renilla luciferase gene (TK promoter-driven) and the Firefly luciferase gene (Mcl-1 promoter-driven). Cells were treated with DMSO (vehicle), Wortmannin (500nM) or PIK-75 (100nM) for 20h following which relative luciferase activity was measured.(C) HEK293T cells were co-transfected as in (C) following which cells were treated with DMSO (vehicle), LY294002 (20μM), PIK-75 (100nM), PI-103 (100nM), IC87114 (5μM), TGX-221 (1μM), AS252424 (100nM), SNS-032 (10μM) or flavopiridol (10μM). After 20h, the relative luciferase activity was determined.(E) Nuclear run-on experiments were performed on nuclei isolated from MV4-11 cells and treated with either DMSO (vehicle), PIK-75 (100nM), LY294002 (20μM), SNS-032 (10μM) or flavopiridol (10μM) for 1h in presence of 100mCi ^{32}P-UTP. Trizol-extracted ^{32}P-labelled RNA was hybridized to Mcl-1 or 18S rRNA cDNAs immobilized to nylon filters. After washing, the filters were exposed to phosphoimager screens and signals quantitated using ImageQuant.

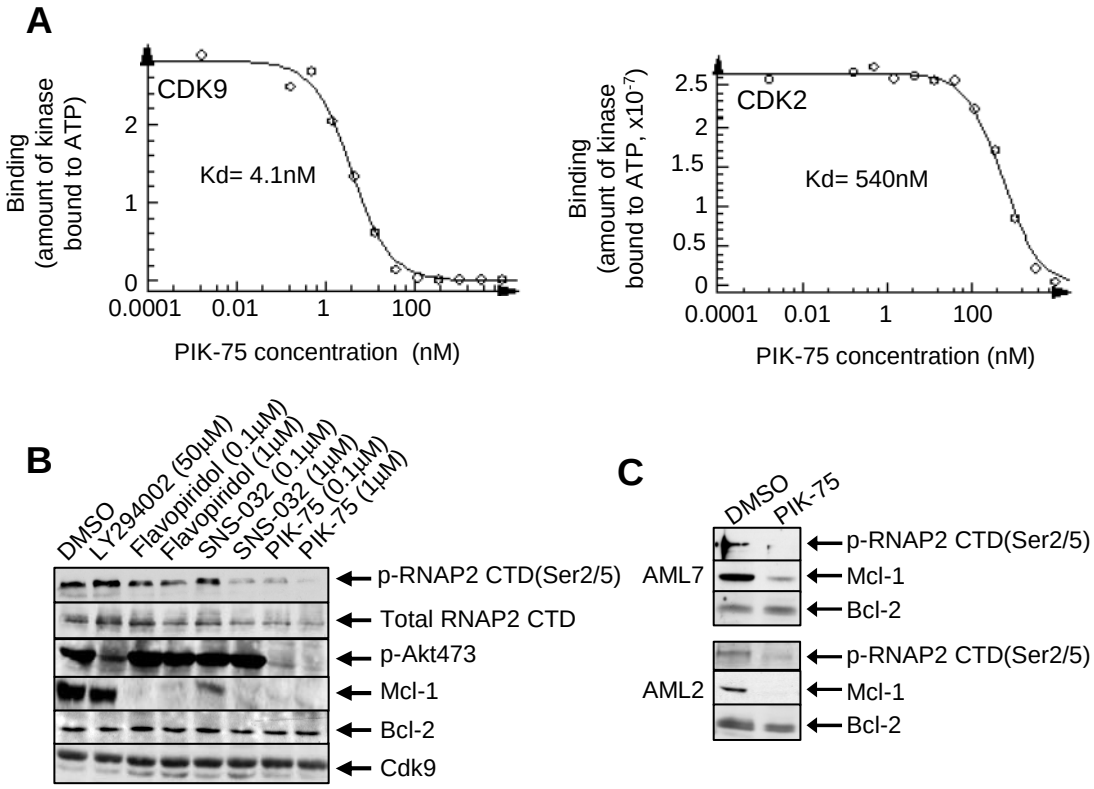

Figure 6.7: PIK-75 is a potent CDK9 kinase inhibitor (A) The binding affinity of PIK-75 to purified recombinant Cdk9 and Cdk2 was measured in a competition assay as described in Materials and Methods (Chapter 2). (B) MV4-11 cells were treated with LY294002, flavopiridol, SNS-032 or PIK-75 at the concentrations shown for 8h following which cell lysates were immunoblotted with the indicated antibodies. (C) Primary AML mononuclear cells were treated with PIK-75 (100nM) or DMSO (vehicle) for 8h following which cell lysates were immunoblotted. The data in panel A was generated by Dr Andrew Wei, Australian Centre for Blood Diseases, Monash University.

6.8 Dual targeting of PI3K and Cdk9 by PIK-75 induces apoptosis in AML cells.

Cdk9 is a key regulator of Mcl-1 gene transcription through its ability to phosphorylate RNAPII, so we therefore considered the possibility that, in addition to its ability to inhibit PI3K p110α, PIK-75 may also directly inhibit Cdk9 kinase activity. *In vitro* kinase assays performed in collaboration with Dr Andrew Wei (Monash University) demonstrated high affinity binding of PIK-75 to the ATP binding pocket of Cdk9 (K_d=4.1nM), whereas the affinity for the related Cdk2 was over 100-fold lower (K_d=540nM)(Figure 6.7A). The binding of PIK-75 to Cdk9 was in a similar range to that previously reported for PI3K p110α (IC50=5.8 nM)(Knight *et al.* 2006). Thus, while PI3K p110α and Cdk9 belong to diverse ancestral branches of the kinome tree and are only 25% identical within their catalytic domains, PIK-75 is able to inhibit both kinases with IC50s in the low nanomolar range.

Consistent with the ability of PIK-75 to inhibit Cdk9 kinase activity, the phosphorylation of RNAPII CTD on Ser2 and Ser5 was also suppressed (Figure 6.7B). Importantly, PIK-75-mediated inhibition of RNAPII CTD phosphorylation as well as Mcl-1 protein down-regulation was similar to that mediated by Flavopiridol and SNS-032 while there was no impact on Bcl-2 levels (Figure 6.7B). Furthermore, because LY294002 had no effect on the phosphorylation of RNAPII CTD (Figure 6.7B), our results indicate that the ability of PIK-75 to inhibit Cdk9 activity was not an indirect consequence of PI3K inhibition. Consistent with these observations in MV4;11 cells, treatment of primary human AML cells with PIK-75 also down-regulated both RNAPII CTD phosphorylation and Mcl-1 protein levels but had no significant impact on Bcl-2 levels (Figure 6.7C). Thus, our findings identify Cdk9 as a biologically important PIK-75 target and that inhibition of Cdk9 results in the blockade of *de novo* Mcl-1 gene transcription leading to the rapid down-regulation of Mcl-1 protein within the cell.

Apart from PIK-75, no other inhibitor targeting the PI3K pathway induced significant apoptosis in AML cells (Figure 6.1,6.2). However, it was not clear whether the ability of

PIK-75 to induce apoptosis was due to the inhibition of PI3K p110α alone or was due to dual inhibition of PI3K p110α *and* Cdk9. We therefore examined the survival of MV4;11 cells following inhibition of either Cdk9 alone (with either SNS-032 or Flavopiridol) or in combination with PI3K inhibition by LY294002. Under conditions where either SNS-032 or Flavopiridol reduced cell survival by ~50%, addition of LY294002 resulted in a further significant reduction in cell viability (Figure 6.8A). We also examined the survival of primary AML cells following siRNA-mediated knockdown of Cdk9 (Figure 6.8B) and PI3K inhibition with LY294002. Cdk9 knockdown alone partly reduced the survival of CD34+CD38-CD123+ AML cells and this effect was significantly enhanced by the subsequent addition of LY294002 (Figure 6.8C). Together, these results show that dual targeting of PI3K and Cdk9 represents an effective approach for the blockade of deregulated cell survival programs in human AML cells.

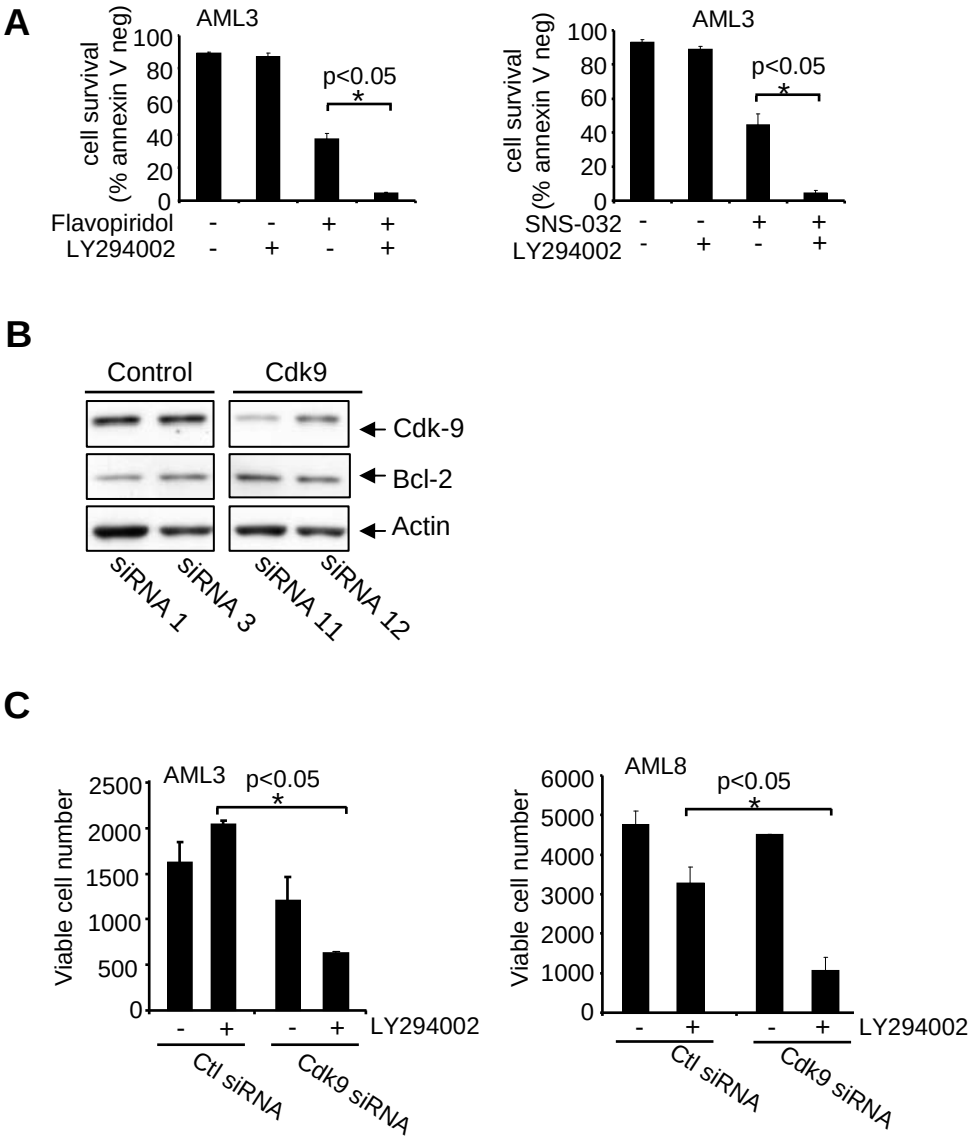

Figure 6.8: Dual Targeting of Cdk9 and PI3K is sufficient to kill AML stem and progenitor cells. FACS-purified primary human AML CD34+CD38-CD123+ LSPCs were treated with 100 nM flavopiridol (left), 100 nM SNS-032 (right) and either DMSO (vehicle) or LY294002 (20 μM) and cell survival was determined at 24h.(B) HEK293T cells were transfected with either 50nM control or Cdk9 specific siRNA (Dharmacon, J0003243-11; target sequence GGACAUGAAGGCUGCUAAU and J003243-12; target sequence UAUAACCGCUGCAAGGGUA). After 48h, cell lysates were immunoblotted for Cdk9.(C) FACS-purified primary human AML CD34+CD38-CD123+ LSPCs were transfected with 100nM siRNA targetting Cdk9 or non-targeting siRNA control +/- LY294002 (20μM) and viable cell number was determined at 72h by using Flow-Count Fluorospheres.

6.9 PIK-75 selectively reduces the clonogenic potential of AML cells in vitro and their engraftment in a mouse xenograftment model.

We then examined the ability of PIK-75 to target the longer-term growth and survival of AML cells either *in vitro* using colony assays or *in vivo* following engraftment in NOD-SCID mice. Our results show PIK-75 significantly reduced leukaemic colony-forming units (CFU-L) from primary AML patient samples (Figure 6.9A). Additionally, PIK-75 treatment of AML blasts derived from a patient with FLT3-ITD+ AML significantly reduced their engraftment in NOD-SCID mice whilst not significantly affecting the engraftment of normal BM progenitors (Figure 6.9B,C). Since PIK-75 was targeting PI3K p110α and Cdk9 each of which has roles in diverse physiological processes, we sought to examine the potential toxicity of PIK-75 *in vivo*. Importantly, no adverse reactions were observed following daily administration of 10mg/kg PIK-75 to mice for 14 days with no significant reduction in circulating blood haemoglobin (Figure 6.10A), platelets (Figure 6.10B), white blood cells (Figure 6.10C), immunophenotypically defined haemopoietic sub-populations in the bone marrow (Figure 6.10D), clonogenic potential of bone marrow-derived cells (Figure 6.10E) or total mouse body weight (Figure 6.10F). In part, the ability of mice to tolerate daily administration of 10mg/kg PIK-75 may be due to its short bioactive window *in vivo* with >90% of drug activity lost 2h after injection (Figure 6.11A). Importantly, we demonstrated in collaboration with Dr Christian Recher (INSERM, Toulouse) that treatment of mice with established disease using a 10 fold lower dose of PIK-75 (1mg/kg) resulted in a significant and rapid decrease in the engraftment of HL-60 AML cells (Figure 6.11B). Together, these results show that PIK-75 can selectively target AML cells without inducing profound toxicity in non-transformed haemopoietic cells.

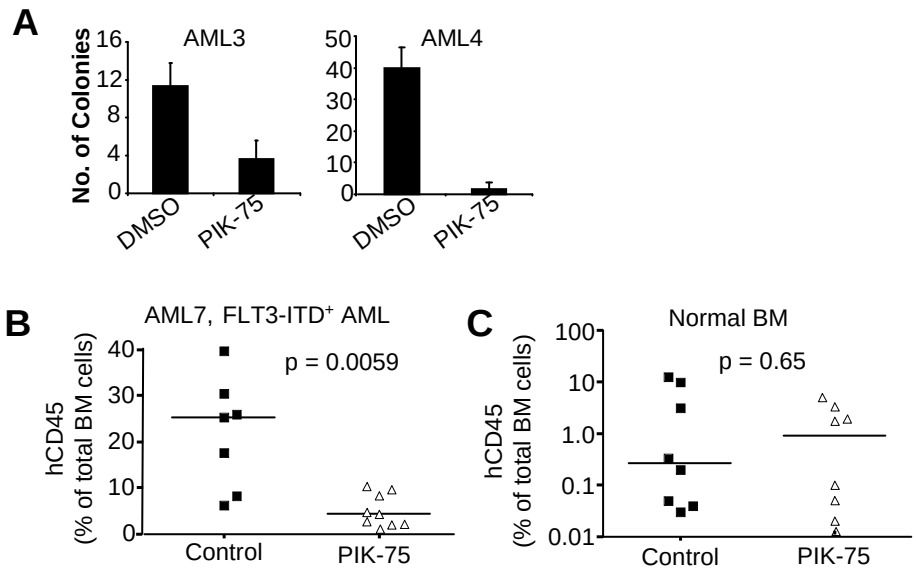

Figure 6.9: PIK-75 selectively reduces the clonogenic potential of AML cells *in vitro* and their engraftment in a mouse xenograftment model. (A) AML blasts from patients AML3 and AML4 were plated in 100nM PIK-75 and colonies were counted at 2 weeks. Error bars represent SEMs.(C) Primary FLT3-ITD+ AML blasts or (D) non-transformed BM from normal donors were incubated in 1μM PIK-75 (△) or DMSO (■) for 3h, washed and then engrafted into NOD-SCID mice. Engraftment was quantified after 4-6 weeks by assessing the percentage of hCD45+ cells in the BM of recipient mice. The data in panels C and D was generated in collaboration with Richard Lock and the engraftment experiments were performed by Erwin Lee at the Children's Cancer Institute Australia for Medical Research, University of New South Wales.

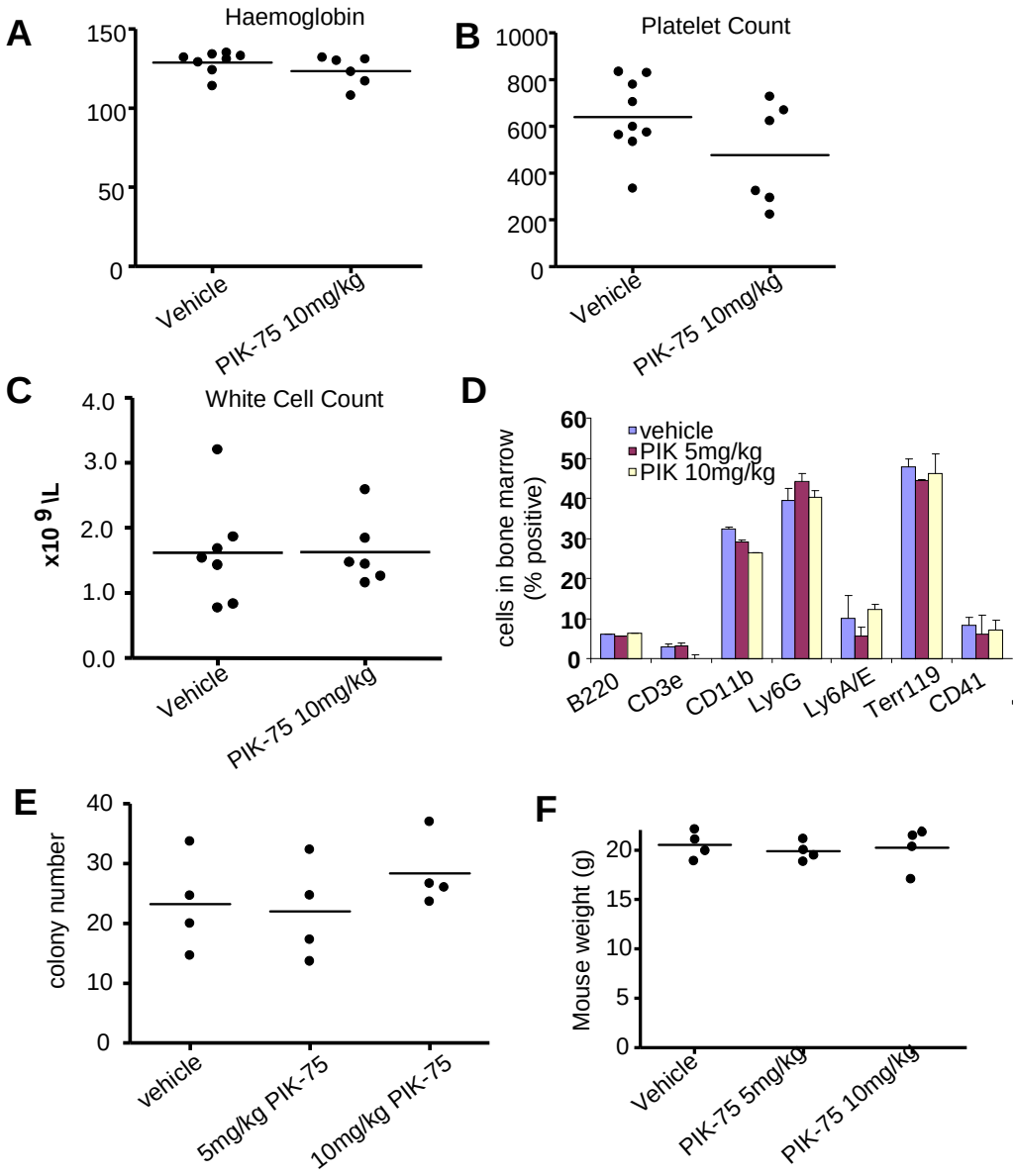

Figure 6.10: Toxicity studies of PIK-75 administered in vivo.(A-F) NOD-SCID mice were injected i.p daily with 10mg/kg PIK-75 for 14 days following which peripheral blood counts were determined on a SYSMEX XE2100 haematology analyser. Histograms show haemoglobin(A), platelet count (B) and white cell count (C). Bone marrow was recovered and stained for the indicated immunophenotypically defined markers with each subpopulation being determined by flow cytometry. (E) Clonogenic assays were performed on bone marrow by plating 50,000cells/ml in soft agar containing 50ng/ml SCF, 8U/ml EPO and 50ng/ml mIL-3. Colonies were counted after 2 weeks using an inverted microscope. (F) NOD-SCID mice were injected i.p daily with 10mg/kg PIK-75 for 14 days with mice being weighed on the last day of injections.

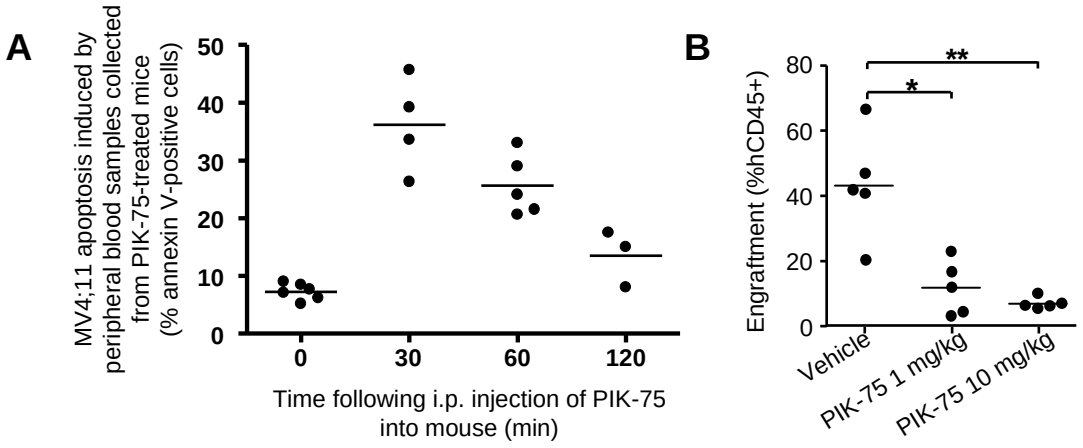

Figure 6.11: PIK-75 selectively reduces AML engraftment in a mouse xenograftment model. (A) Mice were injected i.p. with 10mg/kg of PIK-75 and then sacrificed at the indicated time points with peripheral blood being collected by cardiac puncture into MiniCollect K3 EDTA coated tubes (Greiner Bio-One). Peripheral blood was then diluted 1: 2 with RPMI/1% FCS incubated with MV4;11 cells for 24h following which apoptosis was quantified by annexin V staining and flow cytometry as described for Figure 6.1A. Each dot represents the observed apoptosis of MV4;11 cells following incubation with a peripheral blood sample collected from a separate mouse with the line representing the mean. (B) NOD-SCID mice were engrafted via i.v. injection of HL-60 cells. After disease was established (2 weeks), mice were i.p injected with the indicated concentrations of PIK-75 for 3 days following which engraftment was quantified by assessing the percentage hCD45+ cells in the BM of recipient mice. The data presented in (B) was generated in the laboratory of Dr Christian Récher, INSERM, Toulouse in collaboration with the laboratory of Dr Mark Guthridge.

6.10 Conclusion

Constitutive PI3K signalling and over-expression of Bcl-2 pro-survival proteins are two pivotal mechanisms by which malignant cells acquire the ability to over-ride apoptosis programs. In particular, PI3K and the Mcl-1 pro-survival protein are both deregulated with high frequency in cancer and therapeutics capable of simultaneously targeting both proteins may have clinical potential. We now show that PIK-75, originally identified as a PI3K p110α inhibitor, potently and selectively induced apoptosis in primary CD34+CD38-CD123+ cells from AML patients (Figure 6.1).

The ability of PIK-75 to target AML cells was not solely due to blockade of PI3K p110α, as both pan-specific PI3K inhibitors (LY294002, Wortmannin) and p110α-selective inhibitors (PI-103, YM-024) were significantly less effective at inducing apoptosis (Figure 6.2). In addition to its ability to inhibit p110α, PIK-75 was also able to reduce Mcl-1 protein levels while the levels of both Bcl-xL and Bcl-2 were unaffected (Figure 6.4 and 6.7). Although specific knockdown of Mcl-1 alone using siRNA in primary AML cells had no effect on their survival, combined PI3K inhibition and Mcl-1 knockdown was sufficient for the induction of apoptosis in CD34+CD38-CD123+ cells (Figure 6.5). We have shown that the mechanism by which PIK-75 down-regulates the Mcl-1 protein is via a direct inhibition of Cdk9 kinase activity and a block in the RNAPII-mediated transcription of the Mcl-1 gene (Figure 6.6 and 6.7). Because Mcl-1 protein has a short half-life and is rapidly targeted for degradation by the proteosome, PIK-75-mediated blockade of Mcl-1 transcription through inhibition of Cdk9 led to the rapid and dramatic loss of Mcl-1 protein expression (Figure 6.6).

Other agents that block Cdk9 activity, such as Flavopiridol and SNS-032, have also been reported to rapidly down-regulate Mcl-1 protein levels and induce apoptosis(Chen *et al.* 2005; Chen *et al.* 2009). However unlike PIK-75, neither Flavopiridol nor SNS-032 inhibit PI3K signalling (Figure 6.4 and Figure 6.7) and Phase I/II AML, chronic lymphocytic leukaemia and myeloma trials with these drugs have indicated that despite being well tolerated, only partial and short-term remissions were observed (Lin *et al.* 2009; Tong *et al.* 2010). Such findings would indicate that although Cdk9 represents a tractable therapeutic target with acceptable toxicities, blockade of Cdk9 alone may not be

sufficient to produce long-term clinical responses. In support of such a proposal, we have shown that although PIK-75 was able to inhibit Cdk9, siRNA-mediated knockdown of Cdk9 alone in primary AML patient samples only modestly affected the viability of CD34+CD38-CD123+ AML cells (Figure 6.8). Significantly however, co-targeting of PI3K and Cdk9 using both pharmacological and genetic approaches was consistently more effective at inducing apoptosis in primary AML blasts and CD34+CD38-CD123+ cells than targeting either kinase individually. Thus, dual targeting of PI3K and Cdk9, as occurs with PIK-75, allows the blockade of two key survival pathways in AML and the induction of apoptosis in AML cells.

The highly plastic genetic and epigenetic landscape within transformed cells allows multiple independent signalling pathways to be recruited and corrupted not only during the tumorigenic process, but also in response to cytotoxic and targeted therapies. A clinical consequence of this plasticity is that blockade of a single oncogenic pathway while leaving others unperturbed may allow the survival of a reservoir of cells from which drug resistance and relapse can occur. Although PI3K activation is clearly a key mechanism underpinning oncogenic transformation in diverse tumors including leukaemia, it remains to be determined whether targeting the PI3K pathway alone will provide longer-term improvements in patient outcomes. For example, while therapeutic responses following PI3K inhibition alone in mouse xenograft tumor models have been shown to slow tumor growth, dual PI3K and MEK inhibition resulted in significant tumor regression (Engelman *et al.* 2008; Sos *et al.* 2009). Others have shown that co-targeting PI3K and MEK signalling in melanoma can overcome acquired resistance to B-Raf inhibitors(Villanueva *et al.* 2010). Such findings exemplify an emerging concept that targeting mechanistically independent pathways controlling cell survival will be important in the development of anti-cancer therapies.

Our studies demonstrating the ability of a single compound such as PIK-75 to simultaneously block PI3K p110α and Cdk9 provides proof-of-concept that the search for dual inhibitors capable of selectively targeting malignant cells is a feasible and a clinically attractive goal for pharmaceutical development. Importantly, PIK-75 demonstrated anti-leukaemic activity in an *in vivo* xenograft model at concentrations that were well tolerated by mice with no evidence of overt toxicity (Figure 6.11). The limited toxicity of PIK-75

may be related to the rapid pharmacokinetic clearance of bioactive PIK-75 following injection (Figure 6.11). Significantly, we have shown that this brief window of PIK-75 activity is sufficient for the rapid down-regulation of Mcl-1 protein, blockade of Akt phosphorylation and the induction of apoptosis in primary AML cells (Figure 6.6A). While a number of PI3K inhibitors are currently in preclinical development, to our knowledge, drugs that simultaneously block both p110α and Cdk9 activity have not been reported. The unique ability of PIK-75 to bind two distinct and unrelated kinases with high affinity appears to be due to the flexible hydrazone linker enabling the compound to adopt optimal binding orientations within each kinase ATP binding site (unpublished observation, Michael Parker, St Vincent's Institute of Medical Research). These structural insights may assist in the search for other chemical entities with dual PI3K/Cdk9 targeting properties.

The relative balance between the expression of pro-survival and pro-death Bcl-2 family members sets an intrinsic threshold that controls the cell survival:death axis (Cory and Adams, 2002). Drugs that are capable of targeting members of the Bcl-2 pro-survival family are also under investigation. For example, ABT-737 has been shown to inhibit the prosurvival activity of Bcl-2, Bcl-xL and Bcl-w (Oltersdorf *et al.* 2005). However, it is becoming increasingly clear that a variety of malignancies including chronic and acute leukaemias are resistant to ABT-737, in part due to Mcl-1 overexpression (Aichberger *et al.* 2005; Schwickart *et al.* 2010; van Delft *et al.* 2006; Zhang *et al.* 2002; Zhou *et al.* 1997). Furthermore, Mcl-1 over-expression in a number of solid and haematopoietic malignancies is an independent prognostic indicator of poor patient outcomes (Kaufmann *et al.* 1998). Thus, Mcl-1 represents an important therapeutic target and small molecule inhibitors that block upstream regulators of Mcl-1 expression (as opposed to targeting Mcl-1 directly with small molecule inhibitors such as BH3 mimetics) have the advantage of being able to rapidly deplete cellular pools of Mcl-1 protein due to its very short half-life. Thus, even transient blockade of Cdk9 results in almost complete loss of Mcl-1 and the rapid onset of apoptosis.

Mcl-1 heterodimerizes with, and inactivates, the pro-apoptotic activity of Bak (Willis *et al.* 2005), suggesting that agents able to target Mcl-1 will sensitize cells to Bak-mediated apoptosis. Consistent with such a proposal, we have shown that PIK-75 down-regulates Mcl-1 expression and induces apoptosis in a Bak-dependent manner (Figure 6.3). It would

be predicted that because Bcl-2 does not heterodimerize with Bak (Willis *et al.* 2005), transformed cells over-expressing Bcl-2 would not be resistant to drugs inducing Bak-dependent apoptosis such as PIK-75. Thus, drugs such as PIK-75 that block both Mcl-1 expression through Cdk9 inhibition as well as PI3K signalling may prove effective even in those transformed cells where other members of the Bcl-2 pro-survival family, such as Bcl-2, are over-expressed.

Our findings identify a new approach that enables targeting of AML cells through the dual blockade of two independent biochemical pathways (PI3K and Cdk9 signalling) that converge to promote cell survival. Since both PI3K and Cdk inhibitors are already in trials, our findings provide an immediate clinical rationale for testing the possible efficacy of combining these inhibitors in the treatment of AML. Furthermore, the discovery of PIK-75 as a first-in-class dual PI3K p110α and Cdk9 inhibitor will pave the way for the identification of other dual kinase inhibitors for clinical development in AML.

Chapter 7

DISCUSSION

7.1 Discussion

Deregulated cell survival is a universal hallmark of cancer and targeting cell survival pathways in long-lived cancer-initiating cells is a major goal of current biomedical research (Hanahan and Weinberg, 2000). The body of work presented in this thesis has aimed to gain insight into the signalling pathways associated with cell survival in acute myeloid leukaemia especially with respect to the IL-3/GM-CSF receptor. The over-arching approach employed in this study has been to study IL-3/GM-CSF receptor signalling and function in populations of patient-derived AML cells, including AML $CD34^+CD38^-CD123^+$ cells. Attempts to interfere with these survival signalling pathways *in vitro* and *in vivo* have revealed potential targets for therapeutic development. Four novel targets for AML have been explored and tested in this thesis along with mechanistic insights that have implications for understanding cancer stem cell biology and leukaemogenesis.

The initial observation that serine phosphorylation of the GM-CSF/IL-3 receptor regulates cell survival but not proliferation (Guthridge *et al.* 2006) prompted a careful evaluation of the phosphorylation state and function of GM-CSF/IL-3 receptor in AML. Firstly we confirmed the high expression of the IL-3 receptor α chain on AML $CD34^+CD38^-$ stem/progenitor cells (LSPCs) compared to normal haematopoietic cells and demonstrated a role for IL-3 in signalling cell survival in LSPCs (Chapter 3). We show that IL-3 receptor blocking antibody 7G3 can inhibit the survival of LSPC *in vitro* and reduce homing and engraftment of AML stem cells *in vivo*. These studies were the first in kind to demonstrate proof-of-principle of AML stem cell targeting by using a monoclonal antibody directed against a cytokine receptor. Our critical observation that Serine 585 of the IL-3/GM-CSF receptor was constitutively active in AML (Guthridge *et al.* 2006) sparked a search for the upstream kinase(s) that may be responsible for Ser585 phosphorylation in AML as a possible novel drug target. By testing fractions of AML cytoplasmic extracts for Ser585 kinase activity we made the unexpected discovery that the protein kinase activity of PI3K could function as a Ser585 kinase. This led to experiments supporting an unrecognised role for PI3K in cytokine survival signalling through its protein kinase activity distinct from canonical lipid kinase signalling (Chapter 4). Given the prevalence of constitutive Ser585 phosphorylation in AML we were also interested in downstream transcriptional targets of constitutive Ser585 survival signalling. Global transcriptional profiling of Ser585 mutated

cell-lines versus wild-type led to the discovery of osteopontin (*OPN)* as a Ser585-dependent prognostic factor in AML and the demonstration that targetting *OPN* with siRNA can kill AML $CD34^{+}CD38^{-}$ cells (Chapter 5). Subsequently, our attempts to block PI3K kinase activity in AML LSPCs has unravelled the notion of dual-kinase targetting both PI3K and CDK9 as a possible strategy in leukaemia (Chapter 6).

A central thread that runs throughout the work presented in this thesis is that growth factor receptor signalling pathways associated with survival rather than proliferation appear to be deregulated and active in acute myeloid leukaemia. Whilst the exact molecular mechanisms employed for transduction of these pathways is not understood, they appear to function in a manner distinct from the classical JAK/STAT or Ras/RAF/MAPK associated with growth factor proliferation responses. Yet clearly these alternative pathways have biological and prognostic roles and have led to new possibilities for targeting leukaemia (Figure 7.1). The sections below will discuss the specific contributions to knowledge arising from the observations presented in this thesis and future directions for further research and clinical translation.

The cancer stem cell model predicts that eradication of the leukaemia stem cell should lead eventually to cure (Clarke *et al.* 2006). We and others have noted high expression of the IL-3 receptor α chain on AML stem cells compared with normal haematopoietic stem cells in humans (Jin *et al.* 2009; Jordan *et al.* 2000). Similarly other transmembrane proteins have been expressed including CD44 (Jin *et al.* 2006), CLL-1 (van *et al.* 2007), CD96 (Hosen *et al.* 2007), CD32 (Saito *et al.* 2010), CD25 (Saito *et al.* 2010) and TIM3 (Jan *et al.* 2011). The high expression of CD123/IL-3 receptor implies that IL-3 may be a critical micro-environmental regulator of AML stem cell survival. Our *in vitro* studies with purified AML stem/progenitor cells using 7G3 monoclonal antibody in the absence of cytokine suggest some patient's AML progenitor cells produce autocrine IL-3 that contributes to cell survival (Jin *et al.* 2009). Targetting established AML in xenografts with a blocking mAb directed against the IL-3 receptor suggests that inhibition of IL-3 induced survival of LSCs in the born marrow is potential mechanism to disrupt AML stem cell function. In addition we found that optimal LSC targetting required functioning NK cell activity, indicating innate immune effector mechanisms are also important in designing future LSC directed therapy.

In this regard, the recent finding that CD47, a ligand that inhibits macrophage immune effector function, is also over-expressed on AML stem cells is particularly insightful (Majeti *et al.* 2009). CD47 functions as an inhibitory signal for macrophage phagocytosis by binding to SIRPα and over-expression may serve to protect evolving pre-leukaemic stem/progenitor cells accumulating leukaemogenic mutations from local engulfment. Blocking CD47 with monoclonal antibodies dramatically upregulates murine phagocytosis of human AML blasts (Majeti *et al.* 2009) as well as $CD20^{+}$ lymphoma cells treated with rituximab (Chao *et al.* 2010). It will be exciting in the future to combine CD123 and CD47 blocking monoclonal antibodies *in vivo* and in patient trials.

A search for serine/threonine kinases upstream of βc Ser585 using a semi-purification biochemical technique led to the intriguing observation that LY294002, a non-selective PI3K inhibitor, could block a Ser585 kinase activity present in AML blasts (Chapter 4). We have provided a number of overlapping and complementary approaches that are consistent with Ser585 as a bona fide PI3K extrinsic protein kinase substrate. We have also demonstrated evidence that PI3K protein kinase signalling is active in the context of cell survival at low picomolar levels of cytokine. Whilst difficult to dissect out the contribution of lipid kinase activity versus protein kinase activity in any given biological situation, our observations are nevertheless novel and tantalising. It is possible that PI3K has many protein kinase substrates that are important in cancer signalling but have been overlooked thus far.

Our data thus far is also consistent with the notion of a switch in the activities of PI3K coupling to particular biological processes. Activation of lipid kinase activity after association of PI3K with the inner cell membrane via phosphotyrosine signalling generates secondary messengers associated with robust proliferative signalling(Pawson and Scott, 1997). Activation of the protein kinase activity on substrates such as Ser608 in the inter-SH2 domain of p85 may serve as an intra-molecular brake to restrain lipid signalling(Foukas *et al.* 2004). Our data and others suggest the protein kinase activity can also phosphorylate other serine residues embedded in G-protein coupled receptors and cytokine receptors in a phosphotyrosine-independent manner (Bondeva *et al.* 1998; Naga Prasad *et al.* 2005). The regulation and function of the PI3K activity protein kinase/lipid

kinase switching is likely to be cell-context specific but our studies and others (Foukas *et al.* 2004) indicate cytokines play a role. Future work should examine primary cancer tissues for novel PI3K protein kinase substrates using phosphoproteome peptide trapping.

An additional follow-up experiment arising from this work would be to create a genetic knock-in of PI3K that retains only the protein kinase activity similar to the K941-944A mutant studied in Chapter 4 (Naga Prasad *et al.* 2005). If the above hypothesis is correct then there should be biological phenomena that are evident in the protein-kinase only but not in the kinase-dead mutant. Our prediction would be, based on our own data and the literature thus far, is that the protein kinase activity has a particular role in non-proliferating cells in maintaining cell survival. This is consistent with recent data showing unrestrained Akt signalling, an important signalling node for *lipid kinase* activity, in haematopoietic stem cells results in premature stem cell exhaustion (Kharas *et al.* 2009).

Previously we had shown that Ser585 phosphorylation of the GM-CSF receptor in mononuclear cells from normal donors and factor dependent cell lines CTL-EN and TF-1 occurred with 5 minutes after stimulation by extremely low (picomolar) concentrations of cytokine. This phosphorylation pattern was distinct from that observed with receptor tyrosine phosphorylation and consistently down-regulated at higher (nanomolar) concentrations. We and others have shown that low picomolar concentrations of GM-CSF are associated biologically with eliciting survival responses and Ser585 is required for survival by IL-3 and GM-CSF (Guthridge *et al.* 2006; Guthridge *et al.* 2004; Guthridge *et al.* 2000). We have now confirmed in a panel of 23 AML samples that Ser585 is constituively phosphorylated in the majority of cases (87%) (Chapter 5). In contrast, Tyr577 phosphorylation remained ligand dependent in 17 (94%) of 18 AML samples. The deregulation of Ser585 is demonstrated by a i) increased basal phosphorylation in the absence of factor and ii) a failure to down-regulate with higher concentrations of GM-CSF. To our knowledge this is the first comprehensive analysis of *phosphoserine* cytokine receptor signalling in AML. Other studies have examined JAK2 and STAT5 tyrosine phosphorylation events after GM-CSF stimulation using either phospho-flow or Western blotting and shown marked heterogeneity of response (Gibbs, Jr. *et al.* 2011; Irish *et al.* 2004). Deregulation of phosphoSer585, however, appears to be a consistent finding across range of cytogenetic AML sub-types (Powell *et al.* 2009, Chapter 5). This mandated a

search for novel targets linked to Ser585 signalling in AML.

These findings are intriguing and suggest deregulation of serine kinases and/or phosphatases acting upon the GM-CSF/IL-3 receptor is a common event in AML. This is in direct contrast with other haematological malignancies including myeloproliferative disorders in which deregulated tyrosine kinase activity (JAK2 or BCR-ABL) is clearly driving the leukaemic process. Our data indicates that, in terms of GM-CSF receptor phosphorylation, AML blasts are phenocopying low cytokine stimulation associated with cell survival rather than high dose signalling (nanomolar) associated with rapid cell division. An important deduction from this observation therefore is that targeting AML with JAK2 or other tyrosine kinase inhibitors may be insufficient to obtain an effective therapeutic responses and is consistent with other work using tyrosine kinase inhibitors in the context of *BCR-ABL* and *FLT3-ITD* activating mutations(Mohi *et al.* 2004).

In order to identify critical genes regulated by Ser585 phosphorylation we performed microarray profiling of CTL-EN factor-dependent cell line expressing either Ser585 mutated to glycine or wild-type GM-CSF receptors. We found 138 genes of which 76 were common to both arrays utilised. Gene ontogeny analysis indicated that more than half of the genes performed functions related to cytokine signalling and transcription/translation. Interestingly there was a significant association between the 138 genes and the gene signature produced by the PI3K inhibitor LY294002 (P=0.009). This data is consistent with our previously published association of Ser585-signalling linked to PI3K and underscores the importance of PI3K signalling in AML. Indeed, a major downstream kinase activated by PI3K lipid kinase signalling is Akt and similarly to Ser585 has been shown by ourselves and others to also exhibit constitutive phosphorylation in AML. Unlike solid organ cancers in which activating mutations of PI3K p110α or deletions of PTEN are common, the mode of activation of PI3K in AML is not well understood and remains an major area of interest (Di Cristofano and Pandolfi, 2000).

We also showed that a number of Ser585 transcriptionally regulated genes (*BCL2, VRK1, POLR2C, CDK8* and *FNDC3*) were highly expressed in both AML blasts and purified $CD34^+$ progenitor cells from healthy donors but not in mature $CD14^+$ monocytes. This is also consistent with the constitutive phosphorylation of Ser585 observed in AML and

suggests further functional characterisation of these genes in AML may reveal insights into AML biology and potential targets.

We noted that a secreted gene product, *OPN*, was over-expressed in some but not all AML samples and that is expression was independently regulated from the other genes using pairs plots analysis. *OPN* expression was sensitive to LY294002 and wortmannin treatment suggesting that PI3K signalling can promote *OPN* expression in AML. The broad range of expression prompted us to examine the functional effect of *OPN* expression in AML in terms of survival signalling and clinical outcome after treatment with chemotherapy. We show that blocking *OPN* expression using siRNA in AML blasts and AML stem/progenitor cells resulted in decreased cell survival. These results indicate *OPN* may be a critical leukaemic stem cell survival factor and potential target for AML stem cell therapy.

An emerging body of literature is emphasising the importance of non-cell autonomous factors that may regulate leukaemia and, importantly, leukaemia stem cells. There is evidence that some of these microenvironmental niche factors include cytokines such as IL-3, CXCL12 and $\alpha_4\beta_1$ (Dar *et al.* 2006; Lane *et al.* 2009; Zeng *et al.* 2009). Xenotransplantation assays have demonstrated a role for the endosteal niche in promoting LSC engraftment, quiescence and resistance to Ara C chemotherapy (Ishikawa *et al.* 2007). Our study is the first demonstration that that the gene for osteopontin, which normally lines the endosteal niche(Haylock and Nilsson, 2006; Lin and Yang-Yen, 2001; Nilsson *et al.* 2005), is a survival factor for AML stem/progenitor cells and is associated with poor clinical outcome. In our study we examined enriched AML blasts from bone marrow and hence our data would be in keeping with a role for cell-autonomous expression of osteopontin (i.e. produced by the AML blasts themselves) in promoting chemotherapy resistance. How the protein expression, post-translational modification and regulation of *OPN* in the endosteal niche compare to that produced by blasts remains to be investigated. In future we hope to examine the properties of culturing LSCs in the presence of exogenous purified osteopontin and examine the protein product and translational modification of *OPN* in AML.

There is a great need for prognostic markers in cancer that have both biological function and can be targeted for therapeutics i.e.“druggable”. Normal karyotype AML is a

proportionally large cytogenetic sub-group in adults for which therapy and risk stratification has been difficult for the clinician (Mrozek *et al.* 2007; Tallman, 2008). More recently, improved prognostication has been achieved with the use of *NPM*, *FLT3-ITD* and *CEBP*α mutation analysis, however only *FLT3*, a tyrosine kinase, is druggable (Piloto *et al.* 2007). Our study is significant in that *OPN* gene expression was independent of both *FLT3-ITD* and *NPM* in normal karyotype AML and yet remained strongly associated with poor outcome. We hope, therefore, to incorporate routine testing by RT-PCR of *OPN* in future Australian AML trials and this has been implemented by the Australian Leukaemia Lymphoma Group (Wei, personal communication). This larger patient cohort would grant us the statistical power to investigate the association between particular molecular mutations in AML and *OPN* expression normal karyotype AML.

Careful analysis of the effect of various PI3K isoform-selective inhibitors acting upon purified AML sub-populations led to our observation that a particular p110α inhibitor, PIK-75, now commercially available, had profound effects on LSPCs with low nanomolar efficacy. Our data suggested that one or all of the signalling pathways inhibited by PIK-75 must be essential for AML stem cell survival in our short term survival assay culture conditions. *In vitro* kinase and signalling experiments soon established that unlike other p110α inhibitors, PIK-75 was also a potent CDK9 inhibitor at low nanomolar concentrations. To our knowledge, this is the first demonstration of a combined CDK9/PI3K inhibitor tested against AML stem/progenitor cells and shows proof-of-concept that these two well-established pathways function independently and cooperatively to regulate cell survival.

Dual targeting of kinases is increasingly recognised by the pharmaceutical industry and cancer researchers to be a pre-requisite for an effective anti-cancer strategy. Based on the results of this thesis it is hoped clinical trials examining the use of combining CDK9 inhibitors with PI3K in high risk relapsed/refractory AML patients will be conducted. Future studies aimed at investigating the CDK9 signalling axis in AML and Mcl-1 processing are likely to be fruitful.

7.2 Conclusions

Therapy and clinical outcomes in AML have not altered in over 30 years and 5-year survival remains less than 30%. By carefully analysing in primary AML samples the phosphorylation status of a particular serine residue, namely Ser585 of the GM-CSF receptor, this study has uncovered novel signalling components in the AML cell and contributed to the discovery and pre-clinical validation of a number of potential AML targets, including leukaemic stem cell targets (Figure 7.1). Together these studies underscore the utility of integrating basic discoveries in signal transduction with patient samples and clinical data. By these and similar approaches, the hope of cure in difficult to treat malignancies by targeting cancer stem cells may soon be realised.

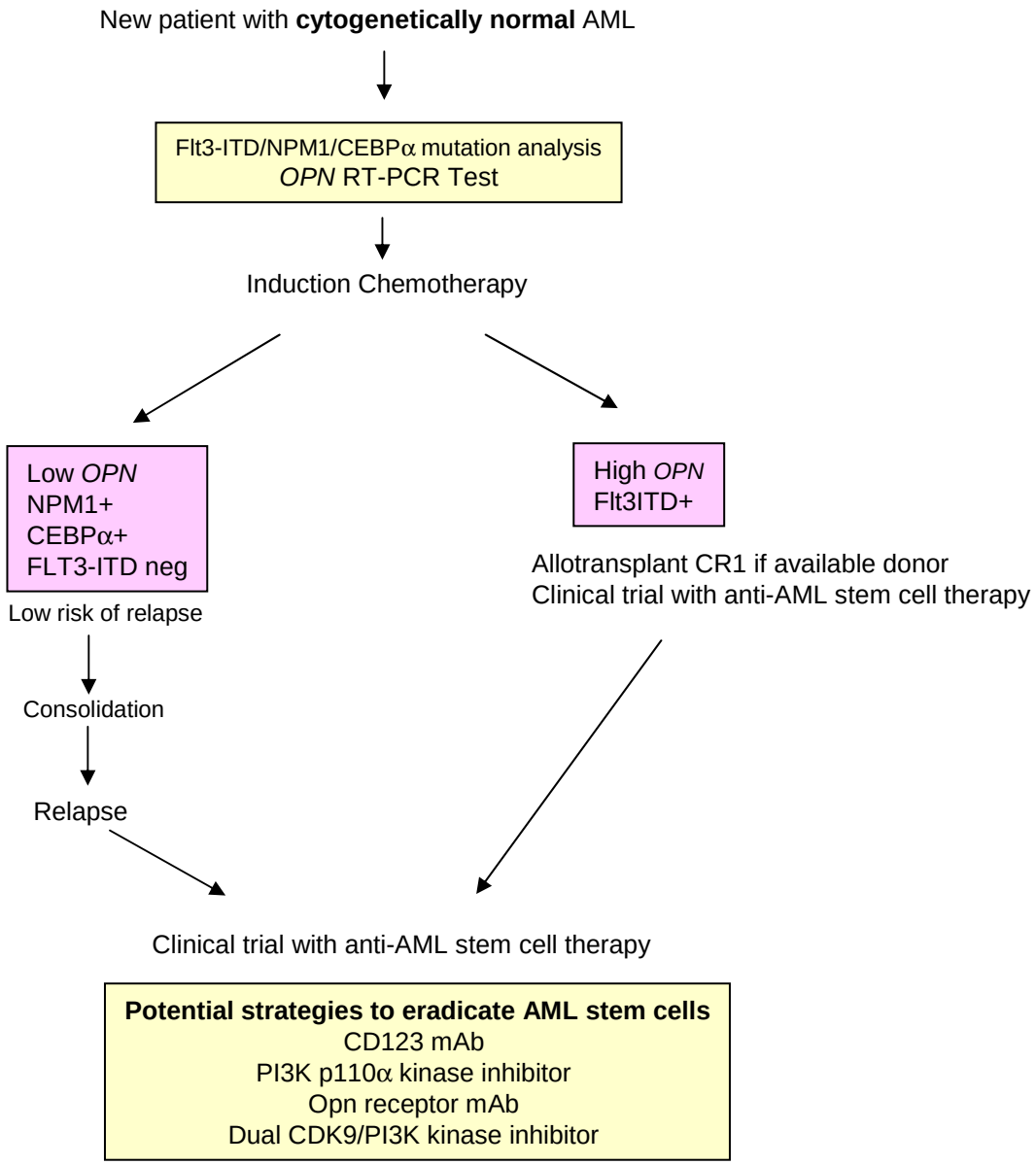

Figure 7.1: Proposed treatment strategy and suggestions for future study for patients with cytogenetically normal AML. Cytogenetically normal AML is a large sub-group of AML that is difficult to prognosticate and manage. The results of Chapter 5 suggest RT-PCR of *OPN* may have additional prognostic information beyond *Flt3/NPM* and *CEBPα* mutation status and this RT-PCR assay is currently being investigated in larger Australia-Leukaemia lymphoma Group (ALLG) AML trials in Australia. Together, these molecular studies would provide useful information to (i) predict which patients are at risk of early relapse and (ii) stratify patients for clinical trials involving novel anti-AML stem cell therapies. Relapsed patients could be considered for a clinical trial examining new therapies to eradicate AML stem cell such as anti-CD123 mAb (Chapter 3, now in Phase I/II clinical trials), PI3K lipid and protein kinase inhibitor as outlined in Chapter 4 (Phase II/III clinical trials), Opn receptor mAb (novel target linked to chemotherapy resistance – see Chapter 5), or dual kinase inhibitor to CDK9 and PI3K (Chapter 6)

Chapter 8

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