DEVELOPMENT OF AN *IN VITRO* RELAPSE MODEL FOR AML

DEVELOPMENT OF AN *IN VITRO* RELAPSE MODEL FOR IDENTIFICATION OF NOVEL THERAPEUTICS IN ACUTE MYELOID LEUKEMIA

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LAY ABSTRACT

AML is a cancer of blood cells characterized by the presence of rapidly dividing cancer cells termed myeloblasts. AML has a high rate of disease relapse. The Bhatia lab modelled AML relapse in a mouse and discovered an unique population of cells that exist prior to relapse termed LRCs. LRCs express distinctive genes that can act as targets for the development of new therapies to prevent relapse. In order to screen potential relapse-preventing compounds, we set out to recapture AML relapse using cells in a dish. AML cells from patients were treated with chemotherapy reagent AraC and the number of cancer progenitors and the expression of specific LRC proteins were measured. AraC did not increase the level of 3 out of 4 LRC proteins studied. We determined the LRCs were not caused by AraC treatment, and the physiology of the bone marrow environment plays an important role in inducing relapse.

ABSTRACT

AML is a cancer of the blood and bone marrow characterized by the presence of highly proliferative and abnormally differentiated myeloblasts. Previous work from the Bhatia lab utilized the orthotopic xenograft model in order to isolate a population of leukemic regenerating cells (LRC) that exists prior to relapse. Affymatrix analysis of LRCs revealed up-regulation of 248 genes that can act as unique targets to prevent relapse. In order to screen compounds against all 248 targets, it is important to develop an *in vitro* model that is able to appropriately recapture the functional and molecular markers of LRCs. Primary AML samples were treated with 5-doses of 0.15 μM, 1 μM AraC, or DMSO control and several outcomes were measured. *In vitro* AraC treatment was not able to recapitulate the progenitor frequency curve and CD34 expression curve observed *in vivo*. Additionally, we were not able to see a consistent increase in select LRC targets DRD2, GLUT2, FUT3, and FASL via flow cytometry. Despite an increase in the mRNA levels of LRC genes 24h after treatment with 0.15 μM AraC, long term analysis could not be completed due to poor RNA quality and low expression of LRC-targets. Primary AML cells were co-culture with mouse MS-5 stromal cell line order to study the effects of mesenchymal stromal cells on AML response to AraC. Co-culture with MS-5 cells had different effects on select primary AML cells. AML 14939 showed an increase in CD34 and LRC targets DRD2 and FUT3 following AraC treatment when co-cultured with MS-5 cells; while A374 showed no differences between DMSO and AraC treated groups. Overall, these findings suggest the LRC signature is not induced by treatment with AraC alone. Complex interactions between AML cells and their bone marrow niche during AraC treatment plays an important role in the development of LRCs prior to AML relapse.

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LIST OF ABBREVIATIONS

7AAD	7-amino actinomycin
AML	Acute myeloid leukemia
AML1-ETO	AML1-Eight-twenty-one oncoprotein
Ang-1	Angiopoietin-1
ANOVA	Analysis of variance
AraC	Cytarabine
BM	Bone marrow
CB	Cord blood
CBFB-MYH11	Core-binding factor subunit beta-myosin 11
CCL3	Chemokine ligand 3
cDNA	Complementary DNA
CFU	Colony forming unit
CR	Complete remission
CXCL12	C-X-C ligand 12
CXCR4	C-X-C receptor 4
DGIdb	Drug genome interaction database
DRD2	Dopamine receptor D2
EDTA	Ethylenediaminetetraacetic acid
FASL	Fas ligand
FBS	Fetal bovine serum
FISH	Fluorescence in situ hybridization
FLT3	Fms like tyrosine kinase 3
FUT3	Fucosyltransferase 3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT2	Glucose transporter 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase
HSC	Hematopoietic stem cell
IL-6	Interleukin-6
ITD	Internal tandem duplications
IUPHAR	International Union of Basic and Clinical Pharmacology
Jag1	Jagged1
LRC	Leukemic regenerating cells
LSC	Leukemic stem cells
LT-HSC	Long term hematopoietic stem cell
MEM	Minimum essential medium
MPB	Mobilized perpherial blood
MRD	Minimal residue disease
MSC	Mesenchymal stem cells

NGS	Next generational sequencing
NOD/SCID	Non-obsese diabetic/Severe combined immunodeficiency
NPM1	Nucleophosmin 1
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PML-RARA	Promyelocytic leukemia-retinoic acid receptor alpha
RBC	Red blood cell
RT-PCR	Quantitative reverse transcriptase polymerase chain reaction
SCF	Stem cell factor
SD	Standard deviation
SEM	Standard error of the mean
SNO	Spindle-like N-cadherin+CD45- osteoblastic
SR1	Stemregenin 1
ST-HSC	Short term hematpoietic stem cell
TPO	Thrombopoietin

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis is completed mainly with the work of Wenqing Wendy Ye with the following contributions from the members in Dr. Mick Bhatia's lab. Jennifer Russell developed the automated 48-well CFU assay utilized for drug screening. Lili Alostovar and Dr. Allison Boyd completed *in vivo* transplantations and isolation of bone marrow cells for *ex vivo* LRC experiments. Dr. Borko Tanasjievic designed primers for PCR analysis.

1.0 INTRODUCTION

1.1 ACUTE MYELOID LEUKEMIA (AML)

1.1.1 Clinical Features and Disease Epidemiology

AML is a cancer of the blood and bone marrow leading to accumulation of highly proliferative, and abnormally differentiated myeloid progenitors (myeloblasts) in the bone marrow, blood, and other parts of the body such as the spleen and liver[1]. Increased proliferation of myeloblasts leads to decreased production of mature myeloid-derived blood cells such as red blood cells, neutrophils, and macrophages resulting in anemia, thrombocytopenia, and neutropenia[2]. Patients typically present with a variety of constitutional symptoms including weakness and fatigue, increased bleeding, petechiae, ecchymosis, and increased likelihood of infections and fever[1]. On history, patients may report increased chest pain due to decrease oxygen delivery to the cardiac tissues, and reoccurring incidence of upper respiratory tract infections due to neutropenia[3].

AML is the most common form of acute leukemia in adults, accounting for over 32% of all leukemia cases in individuals over the age of 20[1]. The overall survival rate of AML is extremely low at 25%. Advancements in cancer therapy have significantly improved the complete remission (CR) rates in AML; however, the rate of refractory disease and relapse remains extremely high. In younger patient populations, 30 – 40% of patients suffering from AML will not obtain CR status [4]. 45 – 60% of patients over the age of 60 will not obtain CR status; a distressing statistic given the median age of AML diagnosis is 70[1]. AML relapse post-consolidation therapy occurs in 20 – 40% of young patients, and 70 – 80% of older patients[2]. Only 10% of relapsed patients achieve secondary CR with intensive induction therapy, and 20 – 30% with autologous-hematopoietic cell transplant[5].

1.1.2 Disease Prognosis and Risk Stratification

Accurate assessment of disease prognosis at the time of diagnosis is critical for determination of treatment regime and patient-centered care. Patient eligibility for intensive induction therapy should be carefully assessed due to the potential side effects associated with anti-neoplasmic therapy. Patient and disease associated factors both play an important role in predicting disease prognosis. Patient associated factors are predictive of treatment-related early death[6]. Older patients (>60 years) may not be ideal candidates for intensive induction therapy due to an increased number of comorbidities such as cardiovascular or metabolic complications and poor performance status as measured by the Eastern Cooperative Oncology Group (ECOG) score[7,8]. Disease associated factors can be used as a predictor of therapeutic resistance and to assess the risk of relapse following induction therapy. A series of investigations including myeloblast morphology, cytogenetic work-up, immunophenotyping via flow cytometry, and determination of molecular point mutations are used in order to assess the disease-associated factors that will play a role in disease prognosis[2]. Several studies have classified AML into favourable, intermediate, and poor prognostic groups based on laboratory analysis of isolated AML myeloblasts[9]. These groupings are summarized in Table 1.1. Disease classification using these features is important in determining type of treatment and predicting success of therapy.

Table 1.1: Risk Stratification of AML

Risk	Cytogenetic and Molecular Genetic Profile
Favourable	AML1-ETO, CBFB-MYH11, NPM1 without FLT3-ITD (normal karyotype), CEBPA (normal karyotype)
Intermediate	NPM1, FLT3-ITD, MLLT3-KMT2A, Normal karyotype
Adverse	GATA2-MECOM (EVI1), DEK-NUP214, KMT2A rearranged, complex

The cytogenetics of the isolated bone marrow myeloblasts are evaluated at the time of diagnosis. Karyotyping and fluorescence *in situ* hybridization (FISH) are performed to determine any cytogenetic abnormalities such as chromosomal translocations within blast populations[10]. Many genetic and chromosomal abnormalities expressed in malignant cells can be associated to specific cell morphology and disease outcomes. AML1-eight-twenty-one-oncogene (AML1-ETO), PML-RARA, and core-binding factor subunit beta-myosin 11 (CBFB-MYH11) are routinely screened for in clinics to build the appropriate treatment plan for the patient. The AML1-ETO chromosomal translocation results in a chimeric protein that prevents normal hematopoietic differentiation leading to the formation of AML[11]. Similarly, the presence of the CBFB-MYH11 translocation also results in an inhibition of hematopoietic differentiation[12]. The presence of the AML1-ETO or CBFB-MYH11 translocation generally have favourable outcomes and patients benefit from allogeneic hematopoietic stem cell transplant as consolidation therapy[13]. Overall, the cytogenetics of AML plays an extremely important role in therapy selection and patient counselling.

Although cytogenetic analysis can provide us with further insight into the prognosis of patients with AML, approximately 50% of AML have a normal karyotype and are

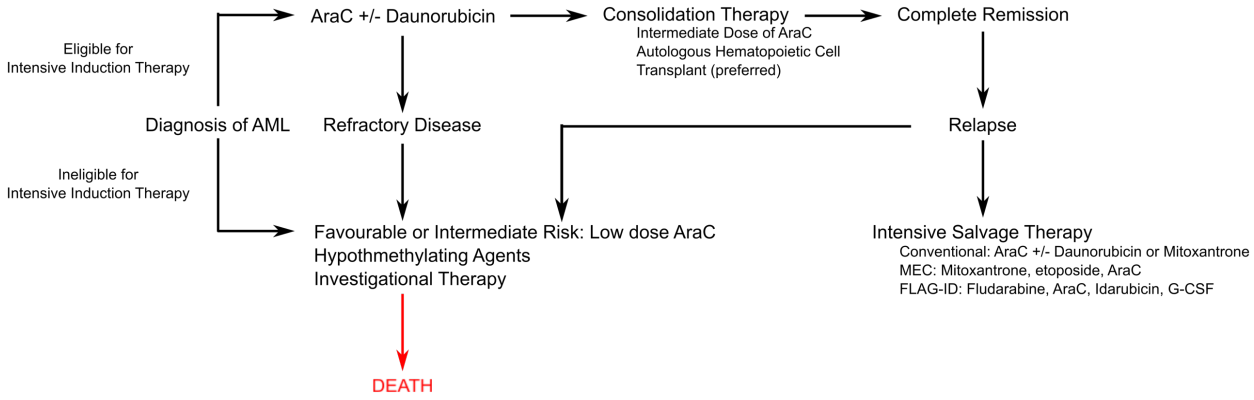
categorized in the intermediate risk group[2]. The development of next-generation sequencing (NGS) has led to the identification of several mutations that play an important prognostic role in AML with normal karyotype. Mutations in several genes have been linked to therapeutic resistance and increased likelihood of disease relapse. Nucleophosmin 1 (NPM1), and Fms like tyrosine kinase 3 (FLT3) mutations have become a part of routine screening upon diagnosis with AML. NPM1 mutations make up the most common molecular genetic abnormality observed in cytogenetically normal AML and is often associated with other mutations[14]. Generally, AML with NPM1 mutations have a favourable outcome and benefit from standard intensive induction therapy[14]. NPM1 is also assessed during remission as a potential mutation observed in the minimal residual disease (MRD). The new expression of NPM1 mutation in the MRD is predictive of disease relapse and therapeutic resistance in secondary leukemia[15]. The role of NPM1 in the development of neoplasms and its contribution of therapeutic resistance is still unclear; however, studies have shown NPM1 to be important in the regulation of tumour suppressor p53[16]. The differential response of NMP1 mutations at diagnosis and relapse to AraC is also uncertain. FLT3 is a proto-onco gene that is associated with cell differentiation, proliferation, and survival[17]. It codes for a tyrosine-kinase receptor that is activated by several cytokines including tumour necrosis factor alpha (TNFα) and transforming growth factor beta (TGFβ)[17]. ITD mutations in FLT3 are the most commonly observed mutations found in AML[13]. Studies have shown AML cases with FLT3 mutations to have poor prognosis[18]. A point mutation at residue D835 in FLT3 is also associated with poor prognosis[13]. FLT3 inhibitors are of high interest and several have been assessed in clinical trials with promising results; however, patients appear to develop

resistance to inhibitor treatment over time[19,20]. The development of FLT3 inhibitor resistance could be attributed to the interaction of FLT3 with other mutations that were identified at diagnosis. Additional mutations such as a TET2, IDH1, and JAK2 have been consistently associated with inferior response to AraC therapy and cancer relapse[8]. Assessment of these genetic mutations will likely be incorporated into the standard diagnostic workup for AML in the upcoming years.

1.1.3 Treatment of AML

The current standard of care for AML is intensive induction therapy, with supportive care supplementation such as replacement of blood products to decrease patient symptoms[4]. AraC is the most common chemotherapeutic reagent used amongst AML patients for intensive induction therapy, and the only drug utilized for monotherapy[4,21]. AraC is commonly combined with an anthracycline drug such as daunorucibin to increase therapeutic effectiveness[22]. Consolidation therapies are used after cancer remission in order to prevent or delay relapse. The goal of consolidation therapy is to maintain a state of remission after intensive induction therapy. Consolidation strategies include chemotherapy as well as autologous hematopoietic-cell transplant[2,23]. Therapy with an intermediate dose of AraC is the preferred route for patients under the age of 60. This regimen was shown to have poor outcomes in older patients[7]. Autologous hematopoietic-cell transplant remains the most effective post-remission therapy for patients who are unlikely to achieve remission with other therapeutic means[24,25]. Due to the difficulty of finding appropriate donors as well as the high toxicity associated with transplantation such as graft versus host disease, autologous hematopoietic-cell transplants are mostly

restricted to patients with high-risk disease[8]. A summary of current clinical strategies for AML can be found in Fig. 1.1. The failure of traditional intensive induction therapy with AraC in relapse patients place a heavy burden on physicians and the health care system. Patients who have relapsed face extremely poor disease prognosis – these patients make up the majority of individuals enrolled in clinical trials and studies of new reagents[5].



[26]**Figure 1.1: Clinical strategies for treatment of AML.** Upon diagnosis of AML, patient eligibility to receive intensive induction therapy is assessed. Factors such as age of the patient, ECOG score, and co-morbidities are all taken into consideration. If the patient is eligible for intensive induction therapy, they will undergo a cycle of 7 days of AraC followed by 3 days of anthracycline drug treatment, most commonly with daunorubicin. If disease remission is achieved, the patient will undergo consolidation therapy consisting of an intermediate dose of AraC, or an autologous hematopoietic cell transplant. Patients who are not eligible for intensive induction therapy, or those who have failed to achieve remission after AraC treatment will be assessed based on disease factors. Those with favourable or intermediate disease risk as determined by cytogenetic and molecular work-up of the leukemia blasts will receive low-dose AraC. Hypomethylating agents and other investigational therapies are also considered at this point. Individuals who are at high risk post-relapse or refractory disease can undergo a number of intensive salvage therapy regimes based on patient drug-tolerance.

1.1.4 Detection and Characterization of the MRD

A population of neoplasmic cells that remain post-consolidation therapy form a MRD that is difficult to detect by current clinical practices[27,28]. Given the high rate of relapse for patients suffering from AML, it is important to understand the molecular mechanisms underlying the formation of MRD and failure of chemotherapy. MRD is detected by

immunophenotyping via flow cytometry; however, due to the heterogeneity of AML myeloblasts and the lack of correlation between surface markers and functional self-renewal capacity, the number of neoplasmic cells that make up the MRD is often under-represented[29-31]. A variety of surface markers are used characterize the blast cells at diagnosis, CR, and relapse. These include CD34, CD38, CD117, CD133 antigens for progenitor cells, CD33, CD15, CD16, CD13 for granulocytic cells, and CD14, CD11c, and CD36 for monocytic cells[32,33]. CD34 expression is utilized heavily in the clinic to identify populations of progenitor cells that can give rise to AML; however, CD34 negative cell populations have also been shown to be able to initiate disease[34]. The limited sensitivity of flow cytometry and cell numbers required can overlook individuals who have a lower blast burden at remission but still capable of disease relapse. Studies have cited up to one third of patients who had negative MRD screens using flow cytometry went on to relapse[15]. There are also difficulties surrounding the accessibility of flow cytometry to all cancer care facilities: specialized core laboratories for the analysis of bone marrow and peripheral blood are required for flow cytometry analysis. RT-PCR is used to detect mutations and chromosomal rearrangements in the MRD in order to predict disease relapse[15]. The heterogeneity of AML makes prognosis assessment of the MRD particularly difficult. Many mutations have been identified in primary AML and the MRD, and more research is required to determine the prognostic value of each individual mutation. Current research is focused on improving the detection of MRD and further characterizing surface marker expression and genomic mutations at diagnosis. A different approach to MRD that could lead to better predictive success involves focus on the evolution of AML myeloblasts in response to chemotherapy.

1.2 THE ROLE OF THE BONE MARROW NICHE IN AML

1.2.1 Cellular Factors in Maintenance of HSCs

The bone marrow microenvironment plays an important functional role in HSC self-renewal and differentiation. Several cell types make up the architecture of the bone marrow niche, including osteoblasts, endothelial cells, non-myelinated Schwaan cells, megakaryocytes, and MSCs[35]. These cell types support HSCs and help regulate the balance between quiescent LT-HSCs and proliferating ST-HSC which are mobilized from the endosteal region to the vascular bone region in order to proliferate and differentiate into mature blood products[35].

A subset of osteoblasts lining the endosteal bone surface termed SNO cells interact with LT-HSCs to maintain their cellular quiescence[36]. Temporary ablation of osteoblast populations in the bone marrow led to a decrease in the total HSCs, decrease in mature blood productions, and an increase in secondary HSC sites such as the liver and spleen[37]. Sinusoidal endothelial cells in the vascular region have also been shown to interact with HSCs and to support HSC differentiation and proliferation[38]. Avecilla *et al* have shown that TPO-independent thrombopoiesis was dependent on the recruitment and interaction of HSCs with sinusoidal cells in the vascular bone region[39]. Another important cell type in the bone marrow niche are the mesenchymal stem cells (MSCs). MSCs in the bone marrow niche give rise to osteoblasts, chondrocytes, endothelial cells, adipocytes and fibroblasts[40]. Selective ablation of nestin+ MSCs have been demonstrated to decrease the number of HSCs and progenitors, with an associated increase of HSC into secondary liver and spleen sites[41].

1.2.2 Non-Cellular Factors in Maintenance of HSCs

In addition to the cellular factors that are important for regulation of HSCs, several soluble factors and signalling pathways have been identified as crucial for HSC survival and regulation of proliferation and differentiation. SCF/c-Kit signalling has been identified as necessary for HSC proliferation and survival[42,43]. The loss of SCF secretion by stromal and osteoblast cells or the loss of c-Kit receptor expression on HSCs has been shown to lead to hematological failure[42]. Another important player in maintenance of HSC quiescence and survival is secretion of Ang-1 by osteoblasts to the Tie2 receptor on HSC[44]. Expansion and proliferation of HSC is regulated by binding of the Jag1 ligand to the Notch receptor on HSCs[45]. Recruitment and mobilization of HSCs to the vascular region is regulated by CXCL12-CXCR4 signalling[46]. HSCs that express high levels of CXCR4 surround the sinusoid endothelial cells in the vascular niche of the bone marrow. Deletion of CXCR4 significantly decreased the number of HSCs and increased the risk of myelotoxic injury in mice[46].

1.2.3 The Bone Marrow Niche in AML

The bone marrow niche also plays a crucial role in the maintenance of LSCs and leukemic cells. Fumihiko et al utilized the AML xenotransplantation model to demonstrate homing of CD34+/CD38- therapy resistant LSC populations to the endosteal, osteoblast-rich section of the bone marrow niche[47]. Interactions between LSCs and cell types within the bone marrow niche alter the niche microenvironment and prevent the engraftment and mobilization of HSCs *in vivo*[48]. LSCs stimulate the formation of dysfunctional osteoblast lineage cells through direct cell-to-cell adhesion supported by secretion of TPO

and chemokine ligand 3 (CCL3) in order to generate a leukemic niche that stimulate their own survival and proliferation[49]. Dysfunctional bone marrow MSCs in the leukemic niche have altered cytogenetics, cytokine secretion of SCF and Jag1, and decreased proliferation and differentiation potential[50,51]. Taken together, all of these alterations lead to a niche environment that is detrimental to HSC and increase survival and proliferation of LSCs and leukemic progenitors.

CXCL12-CXCR4 signalling not only plays an important role in the homing and mobilization of HSCs, LSCs also utilize this signalling pathway in order travel to the bone marrow niche during engraftment experiments[52]. AML cells have increased CXCR4 expression, and have been correlated with poor disease prognosis in AML with FLT3 mutations[53]. Given the importance of CXCL12-CXCR4 signalling in LSC homing, and disease prognosis, CXCR4 inhibitors have been developed and have shown the ability to sensitize AML to novel therapeutics. Inhibition of the CXCR4 signalling pathway with AMD3465 increases AML sensitivity to AraC as well as FLT3 inhibitor sorafenib[54].

1.3 ETIOLOGY OF THERAPY RESISTANCE IN AML

Although disease-specific factors are able to predict the probability of therapeutic resistance and AML relapse, the mechanism by which cancer cells are able to evade current therapies and propagate to form a secondary disease is still up for debate. In this section, we will discuss two common theories to explain the therapeutic resistance and relapse ability of AML myeloblasts.

1.3.1 Cancer Stem Cells Model

An important model in the study of cancer relapse and regeneration is the cancer stem cell (CSC) model (Fig. 1.2). According to the CSC model, a specific group of CSCs with self-renewal capacity evade current cancer therapeutics due to cell cycle quiescence and establish secondary tumours after a remission state has been achieved[55-57]. The CSC model predicts that clinical outcomes should be directly related to the eradication of CSC populations within existing tumours; however, the clinical application of these predictions have not been established[58]. Transplantation experiments performed in severe-combined immune-deficient (SCID) mice has identified a cell capable of initiating AML and confirmed the organization of AML as a hierarchy that originates from a single cell [59,60]. One of the strongest pieces of evidence linking the cancer stem cell theory to the clinic is the prediction that a subset of leukemia stem cells (LSCs) is a rare subset of dormant AML samples that is resistant to initial cytoreductive therapy. These therapy-resistant LSCs may remain following treatment as a MRD, and may propagate at a later time to generate a new tumour. In 2011, Eppert *et al* established a link between the presence of LSCs at diagnosis to disease prognosis and outcome in 16 primary AML samples[61]. Despite encouraging data identifying the existence and clinical relevance of LSCs, there have been limited advances in new therapeutics to target these populations. Patient level application of the molecular LSC signature identified by Epperts *et al* to identify potential therapeutic resistance is technically challenging. Due to the lack of consistent surface markers, LSC populations are difficult to distinguish from proliferative myeloblasts and healthy hematopoietic progenitor cells. LSC populations are also difficult to isolate due to their rarity. It has been reported that only 0.0002 – 0.28% of isolated

bone marrow cells comprise the LSC fraction[62]. More importantly, a therapy that is selective for the LSC fraction but not the healthy hematopoietic progenitor cells have not been identified. Genomic and surface marker analysis has shown striking similarities in expression between healthy hematopoietic progenitors and LSCs[61,63]. Additionally, clinical trials involving granulocyte-colony stimulating factor in combination with AraC to stimulate cell cycle activity in dormant LSCs has not been successful[64].

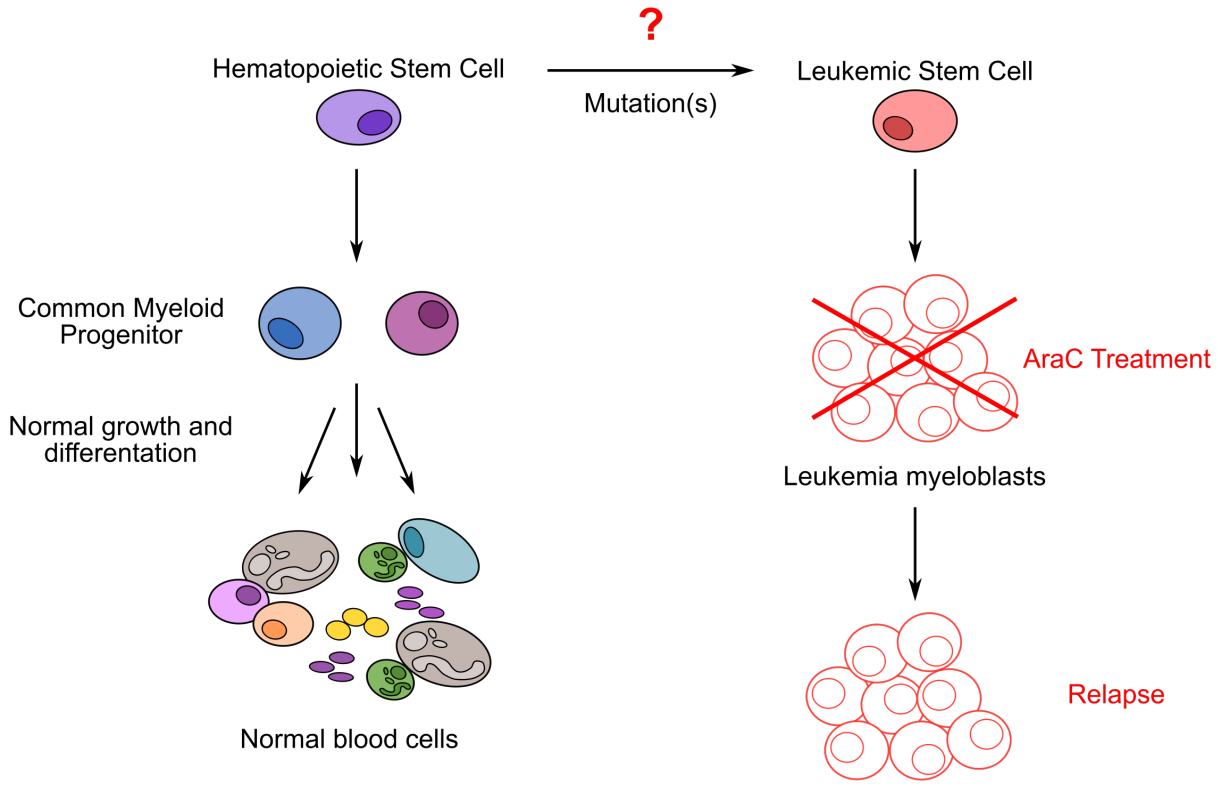


Figure 1.2: Cancer stem cell model of cancer relapse: All cellular components of blood are derived from a common long term haematopoietic stem cell progenitor in the bone marrow. Life-long hematopoiesis is maintained by hierarchical differentiation, ultimately resulting in mature differentiated blood components. In the cancer stem cell hypothesis, HSCs can transfer into LSCs through a serious of genetic mutations. LSCs undergo symmetric division to generate more LSCs, or asymmetric division to generate highly proliferative leukemic progenitors. Chemotherapy is highly effective on rapidly proliferating progenitors, thus allowing LSCs to propagate at a later time to form a secondary tumour.

1.3.2 Clonal Evolution

The clonal evolution model of cancer attributes therapeutic resistance to a population of subclones that may contribute to original primary cancer, or arise post-therapy (Fig. 1.3)[65]. Whole genome sequencing and clonal evolution analysis of primary diagnosis-relapse AML pairs has revealed subclones within the primary tumour at diagnosis that remain post-AraC therapy to regenerate the relapsed disease[66,67]. In 2012, Ding *et al* described the appearance of new cancer subclones when comparing diagnosis versus relapse AML pairs[66]. Despite no specific pattern of mutagenesis, the generation of new therapeutically resistant subclones demonstrate the potential of AraC therapy to induce new somatic mutations that may lead to disease. The mutagenic effect of AraC and other cytoreductive reagents highlight the importance of targeted therapy in cancer and a field of research that needs to be further developed. Targeted therapy towards cancer clones with specific molecular genetic abnormalities has a decreased risk of genetic evolution as these therapies interfere with target signaling as opposed to DNA replication.

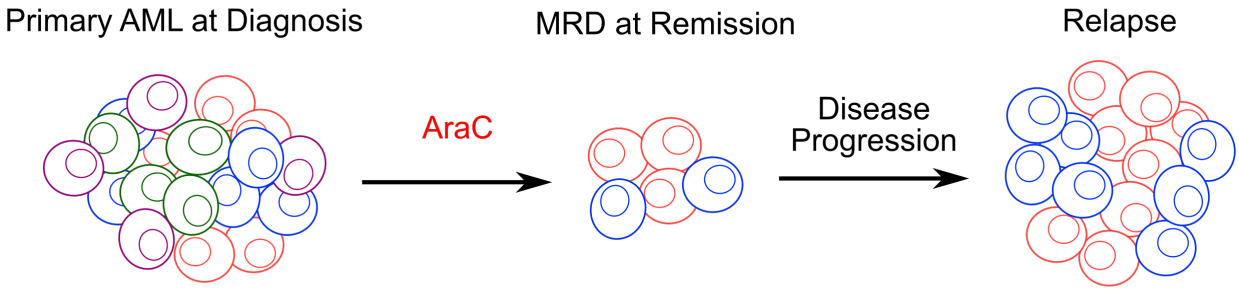


Figure 1.3: Clonal evolution model of cancer relapse[65]: The clonal evolution model posits cancer to be composed of several genetic subclones with different sensitivity to cancer therapeutics. Following chemotherapy, a small population of therapy-resistant subclones remain and propagate to form the secondary cancer.

1.3.3 Altered AraC Metabolism

Differential metabolism of AraC has been studied as a cause of therapeutic resistance and differential cancer response in AML. AraC is rapidly converted to cytosine arabinoside triphosphate (ara-CTP) upon entry into the cell and the active metabolite is able to disrupt DNA synthesis and induce cell death in rapidly dividing cells[68]. In 2006, Yin *et al* were able to generate an AraC resistant cell model of AML by selecting clonal variants that were able to tolerate doses of AraC that were 800 times higher than non-resistant variants[69]. Deoxycytidine kinase (dCK) was identified to be critical in the development of AraC resistance in this model[70]. dCK is an important enzyme that catalyzes the phosphorylation of deoxycytidine in order to generate nucleosides for DNA synthesis[71]. Decrease in dCK levels can be attributed to increased AraC resistance due to decreased levels of ara-CTP available in the cytoplasm[72]. Recently, increased cellular levels of SAM domain and HD domain-containing protein 1 (SAMHD1) has also been shown to be associated with poor AraC response[73,74]. SAMHD1 cleaves and breaks down ara-CTP, and the studies go further to show inhibition of SAMHD1 via genetic knockdown is able to increase AraC sensitivity AML cell lines and transplanted primary AML xenografts[73]. Although SAMHD1 has been shown to be an appropriate biomarker for identifying AraC resistance populations, genetic manipulation is not feasible to achieve on a clinical level, and there are currently no known inhibitors available.

1.4 DRUG DISCOVERY IN AML

The identification of risk-associated cytogenetic mutations have driven the development of novel therapeutics targeting these mutations; however, compounds derived against targets of common AML mutations have not led to significant changes to clinical management[75]. Despite scientific evidence for both clonal evolution and the cancer stem cell models of therapeutic resistance, these models still have not let to significant changes in the clinical management or work-up of newly diagnosed AML. In this section, we discuss some limitations in modelling therapeutic resistance and current clinical advancements in AML therapy.

In vitro and *in vivo* drug screening plays a large role in the discovery of new cancer therapeutics for AML. In 2017, Baccelli *et al* identified a new high-throughput screening platform using primary AML cells have been shown to be successful in identification of novel potential therapies[76]. Moreover, Baccelli *et al* were able to study the synergistic or additive effects of combination therapy on primary AML[76]. It is of interest to study the effects of combination therapy, especially drugs that have synergistic effects, as it allows us to decrease the effect dose of both compounds to minimize the potential side-effects. However, the viability assay presented by Baccelli *et al* only captures global cell death and is not able to specifically identify if the affected cells come from the therapeutically resistant population of AML cells; nor the progenitor LSC cells that will be able to give rise to a secondary cancer. It is of interest to develop a robust, high-throughput assay that is able to capture the effect of drugs on the progenitor fraction.

There have been limited clinical advancements in the treatment of AML despite increased understanding of AML formation and relapse. This may be due to a lack of

appropriate models that accurately reflect clinical relapse. Current *in vivo* and *in vitro* models are able to accurate capture the heterogeneity of AML patients and disease-forming clones within individual engrafting primary AML samples. Rates of AML engraftment in orthotopic mouse xenograft models are not consistent between multiple patient samples, and are also variable between on the strain of mice used for the transplant[62]. Studies have reported up 60% of primary AML samples do not engraft in an orthotopic xenograft mouse model[77]. Due to the genetic heterogeneity of AML reported in patients, this is a large population of patients that we are not able to capture successfully in our model. The generation of pluripotent cells from primary AML is one potential solution to this problem. AML induced pluripotent stem cells (AML-iPSCs) will be able to appropriately capture the range of AML phenotypes and provide a tool to study the disease in all patient samples, regardless of engraftment potential. AML-iPSCs can also be used to study the progression and development of AML phenotypes. However, development of a protocol to general AML-iPSCs have proven to be difficult. Recent publications have reported success in AML-iPSC generation in only mixed-lineage leukemia (MLL)[78,79]. The challenges in achieving programming of all AML subtypes is a roadblock that must be overcome in order to generate an appropriate disease model that can be easily utilized in the laboratory and captures the range of AML phenotypes observed in clinic.

1.5 STUDY RATIONALE

In order to study AML relapse and determine the underlying molecular pathways leading to relapse, previous work from the Bhatia lab utilized the orthotopic xenograph mouse model as a platform to study the progression of AML and AML relapse following cytoreductive therapy with AraC in primary human samples. This model is able to capture the genetic heterogeneity of AML patients, and offers a method to detect low levels of neoplasmic cells that may remain post-therapy via flow cytometry by separating human leukemia cells from their mice hosts using surface markers[60]. Through this model, we have isolated and characterize the transcriptional profile of a unique set of leukemic residue cell (LRC) population that were derived post-AraC therapy in several independent AML patient samples. Globe transcriptome profiling of samples via Affymetrix analysis has revealed 248 up-regulated genes across LRC population. Up-regulation of these genes were not observed prior to AraC exposure, and after AML reoccurrence has been established. This indicates that the LRC signature represents a transient window of opportunity for drug targets to prevent AML relapse. Moreover, gene set enrichment analysis revealed differences between naïve untreated LSCs and LRCs thus further characterizing LRCs as a population of cells that are unique from previously characterized LSCs. 17 out of 248 up-regulated genes have been determined as druggable gene candidates through the Drug Gene Interaction database (DGIdb) (Fig. 1.4) Current work from the Bhatia lab has identified DRD2 inhibitor thioridazine as a LRC-targeting drug. *In vivo* treatment with thioridazine has been shown to reduce leukemic growth rates, as well as progenitor frequency when used in combination with cytoreductive AraC treatment.

This study demonstrates the potential of using the LRC signature to identify novel drug targets as therapies to prevent AML relapse.

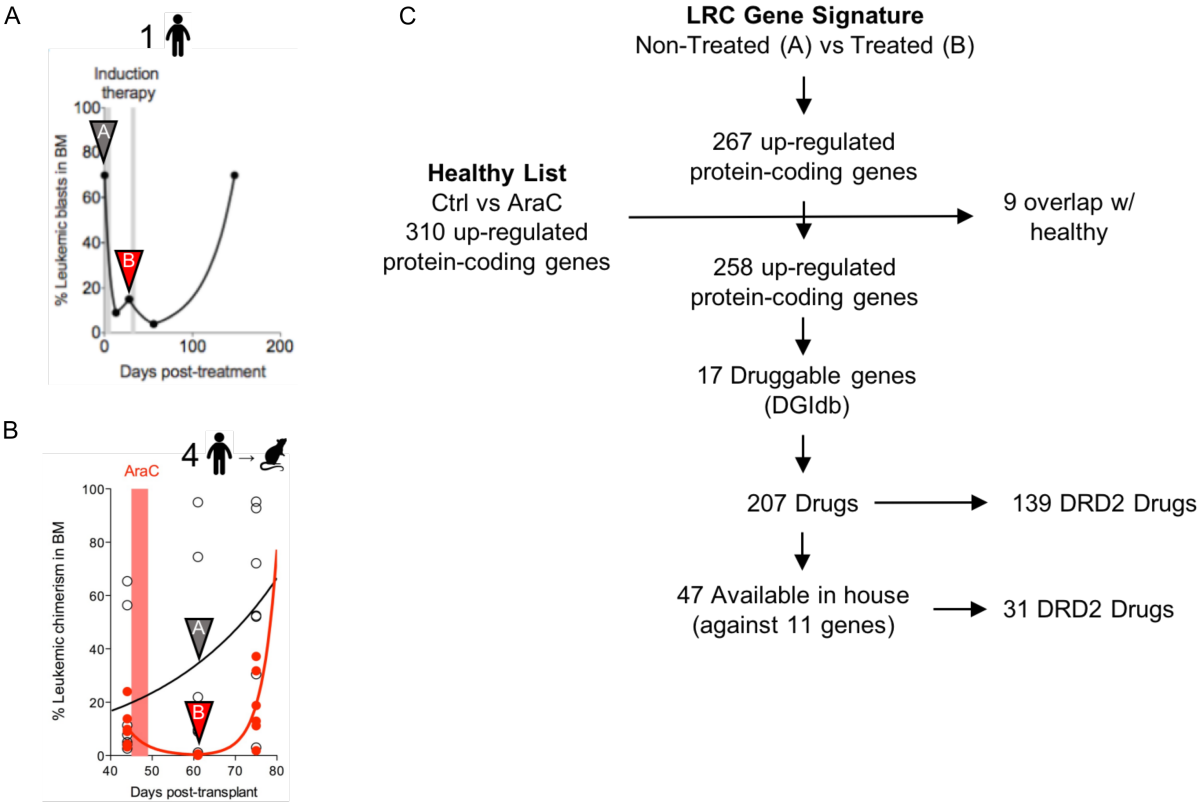


Figure 1.4: Schematic for derivation of LRC-targeting compounds. Affymatrix analysis was performed on mRNA isolated from one patient diagnosis-refractory disease pair (A), and 4 mouse saline-AraC treated sample pairs (B). Healthy saline-AraC transplant pairs were utilized as a control. (C) Analysis revealed 267 up-regulated protein-coding genes, 9 of which overlapped with healthy samples. Out of the remaining 258 genes, 17 were identified as druggable targets with 207 known targeting-compounds using the Drug Genome Interactional database (DGIdb). 47 drugs were identified as available within the SCCRI drug database.

The *in vivo* xenograpt model was successful in identifying a specific target population and gene signature associated with AML relapse. However, due to the rarity of the LRC population, a large number of primary AML cells are required for transplantation in order to achieve the high-throughput screening necessary to efficiently screen all drug candidates. It is also technically challenging, and time-consuming to

transplant a large number of mice to screen potential drugs *in vivo*. Thus is it critical to develop an *in vitro* assay that accurately reflects the molecular signature and functional proteins expressed by the LRCs *in vivo* in order to accurately narrow the number of potential LRC-targeting drugs for *in vivo* testing (Fig. 1.5). We will also screen LRC-targeting compounds against primary AML samples isolated at diagnosis in order to determine any potential effect on non-relapse and refractory AML.

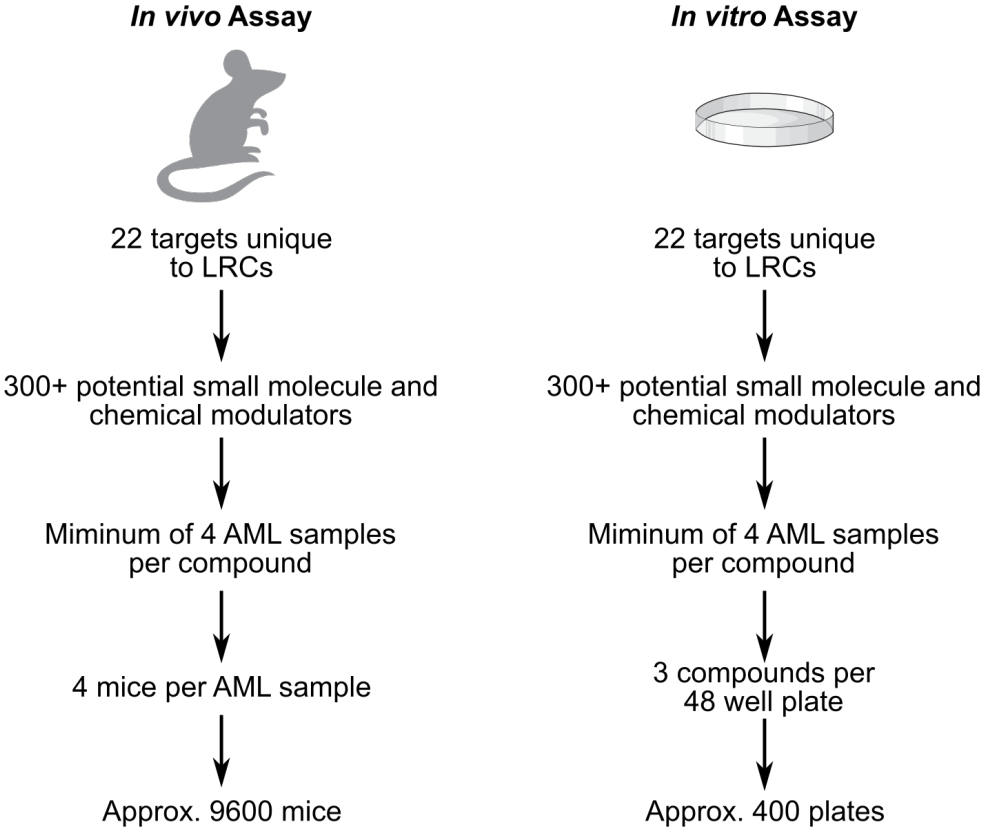


Figure 1.5: Efficacy of *in vivo* versus *in vitro* drug screening. In order to screen 22 unique LRC targets with 300+ potential small molecules and chemical modulators at a minimum of 4 primary AML samples in an *in vivo* model, we would require approximately 9600 mice transplantations. We can achieve higher throughput in an *in vitro* model, where 3 compounds can be screened per 48-well plate, leading to approximately 400 plates.

1.6 STUDY HYPOTHESIS

In vitro treatment with AraC will not be sufficient to recapitulate the LRC signature and remodel AML relapse. Co-culture with mouse MS-5 stromal cells will not be able to capture the multitude of cellular interactions and chemical signalling within the bone marrow microenvironment that may be responsible for the up-regulation of genes identified in the LRC signature.

1.7 STUDY OBJECTIVES

This thesis aims to:

1. *Identify candidate primary AML samples for in vitro relapse model and drug screening assays*

   All experiments proposed in this study will be performed on primary AML samples obtained from consenting patients via peripheral blood apheresis or bone marrow aspiration. 21 primary AML samples were selected and screened for progenitor frequency via 12-well manual colony forming unit (CFU) assay. Samples were selected for screening based on available cell numbers, engraftment information (samples that engraft mice were prioritized), and blast percentage.

2. *To develop an in vitro treatment paradigm that accurately reflects the in vivo AML relapse model*

   Primary AML samples identified in Aim 1 will be treated with 5 doses of low and high concentration AraC. Primary AML cells will also be co-cultured with mouse MS-5 stromal cells. Several outcomes will be measured at each time-point. CFU capacity will be used

as a measure of progenitor frequency and disease regeneration. Flow cytometry will be used to study surface marker expression of key LRC proteins and hematological markers (DRD2, GLUT2, FASL, FUT3, CD34, CD33, CD14, CD45). RT-PCR will be used to assess gene expression of LRC genes.

3. *To determine the effect of candidate LRC targeting compounds on progenitor frequency in diagnostic primary AML samples.*

The first priority of drug screens will be the GPCR targeting compounds. From the list of gene targets, 5 G-protein coupled receptors (GPCRs) associated with AML relapse were selected as prioritized targets for this project. GPCRs are cell surface receptors that play a large variety of roles in regulating downstream intracellular signaling and vital cellular processes thus making them ideal targets for novel drug therapy. A drug response curve with a minimum of 12 concentrations will be performed via an automated high-throughput CFU assay for each LRC targeting compound. A minimum of 2 AML will be screened for each drug. If a drug was effective against AML as measured by the CFU assay, further screening on healthy cord blood (CB) and mobilized peripheral blood (MPB) will be completed.

2.0 MATERIALS AND METHODS

2.1 Primary AML Sample Preparation:

Primary AML samples where obtained from patients via peripheral blood (PB) apheresis or bone marrow (BM) aspiration. All samples were obtained from consenting donors. All protocols have been approved by the Research Ethics Board at McMaster University as well as the London Health Sciences Center at the University of Western Ontario. Umbilical cord blood (CB), and mobilized peripheral blood (MPB) samples from non-disease donors were used as healthy controls. Primary AML samples were cultured in StemSpan™ (Stemcell Technologies) medium supplemented with 100 ng/mL stem cell factor (SCF), 100 ng/mL Fms-related tyrosine kinase 3 ligand (FLT3L), and 20 ng/mL thrombopoietin (TPO).

2.2 Mouse MS-5 Co-Culture:

Mouse MS-5 cells were cultured on 0.1% gelatin (Sigma-Aldrich) coated tissue culture plates in alpha-minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 2 mM sodium pyruvate. MS-5 cells were irradiated at 120 kVp for 4 minutes 47 seconds to inhibit cell growth for co-culture experiments. iMS-5 cells were cultured for 24h prior to co-culture with primary AML cells in StemSpan™ medium supplemented with 100 ng/mL SCF, 100 ng/mL FLT3L, and 20 ng/mL TPO. Co-cultures of primary AML and MS-5 cells were maintained for 24h prior to drug treatment with 0.15 μM AraC, 1 μM AraC, or DMSO control over 5 days. Cells were dissociated into a single cell suspension with TrypLE Express (Invitrogen) prior to flow cytometry analysis.

2.3 Drug Preparation:

LCR-targeting drugs were dissolved to 10 mM in DSMO and stored at -30°C. Drug daughter plates were prepared via serial dilutions to a final concentration of 0.035 – 10 μM in StemSpan™ medium. All drug daughter plates were prepared at 2-times concentration for storage at -30°C to avoid multiple freeze-thaws. The maximum concentration of DMSO in drug treatments was limited to below 0.4% as previous data from the lab has shown higher concentrations of DMSO to be detrimental to colony formation.

2.4 Manual and Automated CFU Assay:

Primary AML samples (1000 – 12000 cells) were suspended in a semi-solid methocellulose medium (MethoCult™ Classic, Stemcell Technologies) using the Microlab NIMBUS liquid handler (Hamilton Robotics) into 48-well non-tissue culture plates. Plates were incubated at 37°C, and 5% CO_2 for 7 – 14 days. CFU assays were stained with 50 nM of calcein green (ThermoFisher) in Hank's Balanced Salt Solution (HBSS) for 60 minutes prior to imaging at 2x focus with the Operetta High Content Screening platform (Perkin Elmer). Colony numbers were quantified via automated analysis using the Acapella Image Analysis system (Perkin Elmer). Colonies are classified based on size, and analysis of red auto-fluorescence and calcein green.

2.5 Flow Cytometry Analysis:

To assess changes in surface marker expression of hematopoietic markers and LRC-targets after AraC exposure, primary AML samples were treated with 5-doses of 0.15 μM AraC, 1 μM AraC, or DMSO control. Cells were washed with 3% phosphate saline buffer/FBS/ethylenediaminetetraacetic acid (EDTA) solution (PEF) and incubated with conjugated antibodies for 15 minutes at room temperature. A list of all antibodies used and their dilution are summarized in Table 2.1. Cells were washed with 3% PEF to reduce unspecific binding prior to staining with 7-AAD viability dye (Beckman Coulter). UltraComp eBeads (Affymetrix eBioscience) were used for compensation analysis. Assessment of surface marker expression via flow cytometry was performed using the BD LSRII Flow Cytometer with BD FACS Diva software. Flow cytometry analysis was performed with the FlowJo 10.2 (FlowJo, LLC). For viability analysis, primary AML cells were treated with 5-doses of 0.15 μM AraC, 1 μM AraC, or DMSO control in StemSpan™ medium supplemented with 100 ng/mL SCF, 100 ng/mL FLT3L, and 20 ng/mL TPO. Cells were washed with 3% PEF to reduce unspecific binding prior to staining with 7-AAD viability dye (Beckman Coulter). Assessment of cell viability and number via flow cytometry was performed using the MACSQuant® Analyzer system (Miltenyi Biotec).

2.5.1 DRD2 and FUT3 Analysis:

In order to assess changes in surface expression of LRC-targeting proteins DRD2 and FUT3, cells were washed with 3% PEF following AraC exposure. Cells were incubated for 15 minutes in a blocking solution consisting of 3% PEF, 7% donkey serum (Jackson ImmunoResearch Laboratories), and Human Fc block (BD Biosciences) in 1:200 dilution. Cells were washed two-times with a solution of 3% PEF supplemented with 7% donkey serum prior to incubation for 45 minutes in primary DRD2 or FUT3 antibody. Primary antibody was washed 2x with washing solution prior to incubation in donkey anti-mouse alexa fluro-647 (ThermoFisher Scientific) for 30 minutes. Cells were incubated for 15 minutes with surface hematological markers as summarized in Table 2.1. Cells were washed with 3% PEF to reduce unspecific binding prior to staining with 7-AAD viability dye (Beckman Coulter).

Table 2.1: Summary of antibodies used in flow cytometry analysis

Antibody	Dilution	Source
CD34-APC	1:200	BD Pharmingen
CD34-PE	1:200	BD Pharmingen
CD34-FITC	1:100	Miltenyi Biotec
CD14-PE	1:100	BD Pharmingen
CD14-FITC	1:100	BD Pharmingen
CD45-V450	1:100	BD Pharmingen
CD45-BV605	1:300	BD Horizon
CD45-A647	1:700	Biolegend
CD33-V450	1:100	BD Horizon
CD33-PE	1:50	BD Pharmingen
FASL-FITC	1:100	ThermoFisher Scientific
GLUT2-A488	1:100	Novus Biologicals
DRD2	1:100	Santa Cruz Biotechnology
FUT3	1:100	Abcam
Donkey anti-Mouse A647	1:1000	ThermoFisher Scientific

2.6 Quantitative and Touchdown PCR Analysis:

Primary AML mRNA was collected after 5-doses of treatment with 0.15 μM AraC, 1 μM AraC, or DMSO control. Total mRNA was isolated in accordance to the Total RNA Purification Micro Kit (Norgen Biotek Corp.) and DNase I (Norgen Biotek Corp.) treated for 15 minutes to digest DNA contamination. mRNA concentration was quantified using the NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). cDNA was generated with 0.5 – 1 mg of total mRNA in accordance to the iScript Reverse Transcription Supermix protocol (Bio-Rad). Quantitative PCR was performed using PowerUp™SYBR™ Green Master Mix (ThermoFisher Scientific). 50 ng of cDNA was amplified per reaction. Primers utilized for PCR are summarized in Table 2.2. Touchdown PCR was performed for FASL, and DRD2 by decreasing the annealing temperature by 0.2 degrees per cycle. Touchdown PCR products were visualized on a 1.5% agarose gel.

Table 2.2: Primer sequences used for PCR analysis

Gene	Primers
CXCR1	F: CCTGGGAAATGACACAGCAA R: CCAAAGGTGTGAGGCAGGAT
GPR119	F: TCTCGGCCCACACAGAAGAC R: GCTGCGGAGGAAGTGACAA
DRD2 (qPCR)	F: CCCACTCCTCTTCGGACTCA R: CGGGTTGGCAATGATGCA
DRD2 (Touchdown)[80]	F: GGTCTACATCAAGATCTACATTGTCCTCC R: TGGCGAGCATCTGAGTGGCTTTCTTCTCC
BDKBR1	F: CCCACTCCTCTTCGGACTCA R: AGCAGGTCCCAGGCTTCTG
GLUT2	F: TGGAATTGACAGGACTCCCAAC R: ATTATTACCTGTTGAGGTGCATTGA
FASL	F: TTGGAGAAGCAAATAGGCCACC R: AGAGGCATGGACCTTGAGTTG
GAPDH (qPCR)	F: CCACATCGCTCAGACACCAT R: GCGCCCAATACGACCACCAAAT
GAPDH (Touchdown)	F: GAAATCCCATCACCAATCTTCCAGG R: GCAATTGAGCCCCAGCCTTCTC
HPRT1	F: AGGGTGTTTATTCCTCATGGACTAA R: TCCTTCATCACATCTCGAGCAA

2.7 In vivo Orthotopic Xenografts (Courtesy of L.A. and A.B.):

8 – 10 week immune-compromised NOD/SCID mice were irradiated and transplanted with 5 – 10 million primary AML cells. Engraftment took place between 3 – 5 weeks. Mice were treated with 50 mg/kg/day of AraC or vehicle control via subcutaneous injection for 5 days. Mice were sacrificed 9 days after the last dose of AraC. LRCs were isolated from the bone marrow and spine via fluorescent activated cell sorting for CD45 and CD34 positive populations. Isolated LRCs were cultured in StemSpan™ medium supplemented with 100 ng/mL of stem cell factor (SCF), 100 ng/mL of Fms-related tyrosine kinase 3 (Flt3) ligand, and 20 ng/mL of thrombopoietin (TPO) for the 24h compound incubation prior to plating in a semi-solid methocellulose medium (MethoCult™ Classic, Stemcell Technologies).

2.8 Statistical Analysis

All results are expressed mean ± SEM unless stated otherwise. All data was generated from a minimum of 2 independent primary AML samples. Data was managed using Excel 15.26 (Microsoft) and Prism 6 (GraphPad Software, Inc.). Statistical significance was derived using repeated measures two-way ANOVA with Bonferroni post-test for pair-wise comparisons. Drug response curves were fitted with the non-linear variable slope log(inhibitor) vs response equation. CFU and CD34 curves were fitted with the exponential growth equation. Results were considered significant when $p<0.05$*.

3.0 RESULTS

3.1 Identification of Primary AML Samples

In order to assess the effect of the LRC-targeting drugs on AML progenitor frequency, the compounds will be screened on primary AML samples obtained from consenting patients via peripheral blood apheresis or bone marrow aspiration. 21 primary AML samples were selected and screened for CFU progenitor frequency via 12-well manual CFU assay. Samples were selected for screening based on available cell numbers, engraftment info (samples that engraft mice were prioritized), and blast percentage. Samples were plated at cell densities of 50000, 25000, 5000, and 1000 per well. Total number of colonies were counted manually at 7 – 14 days. The primary samples were divided into three groups based on CFU progenitor frequency. Group A was defined as samples that formed >1 colony per 2500 cells plated (Fig. 3.1A). Representative wells for Group A samples are presented in Fig. 3.1B. Group B was defined as samples that formed >1 colony per 5000 cells plated (Fig. 3.1C). Group C was defined as samples that did not form colonies at the cell numbers plated. A summary of all screening results is presented in Table 3.1. A final list of 9 selected AML samples was compiled based on screening results as well as previous data available in the lab (Table 3.2). Factors that were taken into consideration for generation of the final list includes available cell numbers, engraftment potential, cytogenetics, and colony formation.

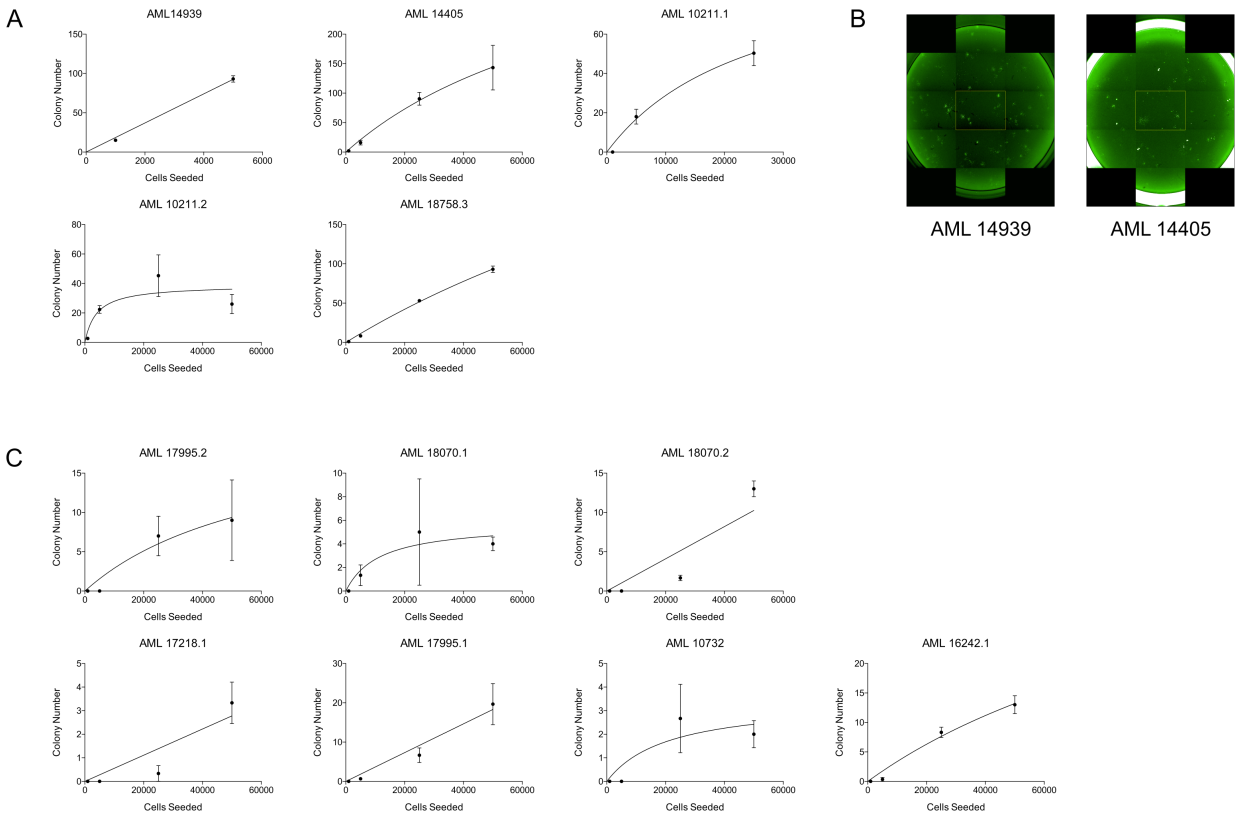


Figure 3.1: Progenitor frequency curves of select primary AML samples. (A) Progenitor frequency curves for primary AML samples that generated 1 or more colonies per 2500 cells plated. 50000, 25000, 5000, and 1000 cells were plated manually in 500 μL MethoCult™ medium and incubated for 7 – 14 days at 37°C prior to manual count. (B) Representative calcein-stained colonies for AML 14939 and AML 14405 plated at 5000 cells/well. (C) Progenitor frequency curves for primary AML samples that generated less than 1 colony per 5000 cells plated.

Table 3.1: Summary of primary AML CFU progenitor frequency screen

	Average Number of Colonies/Cells Plated			
Sample	50000	25000	5000	1000
14939	Overconfluent		89, 101, 89	17, 13
14405	Overconfluent		46, 57, 64	6, 6, 5
A382.1	No colonies			
11333.1	No colonies			
13074.1	1 colony @ 50 000			
17995.2	2, 19, 6	2, 9, 10	No colonies	
16381.2	No colonies			
10211.1	Overconfluent	45, 63, 43	12, 25, 17	No colonies
18070.1	4, 5, 3	1, 14, 0	1, 0, 3	No colonies
12489.2	No colonies			
12489.1	No colonies			
18070.2	12, 14	2, 2, 1	No colonies	
14096.1	1 - 2 colonies		No colonies	
17218.1	2, 5, 3	1	No colonies	
10211.2	13, 34, 31	21, 70, 45	18, 27, 22	3, 2, 3
17995.1	21, 28, 10	3, 9, 8	0, 1, 1	No colonies
17052.2	No colonies			
12503.1	1, 1, 0	0, 0, 2	No colonies	
18758.3	95, 99, 85	56, 52, 51	7, 9, 9	1, 1, 1
10732	2, 3, 1	5, 0, 3	No colonies	
16242.1	12, 16, 11	8, 10, 7	1, 0, 0	No colonies

Table 3.2: Final list of select AML samples utilized in project

Sample	Disease Stage	Source	Cytogenetics/ Molecular	Blast	CD34 %
A374.1	Diagnosis	PB	Complex	NA	80.3
A413	Diagnosis	PB	NA	NA	68.8
14939	Diagnosis	PB	NA	96%	0.20%
10211.1	Diagnosis	PB	NK; NPM1/FLT3-ITD	54%	0.70%
14000.1	Diagnosis	BM	NK/FLT3-ITD	67%	68.3
16406.1	Diagnosis	PB	NK	68%	7.2
10683	Diagnosis	PB	NK	34%	21.2
18070.2	Diagnosis	BM	NA	50%	45
14015	Diagnosis	BM	Abnormal	NA	71.88
18484.2	Diagnosis	PB	NPM1/FLT3-ITD	84%	0.7

3.2 Preliminary Screen of LRC-Targeting Drugs on Primary AML Samples

17 specific LRC signature genes coding for proteins associated with AML relapse has been prioritized for screening in diagnostic primary AML samples. These genes can be targeted by 10 small molecules that may be used as future novel AML therapies. These LRC-targeting drugs and their targets are summarized in Table 3.3. 10 LRC-targeting compounds were selected via *in silico* analysis of Drugbanks, DGIdb, International Union of Basic and Clinical Pharmacology (IUPHAR), Tocris, and Pubmed. Due to the unknown downstream translational and cellular pathways of up-regulated gene targets, both available agonists and antagonists were selected to be screened. A 12-point dose curve was performed on 2 – 3 AML samples to determine if the compound should move forward for further screening and biochemical work-up. Samples were treated for 24h in a liquid StemSpan™ medium with cytokines and seeded in a semi-solid methocellulose medium. A summary of screening results for all LRC-targeting drugs can be found in Fig. 3.2.

Table 3.3: Prioritized LRC-targeting compounds

Drug	Gene Target
Anamorelin	GHSR Agonist
Ibutamoren Mesylate	GHSR Agonist
Semagacestat	GHSR Antagonist
Reparixin	CXCR1 Antagonist
Suramin Sodium Salt	FSHR Antagonist
L-Triiodothyronine	BDKRB1 Antagonist
PSN 632408	GPR119 Agonist
MBX 2982	GPR119 Agonist
AR 231453	GPR119 Agonist
APD-597	GPR119 Agonist

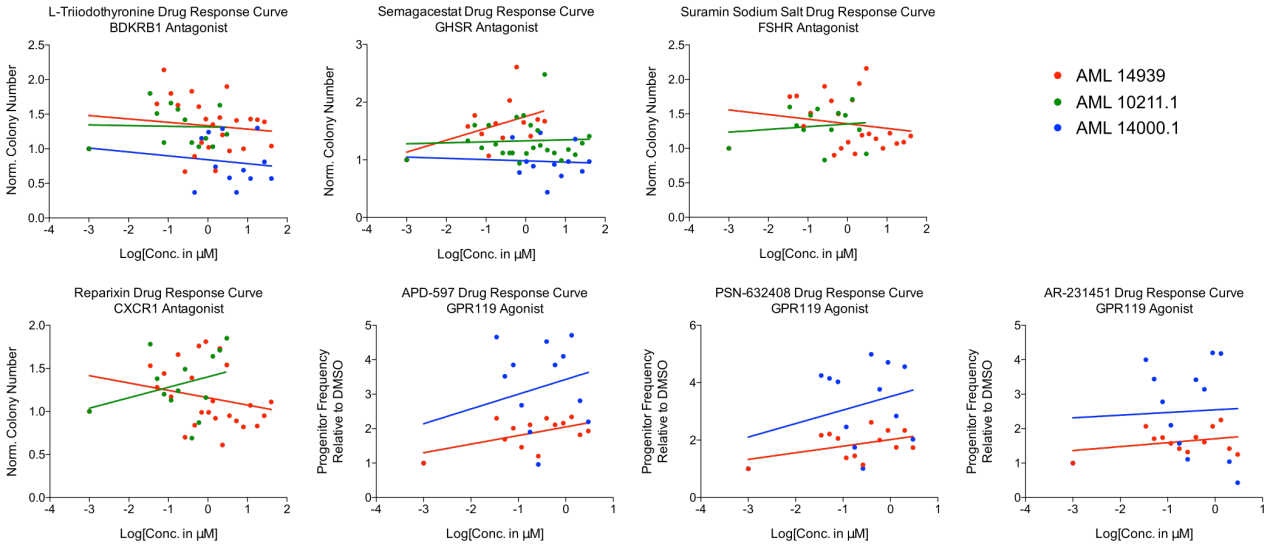


Figure 3.2: Summary of LRC-targeting drug screen in primary AML samples. Drug response curves of 7 LRC-targeting compounds screened on 2 or more primary AML samples. 25000 – 6000 primary AML cells were treated for 24h with 0.035 – 10 µM of selected drugs in StemSpan™ medium supplemented with cytokines prior to re-suspending in MethoCult™. Plates were incubated for 7 – 14 days prior to calcein imaging. All data are normalized over DMSO colony formation.

3.2.1 Anamorelin, a GHSR agonist, decreases progenitor frequency in a cell-dose dependent manner in primary AML samples.

Preliminary screening of LRC-targeting drugs on a minimum of 2 primary AML samples has identified Anamorelin as an active compound of interest. Anamorelin had an EC50 of 0.28 ± 0.079 μM in primary AML samples, and 0.42 ± 0.064 μM in healthy blood samples (Fig. 3.3A). To determine if cell number plays a role in the effectiveness of the drug, a 12-point dose curve was performed with one primary AML samples at 2 different cell doses. Anamorelin had a higher IC50 in higher cell doses, indicating the effects of Anamorelin were cell-number dependent (Fig. 3.3B). In comparison to current AML standard of therapy AraC, Anamorelin has a lower IC50 in AML cells, indicating a lower dose of the drug is able to have the same effect on progenitor frequency *in vitro* (Fig. 3.3C).

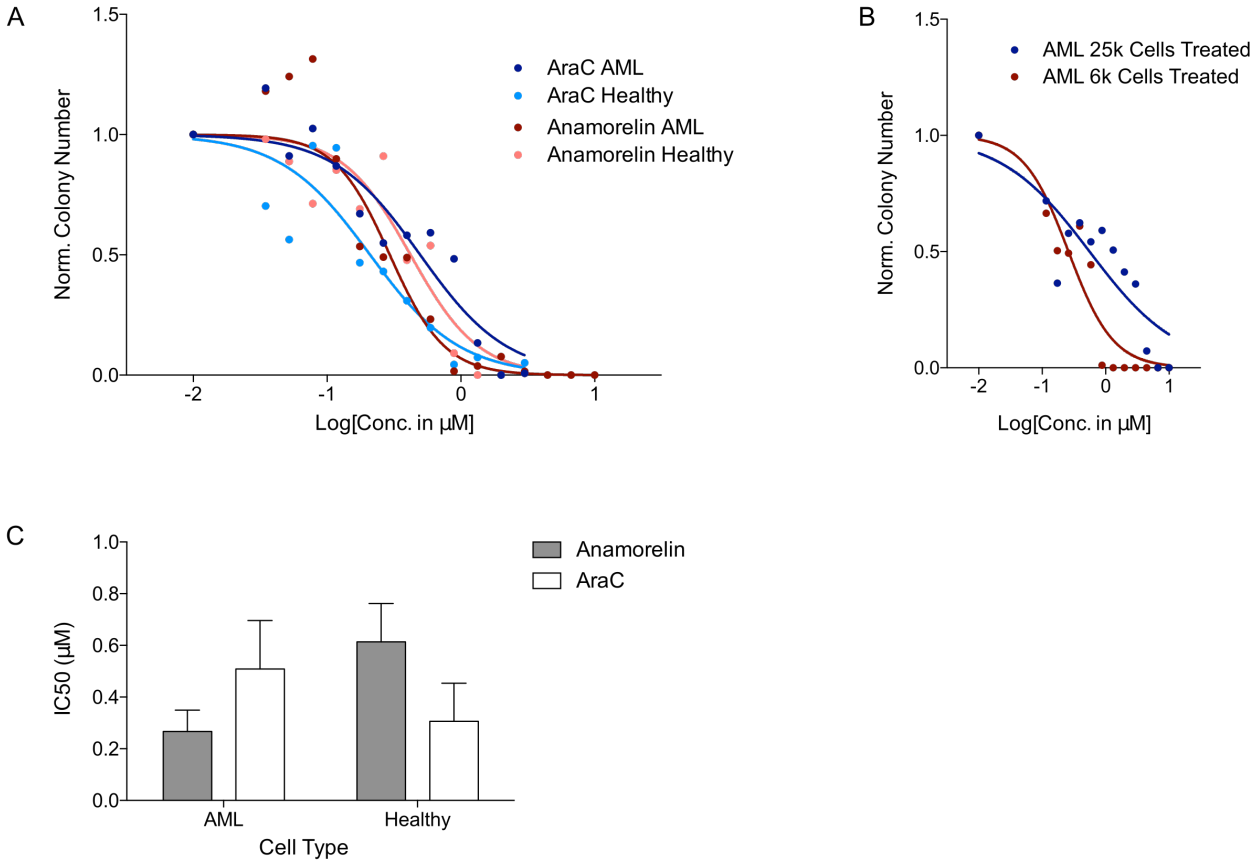


Figure 3.3: Anamorelin decreases progenitor frequency in primary AML samples in a cell-number dependent manner. (A) Dose response curve of Anamorelin versus AraC on primary AML samples and healthy CB or MPB control (n = 3 – 5 independent samples). 6000 – 25000 cells were treated with 0.035 – 10 µM of Anamorelin for 24h in StemSpan™ medium followed by plating in MethoCult™ for 7 – 14 days. (B) Cell-dose dependent drug response curve of Anamorelin on one primary AML. (C) Bar graph representing differences in IC50 between Anamorelin and AraC in healthy and primary AML. There are no significant differences in response to Anamorelin ($p = 0.3493$) or Arac ($p = 0.7643$) between AML and healthy control. There are no significant differences within AML ($p = 0.5666$) or healthy ($p = 0.4832$) groups when comparing the effects of Anamorelin and AraC. Data is presented as mean ± SEM. Two-way ANOVA with Bonferroni post-test was performed for statistical analysis.

3.3 Development of an in vitro AML Relapse Model

One potential reason for the lack of response to LRC-targeting drugs in primary AML cells may be due to the functional and molecular differences between diagnostic AML samples and LRC isolated *in vivo*. The remainder of this results section examine several methods utilized to recapitulate the LRC signature in an *in vitro* model.

3.3.1 In vitro AraC-exposed primary AML are not able to recapitulate the progenitor frequency curves and CD34 surface expression observed after AraC exposure in vivo.

In order to model AML relapse and to recapture the LRC signature *in vitro*, primary AML samples were treated with 5-doses of 0.15 μM AraC, 1 μM AraC, or DMSO control in a liquid StemSpan™ medium supplemented with cytokines (Fig. 3.4A). Number and concentration of AraC doses were selected to match *in vivo* protocols. Colony numbers, cell number, and CD34 surface expression was measured at each time-point. 3125 - 50000 cells were plated for CFU assays based on initial cell numbers plated and the progenitor frequency of selected AML sample. Primary cells were maintained in liquid culture for 8 days total for progenitor assay studies, and up to 19 days for flow cytometry analysis. 0.15 μM and 1 μM of AraC was able to significantly decrease the progenitor frequency (Fig. 3.4B) and cell numbers (Fig. 3.4C) of primary AML cells at every time point. Number of colonies also significantly decreased with time in the DMSO and 0.15 μM AraC group. There were no significant differences in the CD34 expression curve between DMSO, 0.15 μM and 1 μM of AraC (Fig. 3.4D). CD34 expression levels significantly decreased over time in all three groups. Overall, these results demonstrate that *in vitro* treatment with AraC is not able to capture the regeneration of AML progenitors and increase of CD34 expression observed *in vivo*.

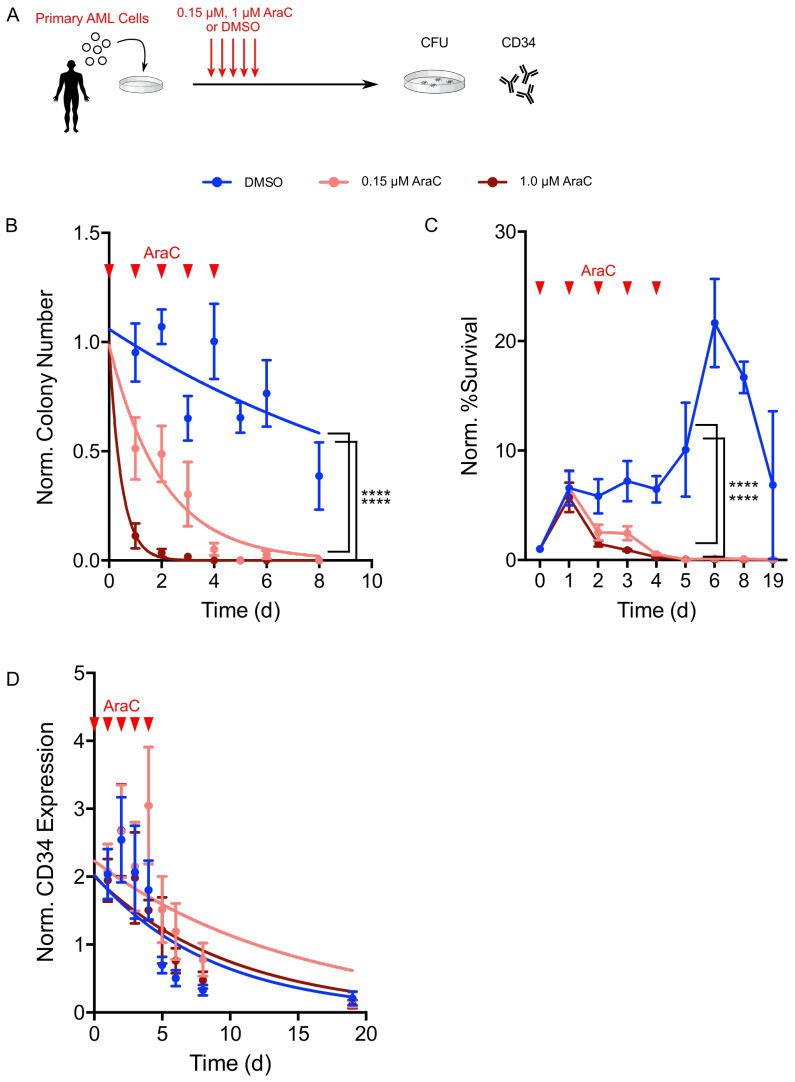


Figure 3.4: *In vitro* 5-day treatment with AraC at two different doses for 8 days does not recapture the progenitor frequency curve or CD34 expression curve observed *in vivo*. (A) Experimental schematic of 5-day AraC treatment. (B) Progenitor frequency curves of primary AML samples treated with DMSO, 0.15 μM and 1 μM of AraC (n = 3 independent samples, 3 technical replicates per sample per timepoint). Differences in progenitor frequency between DMSO, 0.15 μM and 1 μM of AraC groups are statistically significant ($p<0.0001$). Differences in progenitor frequency over time within each treatment group are statistically significant ($p<0.0001$) (C) Cell count curves after treatment with 5 doses of AraC (n = 2 independent samples, 4 technical replicates per sample per timepoint). Differences in cell counts between treatment groups are statistically significant ($p<0.0001$) (D) CD34 expression curve after treatment with 5 doses of AraC (n = 3 independent samples, 2 technical replicates per sample per timepoint). There is no significant difference between DMSO, 0.15 μM and 1 μM AraC groups. CD34 expression significantly decrease over time in all groups ($p<0.0001$) Data is presented as mean ± SEM. Two-way ANOVA with Bonferroni post-test was performed for statistical analysis.

3.3.2 In vitro exposure to AraC does not regulate the surface expression of selected LRC-signature proteins

Due to the differences in progenitor frequency curves between the *in vivo* and *in vitro* AraC treated samples, we were interested in the expression of LRC-signature proteins in *in vitro* AraC-exposed primary AML samples. Primary AML cells were treated *in vitro* with 5 doses of 0.15 μM AraC, 1 μM AraC, or DMSO control in StemSpan™ medium supplemented with cytokines and the expression of 4 LRC targets (DRD2, FUT3, FASL, and GLUT2) was measured prior to culture and at days 1, 2, 3, 4, 5, 6, 8, 12, 16, and 19 (Fig. 3.5A). Cells were fed with StemSpan™ supplemented with cytokines every 48h due to the half-life of TPO, SCF, and FLT3L. We observed differences in regulation of LRC-targeting compounds with AraC. DRD2 and FASL expression did not change with AraC treatment (Fig 3.5B). There was a trend of decreasing GLUT2 expression, and increasing FUT3 expression with AraC (not significant). There were no differences in the expression of these LRC-targeting protein in the CD34+ fraction (Fig. 3.5C).

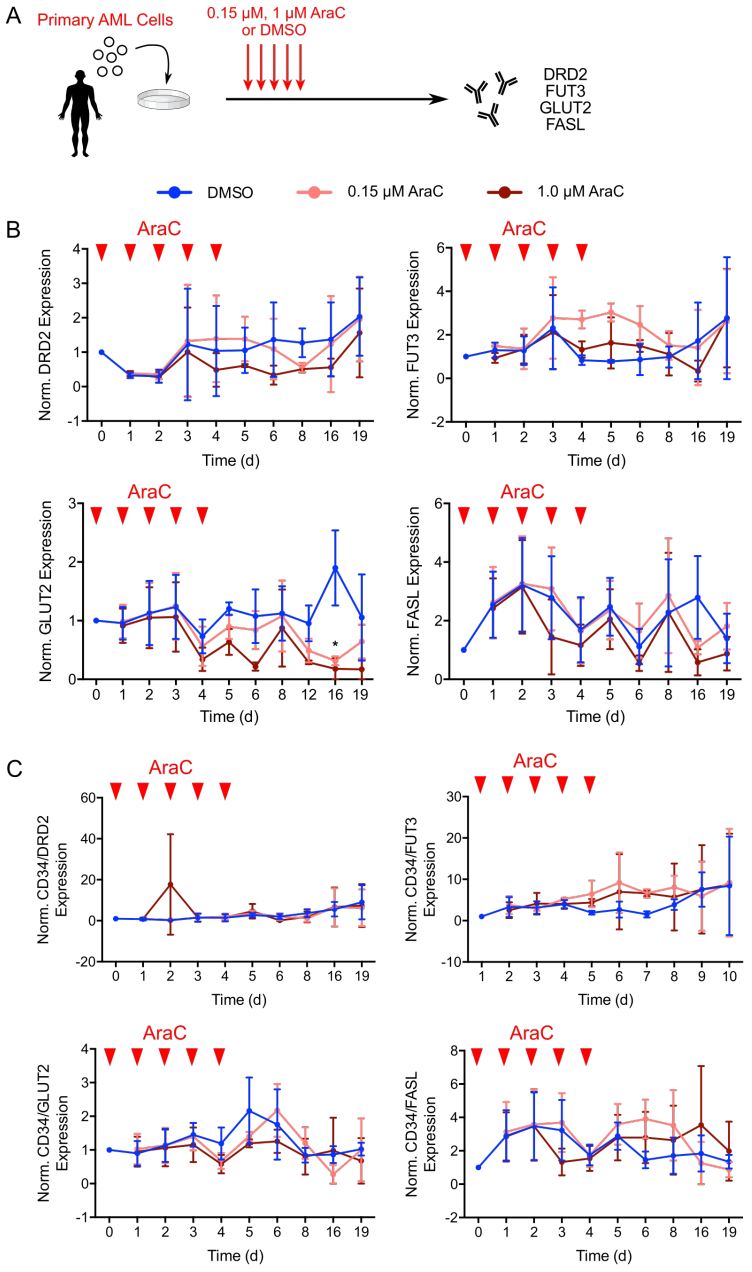


Figure 3.5: *In vitro* AraC treatment has differential effects on expression of LRC-targeting proteins. (A) Experimental schematic of 5-day AraC treatment. Expression of DRD2, FUT3, GLUT2, and FASL over time in total live populations (B) and CD34+ populations (C). Each graph represents 2 – 3 independent AML samples. Changes in DRD2 expression was not significant between treatments (p=0.178) or over time (p=0.2117). Changes in FUT3 expression was not significant between treatment groups (p=0.0781) or over time (p=0.4013). Changes in GLUT2 expression was not significant between treatment groups (p=0.2302), but significantly decreased over time (p=0.0319). Changes in FASL expression was not significant between treatment groups (p=0.8825) and not significant over time (p=0.0581). Data is presented as mean ± SEM. Two-way ANOVA with Bonferroni post-test was performed for statistical analysis.

3.3.3 In vitro exposure to AraC does not increase gene expression of select LRC-signature genes in primary AML samples.

In addition to surface protein expression, we were interested in studying the effects of AraC exposure on mRNA regulation of select LRC-signature genes. Primary AML cells were treated *in vitro* with 5-doses of 0.15 μM AraC, 1 μM AraC, or DMSO control in StemSpan™ medium supplemented with cytokines and mRNA was isolated after 24h. Gene expression of GPR119, BDKBR1, CXCR1, and DRD2 were evaluated via qPCR post-AraC exposure. We observed an increase in mRNA levels of these genes with 0.15 μM of AraC treatment (Fig. 3.6A). However, long term mRNA expression of these genes as well as other genes of interest (GLUT2 and FASL) were not able to be studied in primary AML via qPCR. Touch-down PCR was utilized as an alternative for more specific amplification of these genes. Universal mRNA (UNI) was utilized as a positive control for primer testing in qPCR and touch-down PCR experiments. Touch-down PCR amplification of selected genes was not achieved for DRD2 and GLUT2, and was inconsistent for FASL (Fig. 3.6B). Potential causes to lack of PCR amplification will be addressed in the Discussions section (Chapter 4).

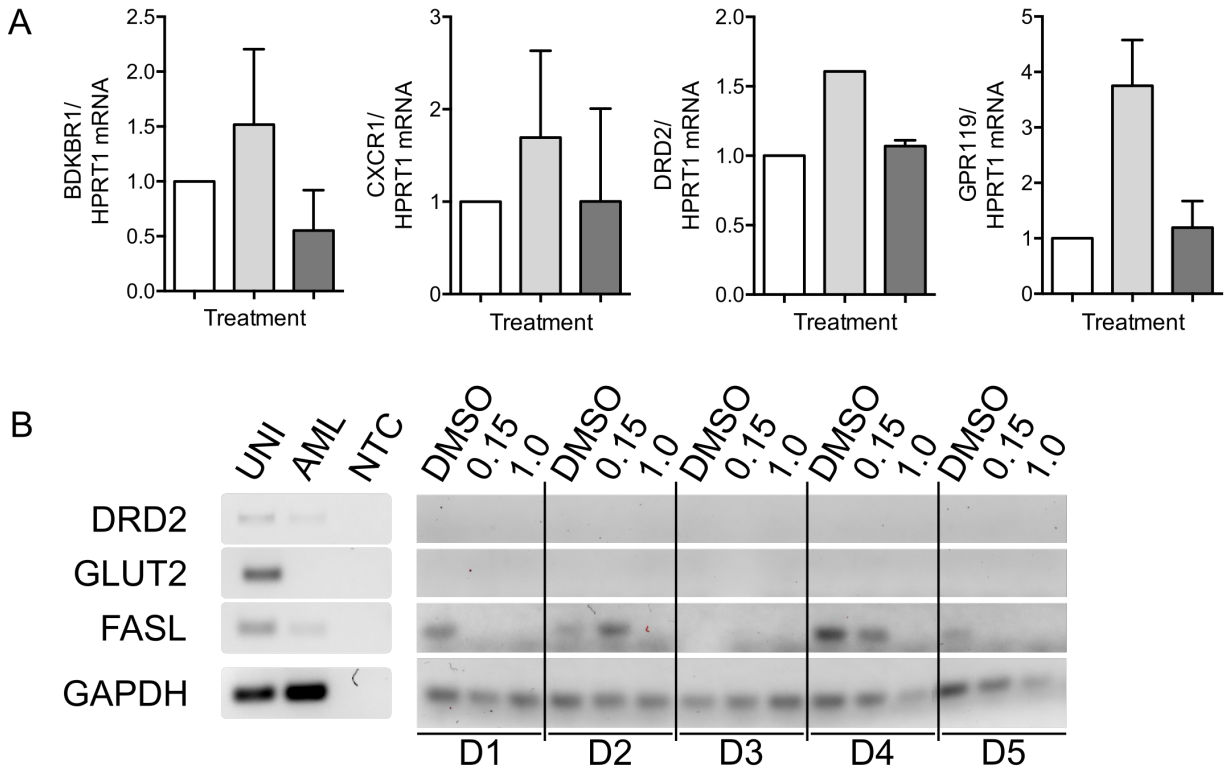


Figure 3.6: mRNA expression of select LRC-genes after *in vitro* AraC exposure. (A) qPCR analysis of BDKRB1, CXCR1, DRD2, and GPR119 after 24h exposure to AraC (n = 2 independent samples). (B) Representative agarose blot of touch-down PCR amplification of DRD2, GLUT2, and FASL. cDNA was generated using 500 ng of RNA and 50 ng/well of cDNA was amplified for PCR reactions.

3.3.4 Co-culture of primary AML samples with mouse MS-5 stromal cell-line

One challenge in recapturing AML relapse and the LRC signature *in vitro* is the lack of bone marrow niche cells that could potentially play an important role in the development of the LRC signature. In order to replicate the bone marrow niche *in vitro*, we co-cultured primary AML samples with irradiated mouse stromal MS-5 cells. 250000 iMS-5 cells were plated in 6-well tissue culture plates coated with 0.1% gelatin for 24h in alpha-MEM medium. After 24h, primary AML cells were co-cultured with iMS-5 cells in StemSpan™ medium supplemented with cytokines for 24h. The cells were subsequently treated with 0.15 μM AraC, 1 μM AraC, or DMSO control. Culture were maintained for 7 days, after which iMS-5 cells began to lift from the gelatin base. Flow cytometry analysis of DRD2 and FUT3 was performed after 24h of co-culture and at day 1, 2, 3, 4, 5, and 8 after AraC treatment (Fig. 3.7A). Co-culture with iMS-5 cells was able to maintain the viability of primary AML cells in comparison to no co-culture conditions (Fig. 3.7B). Co-culture was also able to maintain the level of CD34 at day 9 in comparison to no co-culture conditions (Fig. 3.8C). Co-culture of 2 different primary AML samples showed differential response to AraC treatment. Co-culture of AML 14939 with iMS-5 cells showed decreased sensitivity to AraC, and an increase in CD34 expression (Fig. 3.8B). In the A374 cells, co-culture with iMS-5 cells also decreased sensitivity to AraC but decreased in CD34 expression in comparison to no co-culture conditions (Fig. 3.8A). For LRC target expression, co-culture of AML 14939 with iMS-5 cells showed an increase in DRD2 and FUT3 expression in the total population (Fig. 3.9B) and CD34+ cells (Fig. 3.9D) over time with AraC treatment. However, in AML A374, co-culture with iMS-5s had no effect on DRD2, and FUT3 expression in the total population or CD34+ population (Fig. 3.9A, C).

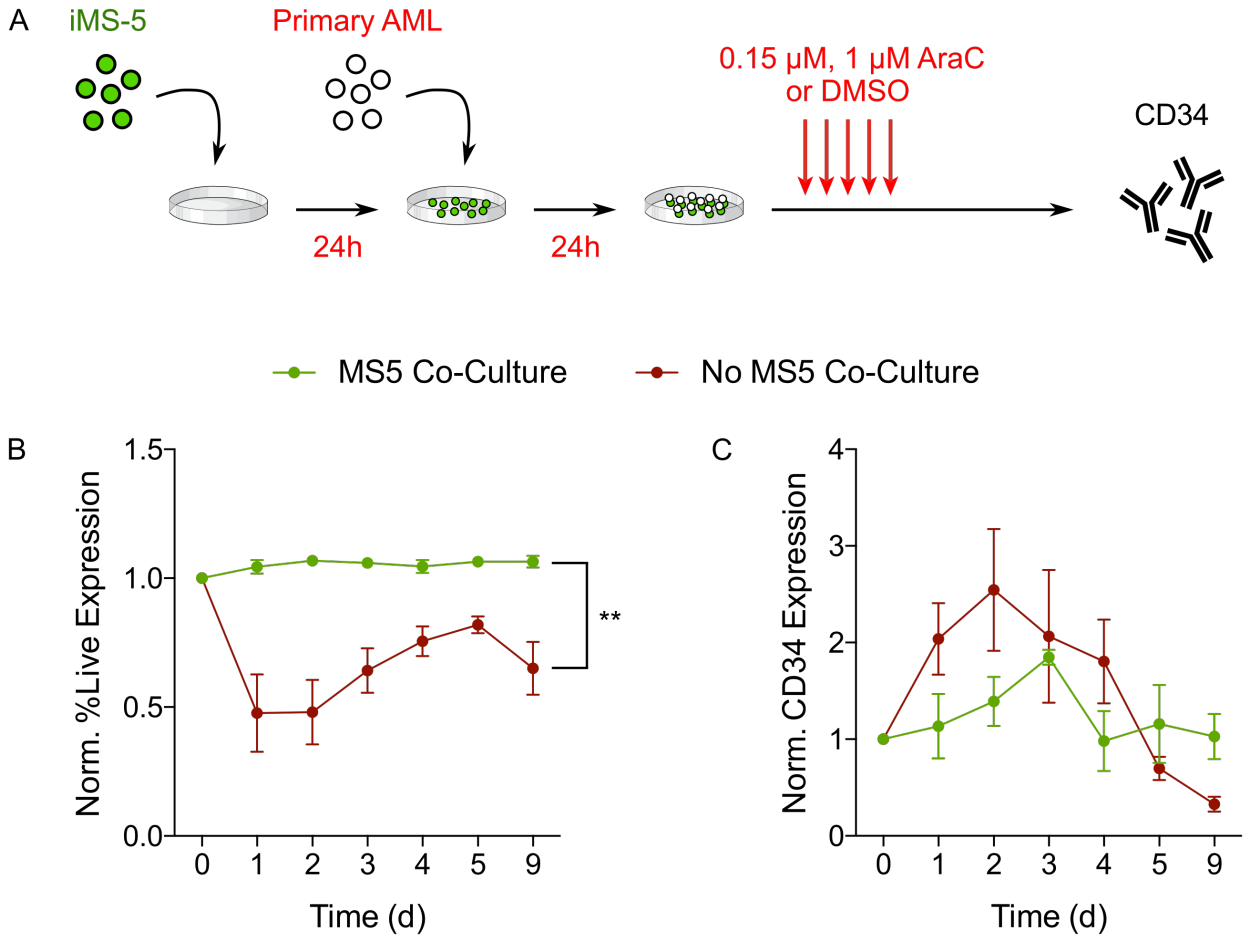


Figure 3.7: Co-culture of primary AML with iMS-5 cells: (A) Experimental schematic of co-culture experiment. Cell viability (B) and CD34 (C) analysis of primary AML cells with and without iMS-5 co-culture (n = 2 independent samples, 2 technical replicates per sample per timepoint). Co-culture significantly increased the viability of primary AML cells in comparison to no co-culture conditions ($p<0.01$).

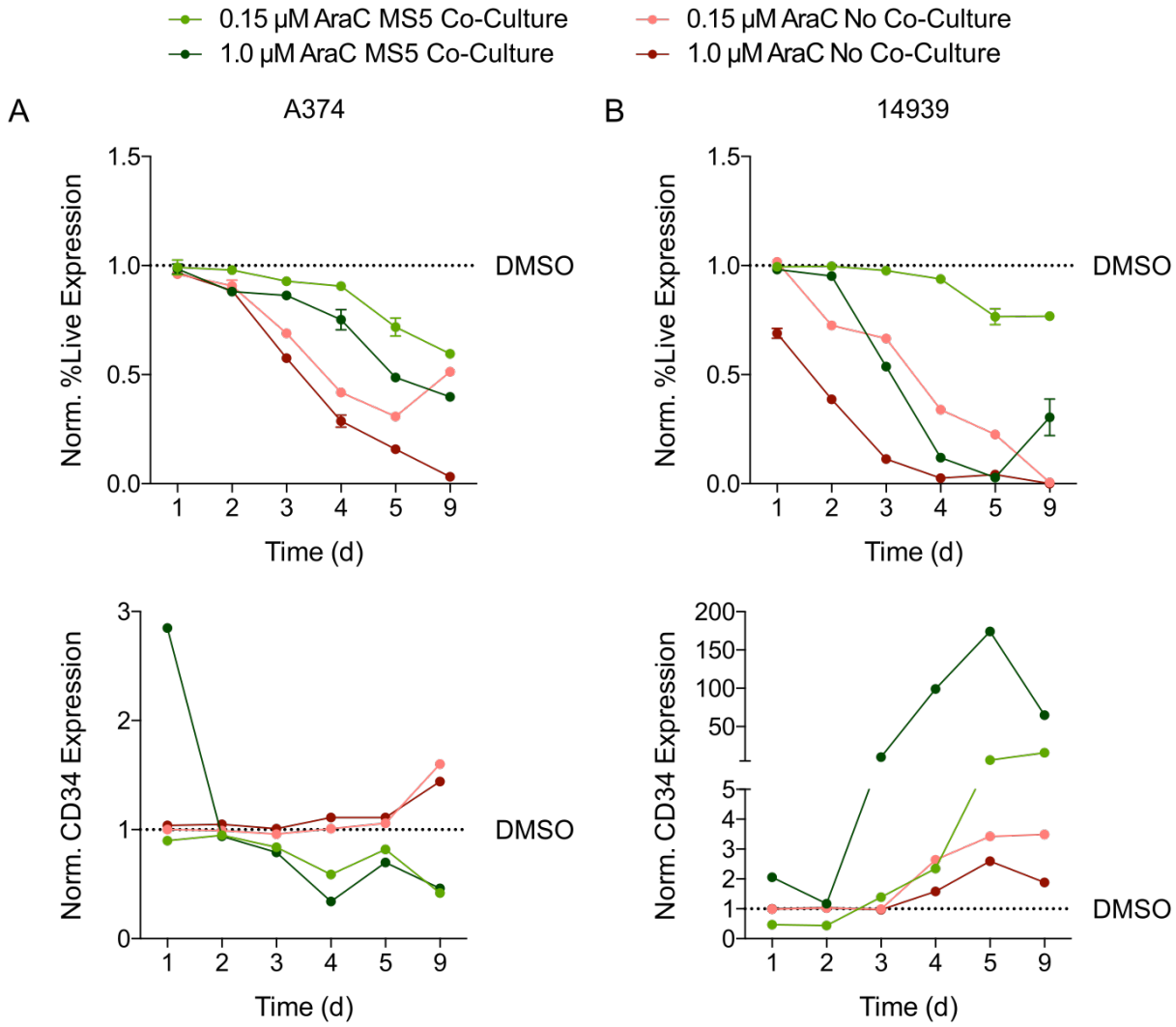


Figure 3.8: Effect of co-culture on CD34 and viability in response to AraC exposure in primary AML. Flow cytometry analysis of cell viability and CD34, after AraC treatment in co-cultured conditions of AML A374 (A), and AML 14939 (B). Statistical analysis could not be performed on one AML replicate.

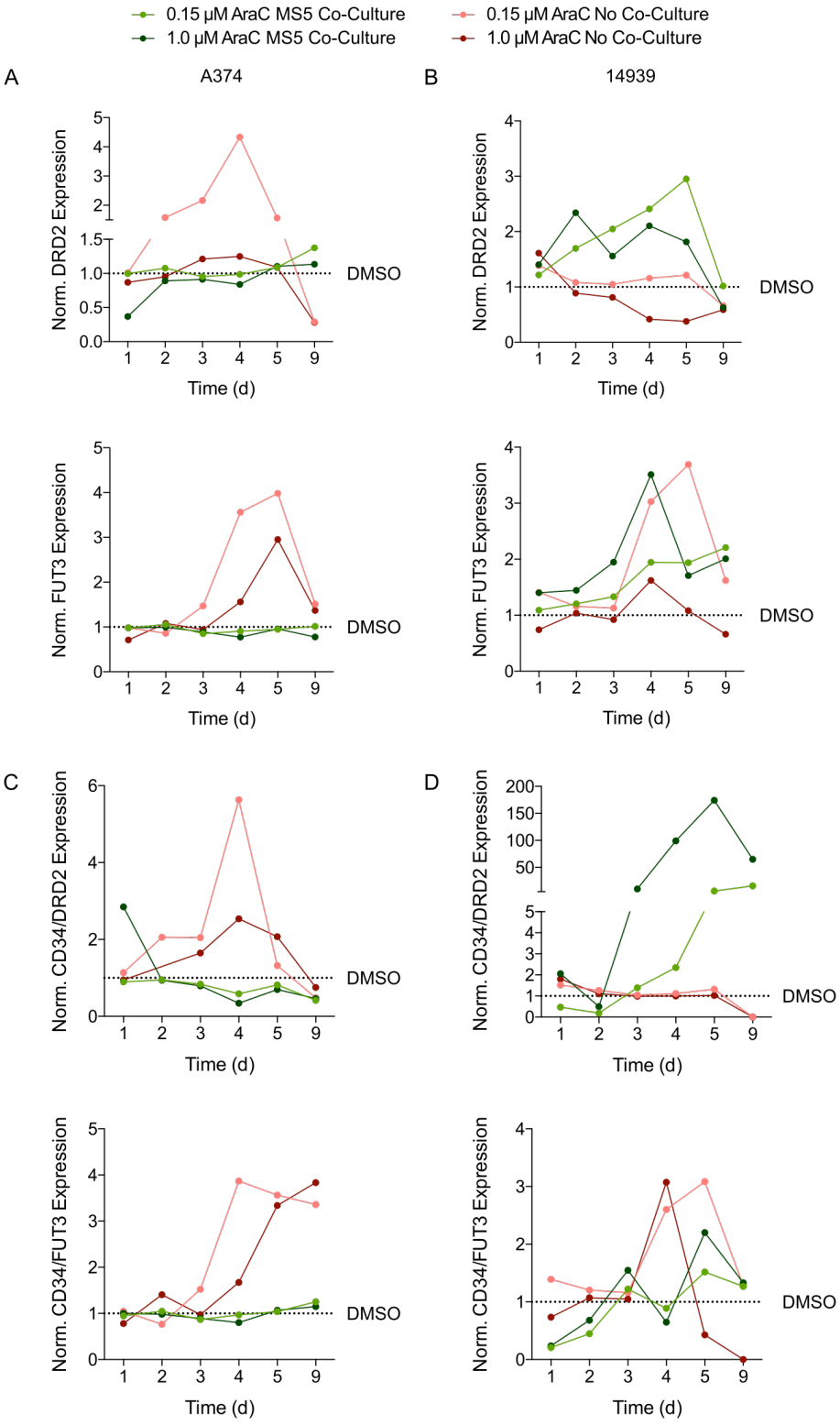
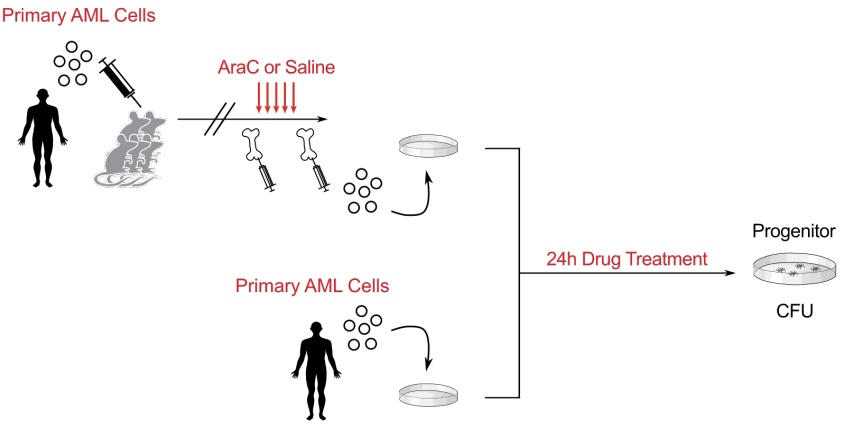


Figure 3.9: Effect of co-culture on LRC expression in response to AraC exposure in primary AML cells. Flow cytometry analysis of DRD2 and FUT3 expression in total and CD34+ cells after AraC treatment in co-cultured conditions of AML A374 (A), and AML 14939 (B). Statistical analysis could not be performed on one AML replicate.

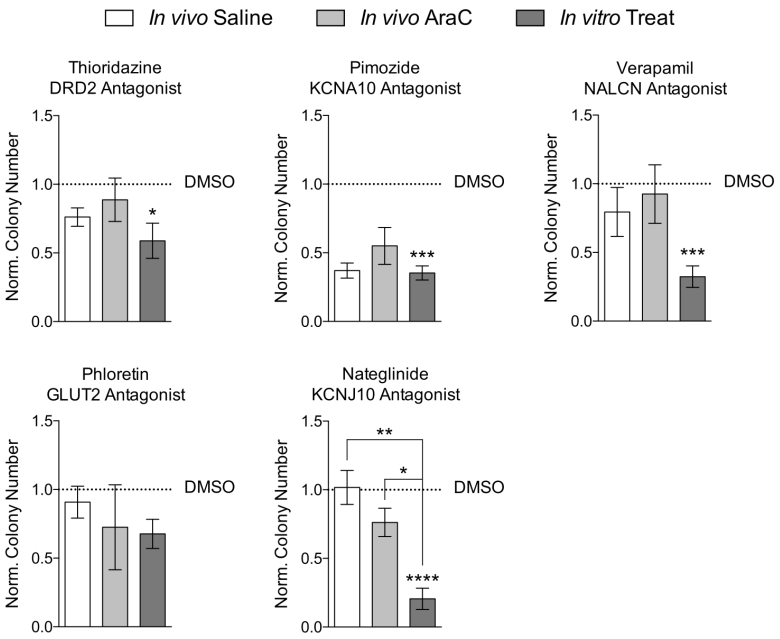
3.3.5 Ex vivo treatment of AraC and saline-exposed LRCs have differential response to LRC-targeting drugs in comparison to in vitro drug treatment in patient-matched sample.

Due to differences between AML progenitor curves observed *in vitro* and *in vivo*, we isolated LRC cells post-AraC therapy from immune-compromised NOD/SCID mice and studied their response to selected LRC-targeting drugs in comparison to the patient-matched *de novo* primary AML sample (Fig. 3.8A). All LRC drugs selected were diluted to 10 μM in DMSO. Drug response curves were not performed due to limited number of LRCs isolated from transplanted mice. 50000 cells/well were treated for 24h in StemSpan™ supplemented with cytokines and plated in MethoCult ™. Wells were incubated for 7 – 14 days. A summary of the LRC-targeting drugs and their targets are summarized in Fig. 3.8B. CFU analysis showed no differences in drug response between cells isolated from Ctrl mice, and LRC isolated from AraC-treated mice (Fig. 3.8C). Higher decreases in progenitor frequency were observed in *in vitro* treated cells.

A



B



C

Gene	Gene Name
DRD2	Dopamine Receptor D2
KCNJ10	K^+ Voltage-gated Channel Subfamily J Member 10
KCNA10	K^+ Voltage-gated Channel Subfamily A Member 10
NALCN	Na^+ Leak Channel, Non-selective
GLUT2	Glucose Transporter 2

Figure 3.10: *Ex vivo* treatment of LRCs with LRC-targeting compounds. (A) Experimental schematic demonstrating the source of cells utilized in the experiment. (B) A summary of LRC-targeting compounds utilized in the experiment. (C) Normalized colony numbers of *in vivo* saline, *in vivo* AraC, and *in vitro* samples after treatment with LRC-targeting compounds. 10 μM of pimozide significantly decreased colony numbers in the *in vivo* isolated saline group and *in vitro* treated group (*p<0.05, ***p<0.001). 10 μM of nateglinide, verapamil, and thioridazine significantly decreased colony numbers in the *in vitro* treated group (*p<0.05, **p<0.02, ***p<0.001, ****p<0.0001). Data is presented as mean ± SEM. One-way ANOVA with Bonferroni post-test was performed for statistical analysis.

4.0 DISCUSSION

Despite advancements in the understanding of AML and AML relapse, there have been very limited progression in the treatment of AML in the clinic. The LRC signature derived from leukemic cells that exist prior to relapse provide an unique window of opportunity to develop new therapeutics that could prevent the relapse of AML. In this thesis, we apply the LRC signature in a drug screening assay and attempt to delineate the molecular pathways underlying the emergence of the LRC signature through development of an *in vitro* model of AML relapse.

Several progenitor assays could be used as a screening platform for drug discovery (Fig. 4.1). *In vivo* transplantation are able to read-out primitive HSCs that are capable for self-renewal and reconstitution of the entire blood lineage – *in vivo* assays derived from patient xenograft samples have been shown to be the most clinically relevant models[81]. The *in vitro* CFU assay is able to measure drug response on common progenitors that have limited differentiation potential and self-renewal capacity[82]. Finally, drug screening in liquid culture captures cell death at the level of the mature blood cells, and is unable to differentiate between drug effects on progenitor and mature cells. The *in vitro* CFU assay was used a surrogate to *in vivo* orthotopic transplant as an preliminary screen for drug potency. 21 primary samples were originally selected to undergo screening, out of which 5 samples were selected as good CFU makers. This demonstrates that not all primary AML samples are capable of colony formation at the cell dose selected. This raises the question: what factors affect the capacity of a cell to form colonies in an CFU assay? Factors to consider include risk stratification based on cytogenetics and molecular information, and expression of CD34. Surface expression CD33 and CD34 have been

shown to be predictive of colony formation[83]. However, samples that had high CFU capacity screened in this thesis did not always have high CD34 expression, implying other factors in the determination of CFU potential. Studies comparing the colony forming capacity of primary AML samples in different risk groups have shown cytogenetic abnormalities to be the most powerful predictor of colony formation and treatment response[84]. Cytogenetic information was not available for a majority of the samples screened. Future screening experiments should focus on samples with cytogenetic abnormalities and high CD34 expression to maximize efforts in screening potential.

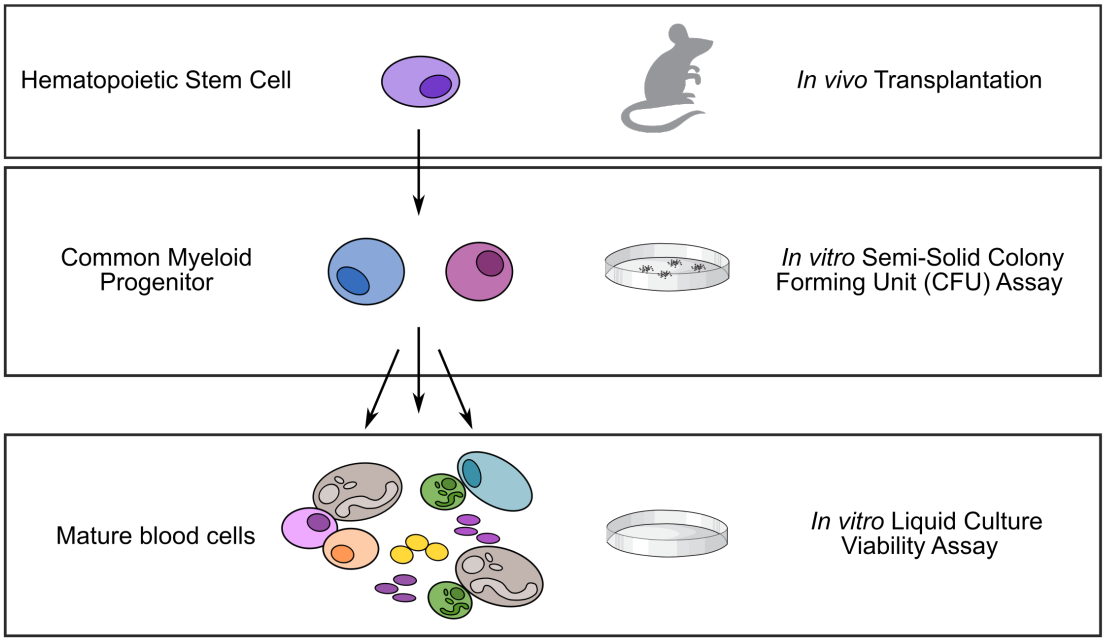


Figure 4.1: Progenitor assays used in the study of the hematopoietic system. Several assays have been developed for the study of the hematopoietic system. In vivo transplantations can capture the entire hierarchy of hematopoiesis and allows for regeneration of all blood constituents. The *in vitro* CFU assay captures progenitor cells with limited differentiation and self-renewal capacity. *In vitro* liquid cultures allows us to study the viability of cells post-drug treatment and does not allow the resolution to capture effects specifically on progenitor cells.

Primary AML samples have been selected to be used in drug screening experiments. There are several advantages that primary AML samples hold over commercially available AML cell models such as the OCI-AML3 cell line. The usage of several primary AML samples in a study is able to more accurately capture the heterogeneity of the disease. Cell models are homogeneous and often clonal in nature. Serial passaging of cell lines can introduce genetic variations that do not remain consistent over time[85]. The OCI-AML3 cell model is derived from a 78-year old patient, and contains a NMP1 mutation[86]. NMP1 occurs in only 20 – 30% of AML cases, and is associated with good disease prognosis[87]. The OCI-AML3 cell line does not appropriately capture the heterogeneity of AML, particularly cases with high risk and poor prognosis, and would not make an appropriate model for drug screening purposes.

The lack of response to several LRC targeting drugs highlight the differences between AML cells at diagnosis and directly prior to relapse, and raises the question of what underlying molecular pathways are responsible for the development of the LRC signature. Potential triggers include intrinsic cellular response to AraC, interactions between leukemic cells and the bone marrow niche environment in response to AraC, paracrine signalling by bone marrow niche cells in response to AraC, or a combination of the above factors. In order to determine if AraC is responsible for inducing the LRC signature, we set out to create an *in vitro* scenario in which primary AML cells are exposed to AraC at similar doses compared to those *in vivo*.

We have demonstrated that cells cultured after 5 treatments of AraC is not able to degenerate in the increase in colonies observed *in vivo*. We also observed a decrease in the percentage of CD34+ over time. CD34 is a marker of human HSCs and is present on

LSC fractions in AML[88]. The decrease in CD34+ can be regarded as a consequence of apoptosis due to suboptimal culture conditions. One major challenge in recapitulating relapse *in vitro* is maintaining primary AML cells in culture. Several methods have been identified in literature for maintenance of primary HSC proliferation and survival in culture. Growth factors such as SCF, TPO, and FLT3 have been identified as critical for maintenance of sensitive HSCs *in vitro*[89]. Other supplements such as stemregenin 1 (SR1) have been shown in literature to be effective at maintaining the CD34+ fraction in culture[90]. Survival of primary AML cells in culture have been previously shown to be correlated with treatment outcome; implicating a cell-intrinsic mechanism of survival[91]. It is not known if specific cytogenetic or molecular aberrations play a role in survival of AML cells *in vitro*. It is not known if AraC-induced development of the LRC signature has occurred *in vitro* but could not be captured in the assay due to culture conditions. Future experiments should explore the possibility of engrafting primary AML samples that have been exposed to AraC *in vitro*. LRCs that may have been generated from AraC exposure will then have optimal *in vivo* conditions to proliferate and grow. The differential response between *ex vivo* isolated LRCs and *in vitro* treated diagnostic AML further highlights the differences between *in vivo* and *in vitro* studies. *In vitro* treated AML show high sensitivity to LRC targeting compounds in comparison to cells isolated from mouse xenotransplants.

In order to tackle the issue of long-term culture conditions described previously, primary AML cells were co-cultured with the mouse MS-5 cell line. The MS-5 cell line is derived from mouse long bone tissue and has been shown to adhere to HSCs and support their survival by secretion of SCF, interleukin-6 (IL-6), and granulocyte-macrophage stimulating factor[92]. Co-culture with MS-5 cells allows for the maintenance and survival of

human CD34+/CD38- populations, as well as the propagation of proliferative progenitors[93]. MSCs have been shown to be important for the survival and proliferation of primary AML cells *in vitro*. Co-culture with MS-5 cells have been show to inhibit apoptosis in primary AML cells[94]. The anti-apoptotic effect of MS-5 co-culture was decreased when cell-to-cell adhesions were inhibited with the use of a filter[94]. Direct contact with MSCs can induce expression and secretion of soluble factors that assist in the maintenance of HSCs[95,96]. The involvement of other cell types critical to the survival and quiescence of HSC was not explored in this study. Osteoblasts in the endosteal niche and endothelial cells in the vascular niche both play important roles in the survival and regulation of HSC activity *in vivo*[97]. Additionally, the interactions between AML with specific cytogenetics and molecular mutations and their bone marrow niche is not well characterized.

DRD2, FUT3, FASL, and GLUT2 are 4 surface receptors and transporters that have been identified in the LRC signature. The physiological function of each receptor can give insight on its function in AML. DRD2 was identified as a promising cancer cell specific target in neoplastic human pluripotent stem cells[98]. Expression of DRD2 has been shown in several tumour types including breast, colon, and leukemia[98]. In 2012, Sachlos et al identified thioridazine as an anti-cancer compound that is selective for cancer stem cells[98]. We observed an overall decrease in DRD2 *in vitro* with 1 μM of AraC treatment over time. This is in contrast to the increase in DRD2 with AraC treatment observed *in vivo*. The increased expression of DRD2 *in vivo* is an AraC-independent event and could potentially be due to interactions of AML cells with other cellular components of the bone marrow niche.

We observed an overall decrease in GLUT2 expression with AraC treatment. GLUT2 is an insulin-independent glucose transporter expressed in the pancreas, liver, and intestines[99]. It is the main transporter responsible for movement of glucose and fructose from the blood into the liver for fuel and storage as glycogen[100]. Overexpression of GLUT2 has been noted in several cancer types including brain, liver, colon, and breast[101]. Inhibition of GLUT2 by phloretin has been shown to induce apoptosis in human liver cancer in both *in vitro* and *in vivo* assays, demonstrating its potential as a therapeutic target in cancer[102]. Expression of GLUT2 has not been studied in HSCs; however, the expression of GLUT2 in primary AML leukemia could be an adaptive mechanism to alter their cellular metabolism and makes them functionally unique from their HSC counterparts. Farge et al have shown chemoresistent populations of AML are dependent on oxidative metabolism and have high expression levels of genes required for oxidative phosphorylation[103]. Alterations in cellular metabolism mediated by transcriptional changes presents one strategy for AML cells to evade chemotherapy.

FASL is a transmembrane protein with an important role in the regulation of cellular apoptosis and immunity[104]. FASL is up-regulated following a lesion to the cell surface that can be caused by DNA damage induced with chemotherapy treatment[105]. Impaired FASL signalling has been implicated in tumorigenesis and can lead to therapeutic resistance in cancer cells[106]. The FASL system is plays an important role in the immune-directed identification of cancer cells – failure in the FASL signalling cascade could lead to evasion of the immune system[107]. Intrinsically, many cancer cell types including colon, and lung have been shown to have increased FASL expression[108-110]. Increased FASL-expression in cancer cells have been show to induce FASL-mediated cell death in lymphocytes that

infiltrate into the tumour, also known as the "Fas-counter attack hypothesis"[108]. The over-expression of FASL in tumour cells does not lead to an increase in FASL-mediated cellular apoptosis, potentially implying impaired FASL signalling in these cells. We observed a 2-fold increase in FASL expression in primary AML cells after 1 day in culture. AraC treatment does not affect the changes in FASL expression over time. This increase in FASL expression with culture is likely correlated with cellular apoptosis due to *in vitro* culture conditions; however, without further mechanistic studies, we cannot confirm is FASL signalling is impaired in AML cells.

Amplification of LRC genes in AML was technically challenging for several reasons. Firstly, the expression of LRC genes are low in the primary AML population. Raw data from flow cytometry experiments presented in this thesis show only 10 – 20% of primary AML cells express DRD2. Additionally, there is little correlation between mRNA and protein expression: this can be attributed to the various levels of protein and mRNA regulation[111-113]. Secondly, it is difficult to obtain large quantities of high quality mRNA for PCR purposes. After several days in culture, as well as AraC treatment, many primary AML cells are undergoing apoptosis leading to the rapid degradation of mRNA[114,115]. Low expression level, low RNA quantity, and low RNA quality are all factors that may have attributed to the lack of amplification of LRC genes in primary AML.

5.1 Conclusion

Therapeutic resistance and relapse presents a major clinical issue in the treatment of AML. Due to the high rates of refractory disease and relapse in AML, it is of great interest to develop an approach to evaluate the chance of therapeutic resistance at the time of diagnosis, and to develop new models and therapies to combat resistance. The LRC signature is able to capable a snapshot in time and allowed for identification of unique targets to prevent AML relapse. An *in vitro* relapse model or another appropriate cell surrogate must be established in order to efficiently screen all potential target molecules. One approach to recapitulating the LRC signature focuses on the role of AraC. The work presented in this thesis demonstrates that *in vitro* treatment with AraC alone cannot induce the functional and molecular changes observed *in vivo*. AraC treatment *in vitro* is not able to recapture the rebounding progenitor frequency curve, increase in CD34 expression, and increase in LRC proteins post-AraC therapy *in vivo*. Technical challenges such as poor mRNA quantity and quality, as well as difficulties in maintaining primary AML cells in culture are confounding factors. This highlights the importance of the *in vivo* bone marrow niche environment as well as the power of *in vivo* xenotransplant models in the study of AML relapse. We will continue to work on developments of new models and gain new scientific insights on the development of therapeutic resistance in AML in hopes of future impact on the lives of patients who suffer from this severe cancer.

5.0 REFERENCES

1 Shipley, J. L. & Butera, J. N. Acute myelogenous leukemia. *Experimental hematology* **37**, 649-658, doi:10.1016/j.exphem.2009.04.002 (2009).

2 Dohner, H., Weisdorf, D. J. & Bloomfield, C. D. Acute Myeloid Leukemia. *The New England journal of medicine* **373**, 1136-1152, doi:10.1056/NEJMra1406184 (2015).

3 De Kouchkovsky, I. & Abdul-Hay, M. 'Acute myeloid leukemia: a comprehensive review and 2016 update'. *Blood Cancer J* **6**, e441, doi:10.1038/bcj.2016.50 (2016).

4 Dohner, H. *et al.* Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **115**, 453-474, doi:10.1182/blood-2009-07-235358 (2010).

5 Stoiser, B. *et al.* Prognosis of patients with a second relapse of acute myeloid leukemia. *Leukemia* **14**, 2059-2063 (2000).

6 Othus, M. *et al.* Declining rates of treatment-related mortality in patients with newly diagnosed AML given 'intense' induction regimens: a report from SWOG and MD Anderson. *Leukemia* **28**, 289-292, doi:10.1038/leu.2013.176 (2014).

7 Dombret, H., Raffoux, E. & Gardin, C. Acute myeloid leukemia in the elderly. *Seminars in oncology* **35**, 430-438, doi:10.1053/j.seminoncol.2008.04.013 (2008).

8 Döhner, H., Weisdorf, D. J. & Bloomfield, C. D. Acute Myeloid Leukemia. *N Engl J Med* **373**, 1136-1152, doi:10.1056/NEJMra1406184 (2015).

9 Byrd, J. C. *et al.* Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood* **100**, 4325-4336, doi:10.1182/blood-2002-03-0772 (2002).

10 Grimwade, D. *et al.* The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood* **92**, 2322-2333 (1998).

11 Licht, J. D. AML1 and the AML1-ETO fusion protein in the pathogenesis of t(8;21) AML. *Oncogene* **20**, 5660-5679, doi:10.1038/sj.onc.1204593 (2001).

12 Walter, R. B. *et al.* Significance of minimal residual disease before myeloablative allogeneic hematopoietic cell transplantation for AML in first and second complete remission. *Blood* **122**, 1813-1821, doi:10.1182/blood-2013-06-506725 (2013).

13 Walter, R. B. *et al.* Resistance prediction in AML: analysis of 4601 patients from MRC/NCRI, HOVON/SAKK, SWOG and MD Anderson Cancer Center. *Leukemia* **29**, 312-320, doi:10.1038/leu.2014.242 (2015).

14 Shayegi, N. *et al.* The level of residual disease based on mutant NPM1 is an independent prognostic factor for relapse and survival in AML. *Blood* **122**, 83-92, doi:10.1182/blood-2012-10-461749 (2013).

15 Grimwade, D. & Freeman, S. D. Defining minimal residual disease in acute myeloid leukemia: which platforms are ready for "prime time"? *Blood* **124**, 3345-3355, doi:10.1182/blood-2014-05-577593 (2014).
16 Ånensen, N. *et al.* Correlation analysis of p53 protein isoforms with NPM1/FLT3 mutations and therapy response in acute myeloid leukemia. *Oncogene* **31**, 1533-1545, doi:10.1038/onc.2011.348 (2012).
17 Gilliland, D. G. & Griffin, J. D. The roles of FLT3 in hematopoiesis and leukemia. *Blood* **100**, 1532-1542, doi:10.1182/blood-2002-02-0492 (2002).
18 Smith, C. C. *et al.* Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia. *Nature* **485**, 260-263, doi:10.1038/nature11016 (2012).
19 Smith, C. C., Lin, K., Stecula, A., Sali, A. & Shah, N. P. FLT3 D835 mutations confer differential resistance to type II FLT3 inhibitors. *Leukemia* **29**, 2390-2392, doi:10.1038/leu.2015.165 (2015).
20 Mony, U., Jawad, M., Seedhouse, C., Russell, N. & Pallis, M. Resistance to FLT3 inhibition in an in vitro model of primary AML cells with a stem cell phenotype in a defined microenvironment. *Leukemia* **22**, 1395-1401, doi:10.1038/leu.2008.125 (2008).
21 Bishop, J. F. *et al.* A randomized study of high-dose cytarabine in induction in acute myeloid leukemia. *Blood* **87**, 1710-1717 (1996).
22 Phillips, G. L. *et al.* High-dose cytarabine and daunorubicin induction and postremission chemotherapy for the treatment of acute myelogenous leukemia in adults. *Blood* **77**, 1429-1435 (1991).
23 Burnett, A. K. *et al.* Randomised comparison of addition of autologous bone-marrow transplantation to intensive chemotherapy for acute myeloid leukaemia in first remission: results of MRC AML 10 trial. UK Medical Research Council Adult and Children's Leukaemia Working Parties. *Lancet* **351**, 700-708 (1998).
24 Koreth, J. *et al.* Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. *Jama* **301**, 2349-2361, doi:10.1001/jama.2009.813 (2009).
25 Litzow, M. R. *et al.* Allogeneic transplantation for therapy-related myelodysplastic syndrome and acute myeloid leukemia. *Blood* **115**, 1850-1857, doi:10.1182/blood-2009-10-249128 (2010).
26 Mangan, J. K. & Luger, S. M. Salvage therapy for relapsed or refractory acute myeloid leukemia. *Ther Adv Hematol* **2**, 73-82, doi:10.1177/2040620711402533 (2011).
27 Venditti, A. *et al.* Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia. *Blood* **96**, 3948-3952 (2000).
28 Paietta, E. Minimal residual disease in acute myeloid leukemia: coming of age. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program* **2012**, 35-42, doi:10.1182/asheducation-2012.1.35 (2012).
29 Buccisano, F. *et al.* Monitoring of minimal residual disease in acute myeloid leukemia. *Current opinion in oncology* **21**, 582-588, doi:10.1097/CCO.0b013e3283311856 (2009).

30 Kern, W., Haferlach, C., Haferlach, T. & Schnittger, S. Monitoring of minimal residual disease in acute myeloid leukemia. *Cancer* **112**, 4-16, doi:10.1002/cncr.23128 (2008).
31 Vidriales, M. B., San-Miguel, J. F., Orfao, A., Coustan-Smith, E. & Campana, D. Minimal residual disease monitoring by flow cytometry. *Best practice & research. Clinical haematology* **16**, 599-612 (2003).
32 D'Arena, G. *et al.* Human umbilical cord blood: immunophenotypic heterogeneity of CD34+ hematopoietic progenitor cells. *Haematologica* **81**, 404-409 (1996).
33 Reading, C. L. *et al.* Expression of unusual immunophenotype combinations in acute myelogenous leukemia. *Blood* **81**, 3083-3090 (1993).
34 Bhatia, M., Bonnet, D., Murdoch, B., Gan, O. I. & Dick, J. E. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. *Nat Med* **4**, 1038-1045, doi:10.1038/2023 (1998).
35 Kopp, H. G., Avecilla, S. T., Hooper, A. T. & Rafii, S. The bone marrow vascular niche: home of HSC differentiation and mobilization. *Physiology (Bethesda)* **20**, 349-356, doi:10.1152/physiol.00025.2005 (2005).
36 Zhang, J. *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* **425**, 836-841, doi:10.1038/nature02041 (2003).
37 Visnjic, D. *et al.* Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood* **103**, 3258-3264, doi:10.1182/blood-2003-11-4011 (2004).
38 Sacchetti, B. *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* **131**, 324-336, doi:10.1016/j.cell.2007.08.025 (2007).
39 Avecilla, S. T. *et al.* Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med* **10**, 64-71, doi:10.1038/nm973 (2004).
40 Minguell, J. J., Erices, A. & Conget, P. Mesenchymal stem cells. *Exp Biol Med (Maywood)* **226**, 507-520 (2001).
41 Méndez-Ferrer, S. *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466**, 829-834, doi:10.1038/nature09262 (2010).
42 Bernstein, A., Forrester, L., Reith, A. D., Dubreuil, P. & Rottapel, R. The murine W/c-kit and Steel loci and the control of hematopoiesis. *Semin Hematol* **28**, 138-142 (1991).
43 Driessen, R. L., Johnston, H. M. & Nilsson, S. K. Membrane-bound stem cell factor is a key regulator in the initial lodgment of stem cells within the endosteal marrow region. *Exp Hematol* **31**, 1284-1291 (2003).
44 Arai, F. *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**, 149-161, doi:10.1016/j.cell.2004.07.004 (2004).
45 Li, L. *et al.* The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. *Immunity* **8**, 43-55 (1998).
46 Sugiyama, T., Kohara, H., Noda, M. & Nagasawa, T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone

marrow stromal cell niches. *Immunity* **25**, 977-988, doi:10.1016/j.immuni.2006.10.016 (2006).
47 Ishikawa, F. *et al.* Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat Biotechnol* **25**, 1315-1321, doi:10.1038/nbt1350 (2007).
48 Colmone, A. *et al.* Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. *Science* **322**, 1861-1865, doi:10.1126/science.1164390 (2008).
49 Schepers, K. *et al.* Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. *Cell Stem Cell* **13**, 285-299, doi:10.1016/j.stem.2013.06.009 (2013).
50 Huang, J. C. *et al.* Mesenchymal stromal cells derived from acute myeloid leukemia bone marrow exhibit aberrant cytogenetics and cytokine elaboration. *Blood Cancer J* **5**, e302, doi:10.1038/bcj.2015.17 (2015).
51 Geyh, S. *et al.* Functional inhibition of mesenchymal stromal cells in acute myeloid leukemia. *Leukemia* **30**, 683-691, doi:10.1038/leu.2015.325 (2016).
52 Tavor, S. *et al.* CXCR4 regulates migration and development of human acute myelogenous leukemia stem cells in transplanted NOD/SCID mice. *Cancer Res* **64**, 2817-2824 (2004).
53 Konoplev, S. *et al.* Overexpression of CXCR4 predicts adverse overall and event-free survival in patients with unmutated FLT3 acute myeloid leukemia with normal karyotype. *Cancer* **109**, 1152-1156, doi:10.1002/cncr.22510 (2007).
54 Zeng, Z. *et al.* Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. *Blood* **113**, 6215-6224, doi:10.1182/blood-2008-05-158311 (2009).
55 Hope, K. J., Jin, L. & Dick, J. E. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nature immunology* **5**, 738-743, doi:10.1038/ni1080 (2004).
56 Jordan, C. T., Guzman, M. L. & Noble, M. Cancer stem cells. *The New England journal of medicine* **355**, 1253-1261, doi:10.1056/NEJMra061808 (2006).
57 Saito, Y. *et al.* Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. *Sci Transl Med* **2**, 17ra19, doi:10.1126/scitranslmed.3000349 (2010).
58 Nassar, D. & Blanpain, C. Cancer Stem Cells: Basic Concepts and Therapeutic Implications. *Annual review of pathology* **11**, 47-76, doi:10.1146/annurev-pathol-012615-044438 (2016).
59 Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3**, 730-737 (1997).
60 Lapidot, T. *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**, 645-648, doi:10.1038/367645a0 (1994).
61 Eppert, K. *et al.* Stem cell gene expression programs influence clinical outcome in human leukemia. *Nat Med* **17**, 1086-1093, doi:10.1038/nm.2415 (2011).

62 Vargaftig, J. *et al.* Frequency of leukemic initiating cells does not depend on the xenotransplantation model used. *Leukemia* **26**, 858-860, doi:10.1038/leu.2011.250 (2012).
63 Walter, R. B. *et al.* Effect of genetic profiling on prediction of therapeutic resistance and survival in adult acute myeloid leukemia. *Leukemia* **29**, 2104-2107, doi:10.1038/leu.2015.76 (2015).
64 Löwenberg, B. *et al.* Effect of priming with granulocyte colony-stimulating factor on the outcome of chemotherapy for acute myeloid leukemia. *N Engl J Med* **349**, 743-752, doi:10.1056/NEJMoa025406 (2003).
65 Greaves, M. & Maley, C. C. Clonal evolution in cancer. *Nature* **481**, 306-313, doi:10.1038/nature10762 (2012).
66 Ding, L. *et al.* Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature* **481**, 506-510, doi:10.1038/nature10738 (2012).
67 Parkin, B. *et al.* Clonal evolution and devolution after chemotherapy in adult acute myelogenous leukemia. *Blood* **121**, 369-377, doi:10.1182/blood-2012-04-427039 (2013).
68 Cohen, S. S. The mechanisms of lethal action of arabinosyl cytosine (araC) and arabinosyl adenine (araA). *Cancer* **40**, 509-518 (1977).
69 Yin, B., Kogan, S. C., Dickins, R. A., Lowe, S. W. & Largaespada, D. A. Trp53 loss during in vitro selection contributes to acquired Ara-C resistance in acute myeloid leukemia. *Exp Hematol* **34**, 631-641, doi:10.1016/j.exphem.2006.01.015 (2006).
70 Rathe, S. K. & Largaespada, D. A. Deoxycytidine kinase is downregulated in Ara-C-resistant acute myeloid leukemia murine cell lines. *Leukemia* **24**, 1513-1515, doi:10.1038/leu.2010.88 (2010).
71 Kim, M. Y. & Ives, D. H. Human deoxycytidine kinase: kinetic mechanism and end product regulation. *Biochemistry* **28**, 9043-9047 (1989).
72 Veuger, M. J., Honders, M. W., Spoelder, H. E., Willemze, R. & Barge, R. M. Inactivation of deoxycytidine kinase and overexpression of P-glycoprotein in AraC and daunorubicin double resistant leukemic cell lines. *Leuk Res* **27**, 445-453 (2003).
73 Herold, N. *et al.* Targeting SAMHD1 with the Vpx protein to improve cytarabine therapy for hematological malignancies. *Nat Med* **23**, 256-263, doi:10.1038/nm.4265 (2017).
74 Schneider, C. *et al.* SAMHD1 is a biomarker for cytarabine response and a therapeutic target in acute myeloid leukemia. *Nat Med* **23**, 250-255, doi:10.1038/nm.4255 (2017).
75 Stein, E. M. & Tallman, M. S. Emerging therapeutic drugs for AML. *Blood* **127**, 71-78, doi:10.1182/blood-2015-07-604538 (2016).
76 Baccelli, I. *et al.* A novel approach for the identification of efficient combination therapies in primary human acute myeloid leukemia specimens. *Blood Cancer J* **7**, e529, doi:10.1038/bcj.2017.10 (2017).
77 Pabst, C. *et al.* Identification of small molecules that support human leukemia stem cell activity ex vivo. *Nat Methods* **11**, 436-442, doi:10.1038/nmeth.2847 (2014).

78 Chao, M. P. *et al.* Human AML-iPSCs Reacquire Leukemic Properties after Differentiation and Model Clonal Variation of Disease. *Cell Stem Cell*, doi:10.1016/j.stem.2016.11.018 (2016).
79 Kotini, A. G. *et al.* Stage-Specific Human Induced Pluripotent Stem Cells Map the Progression of Myeloid Transformation to Transplantable Leukemia. *Cell Stem Cell*, doi:10.1016/j.stem.2017.01.009 (2017).
80 Wu, K. D. *et al.* Expression and localization of human dopamine D2 and D4 receptor mRNA in the adrenal gland, aldosterone-producing adenoma, and pheochromocytoma. *J Clin Endocrinol Metab* **86**, 4460-4467, doi:10.1210/jcem.86.9.7859 (2001).
81 Gao, H. *et al.* High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response. *Nat Med* **21**, 1318-1325, doi:10.1038/nm.3954 (2015).
82 Entringer, M. A., Robinson, W. A. & Kurnick, J. E. Colony growth of normal human bone marrow in agar-gel. *Exp Hematol* **5**, 125-135 (1977).
83 Andrews, R. G., Singer, J. W. & Bernstein, I. D. Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties. *J Exp Med* **169**, 1721-1731 (1989).
84 Ohler, L. & Geissler, K. Semi-solid colony growth in acute myeloid leukemia and its relation to cytogenetic risk groups. *Leuk Lymphoma* **43**, 1743-1747, doi:10.1080/1042819021000006484 (2002).
85 Ross, D. T. *et al.* Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* **24**, 227-235, doi:10.1038/73432 (2000).
86 Hollink, I. H. *et al.* Low frequency of DNMT3A mutations in pediatric AML, and the identification of the OCI-AML3 cell line as an in vitro model. *Leukemia* **26**, 371-373, doi:10.1038/leu.2011.210 (2012).
87 Heath, E. M. *et al.* Biological and clinical consequences of NPM1 mutations in AML. *Leukemia* **31**, 798-807, doi:10.1038/leu.2017.30 (2017).
88 Greaves, M. F. *et al.* Molecular features of CD34: a hemopoietic progenitor cell-associated molecule. *Leukemia* **6 Suppl 1**, 31-36 (1992).
89 Yao, C. L. *et al.* Characterization of serum-free ex vivo-expanded hematopoietic stem cells derived from human umbilical cord blood CD133(+) cells. *Stem Cells Dev* **15**, 70-78, doi:10.1089/scd.2006.15.70 (2006).
90 Boitano, A. E. *et al.* Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science* **329**, 1345-1348, doi:10.1126/science.1191536 (2010).
91 Maha, A., Cheong, S. K., Leong, C. F. & Seow, H. F. Cell viability of acute myeloid leukaemia blasts in culture correlates with treatment outcome. *Hematology* **13**, 13-20, doi:10.1179/102453308X315762 (2008).
92 Suzuki, J., Fujita, J., Taniguchi, S., Sugimoto, K. & Mori, K. J. Characterization of murine hemopoietic-supportive (MS-1 and MS-5) and non-supportive (MS-K) cell lines. *Leukemia* **6**, 452-458 (1992).
93 Issaad, C., Croisille, L., Katz, A., Vainchenker, W. & Coulombel, L. A murine stromal cell line allows the proliferation of very primitive human CD34++/CD38-

progenitor cells in long-term cultures and semisolid assays. *Blood* **81**, 2916-2924 (1993).
94 Konopleva, M. *et al.* Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. *Leukemia* **16**, 1713-1724, doi:10.1038/sj.leu.2402608 (2002).
95 Gottschling, S. *et al.* Human mesenchymal stromal cells regulate initial self-renewing divisions of hematopoietic progenitor cells by a beta1-integrin-dependent mechanism. *Stem Cells* **25**, 798-806, doi:10.1634/stemcells.2006-0513 (2007).
96 Alakel, N. *et al.* Direct contact with mesenchymal stromal cells affects migratory behavior and gene expression profile of CD133+ hematopoietic stem cells during ex vivo expansion. *Exp Hematol* **37**, 504-513, doi:10.1016/j.exphem.2008.12.005 (2009).
97 Zhou, H. S., Carter, B. Z. & Andreeff, M. Bone marrow niche-mediated survival of leukemia stem cells in acute myeloid leukemia: Yin and Yang. *Cancer Biol Med* **13**, 248-259, doi:10.20892/j.issn.2095-3941.2016.0023 (2016).
98 Sachlos, E. *et al.* Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells. *Cell* **149**, 1284-1297, doi:10.1016/j.cell.2012.03.049 (2012).
99 Kellett, G. L., Brot-Laroche, E., Mace, O. J. & Leturque, A. Sugar absorption in the intestine: the role of GLUT2. *Annu Rev Nutr* **28**, 35-54, doi:10.1146/annurev.nutr.28.061807.155518 (2008).
100 Cheeseman, C. I. GLUT2 is the transporter for fructose across the rat intestinal basolateral membrane. *Gastroenterology* **105**, 1050-1056 (1993).
101 Yamamoto, T. *et al.* Over-expression of facilitative glucose transporter genes in human cancer. *Biochem Biophys Res Commun* **170**, 223-230 (1990).
102 Wu, C. H. *et al.* In vitro and in vivo study of phloretin-induced apoptosis in human liver cancer cells involving inhibition of type II glucose transporter. *Int J Cancer* **124**, 2210-2219, doi:10.1002/ijc.24189 (2009).
103 Farge, T. *et al.* Chemotherapy-Resistant Human Acute Myeloid Leukemia Cells Are Not Enriched for Leukemic Stem Cells but Require Oxidative Metabolism. *Cancer Discov* **7**, 716-735, doi:10.1158/2159-8290.CD-16-0441 (2017).
104 O'Brien, D. I. *et al.* Targeting the Fas/Fas ligand pathway in cancer. *Expert Opin Ther Targets* **9**, 1031-1044, doi:10.1517/14728222.9.5.1031 (2005).
105 Müller, M. *et al.* p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. *J Exp Med* **188**, 2033-2045 (1998).
106 Muppidi, J. R. & Siegel, R. M. Ligand-independent redistribution of Fas (CD95) into lipid rafts mediates clonotypic T cell death. *Nat Immunol* **5**, 182-189, doi:10.1038/ni1024 (2004).
107 Maecker, H. L., Yun, Z., Maecker, H. T. & Giaccia, A. J. Epigenetic changes in tumor Fas levels determine immune escape and response to therapy. *Cancer Cell* **2**, 139-148 (2002).
108 O'Connell, J., O'Sullivan, G. C., Collins, J. K. & Shanahan, F. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. *J Exp Med* **184**, 1075-1082 (1996).

109 Bennett, M. W. *et al.* Expression of Fas ligand by human gastric adenocarcinomas: a potential mechanism of immune escape in stomach cancer. *Gut* **44**, 156-162 (1999).
110 Elnemr, A. *et al.* Human pancreatic cancer cells express non-functional Fas receptors and counterattack lymphocytes by expressing Fas ligand; a potential mechanism for immune escape. *Int J Oncol* **18**, 33-39 (2001).
111 de Sousa Abreu, R., Penalva, L. O., Marcotte, E. M. & Vogel, C. Global signatures of protein and mRNA expression levels. *Mol Biosyst* **5**, 1512-1526, doi:10.1039/b908315d (2009).
112 Vogel, C. & Marcotte, E. M. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* **13**, 227-232, doi:10.1038/nrg3185 (2012).
113 Maier, T., Güell, M. & Serrano, L. Correlation of mRNA and protein in complex biological samples. *FEBS Lett* **583**, 3966-3973, doi:10.1016/j.febslet.2009.10.036 (2009).
114 Del Prete, M. J. *et al.* Degradation of cellular mRNA is a general early apoptosis-induced event. *FASEB J* **16**, 2003-2005, doi:10.1096/fj.02-0392fje (2002).
115 Thomas, M. P. *et al.* Apoptosis Triggers Specific, Rapid, and Global mRNA Decay with 3' Uridylated Intermediates Degraded by DIS3L2. *Cell Rep* **11**, 1079-1089, doi:10.1016/j.celrep.2015.04.026 (2015).