THE ANTAGONISM OF AHR AS A NOVEL ANTI-LEUKEMIC STRATEGY

TARGETING THE ANTAGONISM OF AHR BY MSI2 AS A NOVEL ANTI-LEUKEMIC STRATEGY IN HUMAN ACUTE MYELOID LEUKEMIA

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TITLE: Targeting the antagonism of AHR by MSI2 as a novel anti-leukemic strategy in human acute myeloid leukemia

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LAY ABSTRACT

The human blood system is sustained by a population of blood stem cells that are tightly regulated in their production of stem and differentiated cells. The Musashi-2 (MSI2) protein is a key regulator of blood stem cell identity through its inhibition of the aryl hydrocarbon receptor (AHR) signaling pathway. When there is dysregulation of blood cell homeostasis, blood malignancies such as acute myeloid leukemia (AML) may arise. In this work, the relationship between MSI2 and the AHR signaling pathway was explored within a myeloid leukemic context. It was shown that MSI2 imposes inhibitory effects on AHR to promote disease progression and that its reduction could help alleviate disease burden. Additionally, it was found that activation of the AHR signaling pathway could overcome the MSI2 differentiation block to create a therapeutic effect. Overall, the results of this project shed light on novel therapeutic strategies and targets for the treatment of AML.

ABSTRACT

Acute myeloid leukemia (AML) is an aggressive malignancy of the hematopoietic system, characterized by the accumulation of abnormally differentiated blast cells that is driven by leukemic stem cells (LSCs). In murine AML, Musashi-2 (MSI2), an RNA-binding protein and positive regulator of stemness, has been implicated in the propagation of disease. While its enhanced expression correlated with poor disease outcome for human AML patients, no study has yet examined its actual functional role in human leukemia.

In normal human hematopoietic stem cells (HSCs), we have recently reported the inhibitory effects of MSI2 on the pro-differentiative aryl hydrocarbon receptor (AHR) signaling pathway as a mechanism for promoting self-renewal in HSCs. We hypothesized that elevated MSI2 is critical for maintenance of human AML and promotes unrestrained self-renewal of LSCs in part through constitutive repression of AHR signaling. Our work aimed to unravel the relationship between MSI2 and AHR in the human leukemic context and to determine if activation of AHR signaling can promote differentiation.

Results confirmed that MSI2 is preferentially expressed in primary patient LSCs and is negatively correlated with the expression of AHR gene targets. Upon lentiviral knockdown of MSI2 in-vitro and in-vivo, leukemic growth was compromised and increased AHR signaling was observed. Circumventing the inhibitory role of MSI2 in AML, activation of AHR with a potent agonist impaired leukemic progenitor activity and proliferation. In-vivo studies employing reconstitution of immunodeficient mice with

primary AML samples showed impairment of AML engraftment for a significant proportion of tested samples upon treatment with an AHR agonist.

Overall, our findings from this project indicated that MSI2 is required for human AML propagation and that a decrease in MSI2 inhibitory effects on AHR signaling or direct activation of the AHR signaling pathway via a potent agonist can promote AML cell differentiation and loss.

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LIST OF ABBREVIATIONS

7AAD	7-aminoactinomycin D
AHR	Aryl hydrocarbon receptor
AHRC	Aryl hydrocarbon receptor complex
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
ARNT	Aryl hydrocarbon receptor nuclear translocator
BFU-E	Burst-forming unit-erythroid
bHLH	Basic helix-loop-helix
CFU	Colony-forming unit
CFU-G	Colony-forming unit-granulocyte
CFU-GEMM	Colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM	Colony-forming unit-granulocyte, macrophage
CFU-M	Colony-forming unit-macrophage
CFU-Mk	Colony-forming unit-megakaryocyte
CFU-S	Colony-forming unit spleen
ChIP-seq	Chromatin-immunoprecipitation sequencing
CLP	Common lymphoid progenitor
CLIP-seq	Cross-linking immunoprecipitation sequencing
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CNS	Central nervous system
CSC	Cancer stem cell
CYP	Cytochrome P450
d-MSI	Drosophila Musashi
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
FAB	French-American-British
FACS	Fluorescence-activate cell sorting
FICZ	6-formylindolo-[3,2-b] carbazole
GFP	Green fluorescent protein
GMP	Granulocyte-macrophage progenitor
GSEA	Gene set enrichment analysis
GTP	Guanine tri-phosphate
HAH	Halogenated aromatic hydrocarbons
HLH	Helix-loop-helix
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
HSP90	Heat-shock protein 90
iPSC	Induced pluripotent stem cells
IT-HSC	Intermediate-term hematopoietic stem cells
KD	Knock-down
LAK	Lymphokine-activated killer
LSC	Leukemic stem cell
LSC17	Leukemic stem cell 17-gene signature
LSC-R	Leukemic stem cell-related
LT-HSC	Long-term hematopoietic stem cell
LTC-IC	Long-term culture-initiating cell
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte-erythrocyte progenitor
MILE	Microarray Innovation in Leukemia
MPP	Multipotent progenitor cell
mPB	Mobilized peripheral blood
mRNA	Messenger RNA

MSI1	Musashi-1
MSI2	Musashi-2
NES	Normalized enrichment score
NK	Natural killer
NOD-SCID	Non-obese diabetic severe combined immune-deficient
NSC	Neural stem cell
NSG	NOD-*Scid* gamma
PABP	Poly-A binding protein
PAH	Polyaromatic hydrocarbon
RA	Retinoic acid
RAR	Retinoic acid receptor
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RBP	RNA-binding protein
RIP	RNA-immunoprecipitation
RNP1	Ribonucleoprotein 1
RNP2	Ribonucleoprotein 2
RRM	RNA recognition motif
SCID	Severe combined immune-deficient
shControl	Short hairpin RNA, targeting RFP
shRNA	Short hairpin RNA
shMSI2	Short hairpin RNA, targeting MSI2
SR1	StemReginin-1
ST-HSC	Short-term hematopoietic stem cell
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
tRNA	Transferase-RNA
TRP	Tryptophan

TTK	Tramtrack
UTR	Untranslated region
UV	Ultra-violet
WHO	World Health Organization
XAP2	Hepatitis B X-associated protein 2
XRE	Xenobiotic response element

DECLARATION OF ACADEMIC ACHIEVEMENT

This project was supervised by Dr. Kristin Hope. She participated in the designing of experiments and performed the intrafemoral injections of animals. Dr. Nicholas Holzapfel created the MSI2 KD lentiviral vector and carried out majority of the MSI2 KD in-vitro/in-vivo experiments in primary AML samples. Dr. Stefan Rentas aided in the design and analysis of experiments, performed the analyses of AHR expression in the MILE study and conducted the immunofluorescence staining. I contributed to the design and execution of experiments presented, performed data analysis and writing of all sections of this thesis unless otherwise noted.

CHAPTER 1: INTRODUCTION

1. Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is an aggressive hematopoietic malignancy that is characterized by clonal expansion of abnormally differentiated or poorly differentiated myeloid cells in the bone marrow, peripheral blood and other tissues (Dohner et. al., 2015; O'Donnell et. al., 2012).

1.1 Epidemiology of AML

AML is the most common form of adult acute leukemia. In 2016, the Surveillance, Epidemiology and End Results (SEER) Program reported approximately 20,000 new AML cases, constituting 33% of all new leukemia diagnoses and 1.2% of all new cancer cases (SEER, 2016). It is the leading cause of death due to hematopoietic malignancies, accounting for 43% of all leukemia deaths and 1.8% of all cancer cases in North America (O'Donnell et. al., 2012; SEER, 2016).

In AML, the highest incidences are seen in individuals with increased age. The median age for diagnosis is 67 years, with over 54% of the patients being diagnosed at 65 years or older (O'Donnell et. al., 2012).

1.2 Diagnosis for AML

A full diagnostic procedure for AML include morphological assessment of cell smears from the bone marrow and peripheral blood, analysis of leukemic biomarkers and cell-surface markers and cytogenetic screening for genetic lesions (Dohner et. al., 2015).

Patient bone marrow and peripheral blood smears are examined with the Wright-Giemsa stain for visualization of cell morphology. An AML case is suspected if the presence of undifferentiated, myeloid blast cells is detected and greater than 20% in either the bone marrow or peripheral blood (Dohner et. al., 2010). A blast cell is defined as a hematopoietic tumour cell with a primitive morphology (Huntly and Gilliland, 2005). Expression of biomarkers such as early hematopoiesis-associated antigens or lack of expression of myeloid and monocytic maturation markers on hematopoietic cells can also be an indicator of AML (Dohner et. al., 2010). Finally, a mandatory cytogenetics screen searching for chromosome abnormalities or genetic lesions can confirm the diagnosis, since over 50% of all adult AML cases have an abnormal karyotype or carry a genetic mutation. Common lesions and molecular markers such as *FLT3*, *c-KIT*, *NPM1*, and *CEBPA* gene mutations are prognostic and predict the overall therapeutic response and patient survival outcomes (O'Donnell et. al., 2012).

1.3 Classification systems of AML

The multidisciplinary procedures in place for AML diagnosis allow for an accurate classification of the disease according to the French-American-British (FAB) system and the World Health Organization (WHO) classification system. Established in 1976, the FAB system was the first to divide AML into seven unique subtypes (M1 to M7) based on the cytochemical stains and morphology of patient blood smears (O'Donnell et. al., 2012) (Table 1). As genetics emerged as a prominent factor in disease, the WHO updated the classification system in 1999 to incorporate cytogenetics to help refine the subtypes (Dohner et. al., 2010) (Table 2).

1.4 Therapeutic strategies for AML

Over the past 30 years, the standard therapeutic strategy for treating AML patients has not changed substantially. Eligibility of the patient to undergo intensive induction chemotherapy and post-remission therapy is determined at the initial assessment and diagnosis (Dohner et. al., 2015). The therapeutic divergence point is 60 years of age as increased age is associated with higher prevalence of unfavourable cytogenetics, prior hematologic malignancies, higher incidence of multidrug resistance and increased frequency of comorbid medical conditions (O'Donnell et. al., 2012). Younger patients that qualify for the intensive induction chemotherapy receive high doses of anthracycline with cytarabine (Dohner et. al., 2010). Older patients may receive supportive care, low-dose cytarabine and/or hypomethylating agents (Dohner et. al., 2015).

Complete remission is defined by a blast count of <5% in the bone marrow (O'Donnell et. al., 2012). It is achieved by 40% of patients younger than 60 years of age and 5-15% of patients older than 65 years of age. Post-remission therapy is known as consolidation therapy, which involves a low to intermediate dose of cytarabine or a hematopoietic stem cell transplant (Dohner et. al., 2015). Those that do not undergo post-remission therapy experience relapse within 6-9 months after initial chemotherapy (O'Donnell et. al., 2012). However, even with post-remission therapies, approximately 70% of patients in complete remission relapse within the first three years with a poor prognosis and decreased overall survival rate due to resistance to therapy (SEER, 2016; Dohner et. al., 2010).

New treatments for AML are currently being explored to target signaling pathways, epigenetic regulation, nuclear export and antigens specific to leukemic cells in hopes of reducing the therapeutic resistance of AML, but not many have successfully been translated into the clinic. One major current challenge is the identification of predictors for a response to specific agents that will allow for combinatorial therapies with pre-existing chemotherapies (Dohner et. al., 2015).

2. The Hematopoietic System

The human hematopoietic system is one of the most well understood systems of the body (Doulatov et. al., 2012). It functions to provide the organism with a diversity of lymphoid and myeloid hematopoietic cells throughout its lifetime (Seita and Weissman, 2010). Lymphoid hematopoietic cells are NK, B- and T-cells that have adaptive and innate immune functions. Myeloid hematopoietic cells include many distinct, short-lived cell types such as granulocytes, monocytes, erythrocytes and megakaryocytes (Doulatov et. al., 2012). Aside from function, these cells vary in their lifespan, which ranges from a few days to several years (Cumano and Godin, 2007). Thus, the hematopoietic system must have high regenerative power to replenish over a trillion cells daily (Doulatov et. al., 2012). This constant turnover relies heavily on a rare population of primitive cells found in the bone marrow that possess self-renewal and multilineage differentiation potential (Doulatov et. al., 2012; Orkin and Zon, 2008). These cells are termed hematopoietic stem cells (HSCs)

which undergo the process of hematopoiesis – the expansion and differentiation of HSCs into mature, functioning hematopoietic cells of the body.

2.1 The discovery of hematopoietic stem cells

The hierarchical organization of the hematopoietic system was first postulated at the beginning of the 20th century by a Russian biologist, Dr. Alexander Maximow, when multiple cellular morphologies were observed in the bone marrow. It was theorized that there was a common ancestral cell that gave rise to the variety of cells detected (Maximow, 1909). However, at this moment in history, there was no direct scientific evidence to support the developmental hierarchy of the hematopoietic system.

By the mid-20th century, the lethal consequences of irradiation on the body urged for a deeper understanding of the hematopoietic system (Morrison et. al., 1995; O'Donnell et. al., 2012). Many exposed to irradiation suffered from bone marrow failure and ultimately, death. However, bone marrow transplants from healthy, unexposed donors rescued these defects, suggesting that there were blood-forming cells in the bone marrow that could regenerate the hematopoietic system (Lorenz, et. al., 1951). This was direct evidence for the existence of an HSC, but it was unclear whether there were one or multiple primitive cells for all the cells of the hematopoietic system.

Finally, a major scientific breakthrough occurred in 1961, when Dr. James Till and Dr. Ernest McCulloch moved the hematopoietic field towards a more functional focus. They provided experimental evidence for the existence of HSCs through an in-vivo assay, in which mouse bone marrow cells were injected into irradiated recipients and formed

colonies in their spleens. These colonies were quantifiable and appeared as gross nodules that were discrete, round and grey. Histology showed that these colonies consisted of rapidly, proliferative hematopoietic tissue with many undifferentiated cells that over time, became more mature. Till and McCulloch termed these mouse bone marrow cells as colony-forming unit spleens (CFU-S), which could give rise to not only colonies, but also produce a population of differentiated hematopoietic cells (Till and McCulloch, 1961).

In the late 20th century, human HSCs were being explored both in-vitro and in-vivo. In 1970, Dr. Beverly Pike and Dr. William Robinson found that human progenitor cells cultured on feeder layers had the ability to form colonies of differentiated cells in-vitro, on agar (Pike and Robinson, 1970). Closely following this, Dr. John Dick and his colleagues worked with humanized, immune-deficient mouse models to study human bone marrow cells injected intravenously and the ability to achieve long-term reconstitution of the hematopoietic system (Kamel-Reid and Dick, 1988; Bonnet and Dick, 1997).

The scientific observations and breakthroughs that led to the discovery and functional studies of mouse and human HSCs in the past century have greatly contributed to defining this rare cell population. As a result, we now appreciate that the HSC is a primitive cell of the hematopoietic tissue with self-renewal and multipotent differentiation potential (Orkin and Zon, 2008; Reya et. al., 2001; Krause et. al., 2001; Seita and Weissman, 2010). The self-renewal capacity inherent in HSCs confers the ability to give rise to progeny that has the identity of an HSC without undergoing differentiation. Multipotency, on the other hand, is the ability of an HSC to undergo maturation processes to become a functioning cell of the hematopoietic system (Seita and Weissman, 2010). The

HSC is crucial to the lifelong survival of an organism, and since its discovery, much effort has been devoted to its understanding as a therapeutic target in regenerative medicine.

2.2 The hematopoietic hierarchy

With the discovery of the HSC came an understanding of the larger hematopoietic developmental hierarchy. The objective of this hierarchy is to produce mature hematopoietic cells that can carry out specific functions of the body from one common progenitor cell – the HSC. Therefore, progression through the hierarchy includes a series of processes that restrict an HSC's ability to self-renew and its multipotency while increasing its differentiation potential (Orkin and Zon, 2008; Reya et. al., 2001; Seita and Weissman, 2010; Doulatov et. al., 2012).

The developmental hierarchy is organized as a triangle. At its apex sits the HSC, representing the most primitive form of a hematopoietic cell with the multipotent ability to produce all hematopoietic lineages (Kondo, 2010; Orkin and Zon, 2008; Chao et. al., 2008; Morrison et. al. 2003). Downstream is the multipotent progenitor (MPP), which has multipotent differentiation potential but lacks the self-renewal capability of an HSC. Continuing through the process of hematopoiesis, the MPP commits to the myeloid or lymphoid lineage and eventually becomes a mature, functioning cell at the base of the hematopoietic hierarchy (Orkin and Zon, 2008; Chao et. al., 2008).

There currently stand two models for the differentiation process of HSCs – a classical model and an alternative model. The classical model is a step-wise bifurcation of the lymphoid and myeloid lineages at the early stage of MPPs in the hematopoietic

hierarchy, producing a common lymphoid progenitor (CLP) and a common myeloid progenitor (CMP). CLPs further produce B-cell precursors and thymic progenitors. CMPs give rise to granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs). These progenitor cells then undergo further commitment decisions to differentiate into mature cells of their respective lineages (Reya et. al., 2001; Akashi et. al., 2000; Kondo, 2010). The alternative model suggests that the lymphoid and myeloid fates are coupled for lymphoid progenitors and that these progenitors have myelo-lymphoid potential since no single cell was found to be restricted to B- and T-cell output. The model postulates that these multipotent progenitors do not undergo a lineage bifurcation, but rather are primed for the lymphoid lineage with myeloid potential (Kawamoto et. al., 2010). Through changes in epigenetics and gene expression, these progenitors lose the myeloid potential and mature as a lymphoid cell (Kawamoto et. al., 2010; Mansson et. al., 2007). In this alternative model, myeloid progenitors follow the classical model of lineage bifurcation (Doulatov et. al., 2012).

2.3 Properties of an HSC

As previously discussed, the HSC had two definitive properties – self-renewal and multipotent differentiation potential. However, there are other characteristics that are unique to HSCs.

Unlike differentiated cells, HSCs have a high proliferative capacity and can produce a vast number of hematopoietic cells for months to years to the lifetime of the organism.

Thus, HSCs are categorized as short-term HSCs (ST-HSCs), intermediate-term HSCs (IT-HSCs) or long-term HSCs (LT-HSCs) (Doulatov et. al., 2012; Beneveniste et al., 2010). In an in-vivo functional assay, transplanted HSCs will recapitulate the hematopoietic system of an irradiated recipient, ST-HSCs are transient and can maintain a graft for approximately 6-8 weeks (Reya et. al., 2001) while LT-HSCs will provide support for longer than 12-16 weeks (Doulatov et. al., 2012). Without the self-renewal property of HSCs, an organism is unable to sustain its hematopoietic system longer than the lifetime of its mature, differentiated blood cells.

Another important property of HSCs is their quiescence. Despite their proliferative capacity, most HSCs are maintained in the G_0 phase of the cell cycle, with <2% of the HSCs population entering mitosis daily to replenish the system (Chao et. al., 2008; Ogawa, 1993; Morrison and Weissman, 1994; Kondo et. al. 1997, Akashi et. al., 2000). Experimental data shows that mouse LT-HSCs divide every 30-50 days (Cheshier et. al., 1999; Kiel et. al., 2007), with a more dormant population that divides less than five times in a mouse's lifetime (Foudi et. al., 2009; Wilson et. al., 2008). Human HSCs have been predicted to divide every 175-350 days (Shepherd et. al., 2004; Catlin et. al., 2011). The balance between quiescence and mitosis of HSCs is determined by the organism's needs. Under healthy conditions, HSCs are quiescent and the pool of hematopoietic cells are relatively stable (Cheshier et. al., 1999). Upon stress or injury, fluctuations in cell numbers induce HSCs to enter mitosis to re-establish the pool of differentiated hematopoietic cells (Chao et. al., 2008).

When an HSC enters the mitotic stage, it undergoes fate commitment with the following choices: 1) self-renew as an HSC 2) differentiate to a committed lineage progenitor or 3) become apoptotic (Passegue et. al., 2003). It is important when the HSCs pool is mitotically active that half of its cell divisions are self-renewing to maintain the HSCs reservoir (Passegue et. al., 2003; Chao et. al., 2008). The fate decisions of an HSC can be influenced by gene expression, epigenetics, and environmental stimuli and is upheld by a network of transcription factors, transcriptional and translational regulators and signaling pathways.

Thus, HSCs exist as a complex entity, balancing between self-renewal and differentiation, quiescence and mitosis, but it is these properties of the HSC that allows for a lifetime's support of the hematopoietic system.

2.4 Experimental assays for studying HSCs

The isolation of a pure HSCs population is integral for understanding their developmental biology, role and clinical use in the hematopoietic system. The first use of monoclonal antibodies targeting cell surface antigens specific to HSCs for purification was by Dr. Gerald Spangrude in 1988. They employed limiting dilution assays and magnetic cell separation techniques to determine and isolate the HSCs and progenitors. Spangrude and colleagues discovered that mouse HSCs can be identified by a Thy-1^{lo}Lin^{-}Sca1^{+} cell surface phenotype (Spangrude et. al., 1988). Similarly, human HSCs can be purified as the Lin^{-}CD34^{+}CD38^{-}CD90^{+}CD45RA- population (Majeti et. al., 2007; Chao et. al., 2008). The identification of a human HSCs cell surface antigen signature first began with the

enrichment for CD34, a ligand for L-selectin that is expressed on 0.5-5% of all hematopoietic cells in the human fetal liver, cord blood and adult bone marrow (Civin et. al., 1984; DiGiusto et. al., 1994; Krause et. al., 1996). Cells expressing CD34 were found to be heterogeneous but to have multipotent differential potential that was representative of a primitive population within the hierarchy (Seita and Weissman, 2010). The majority of CD34+ cells were also found to express CD38, a transmembrane glycoprotein involved in signal transduction (Mehta et. al., 1996). However, the most multipotent CD34+ cells, giving rise to multilineage cells were in the CD38- fraction of the population (Baum et. al., 1992; Huang and Terstappen, 1994; Miller et. al., 1999). Through mouse experimental studies, CD34+CD38- cells expressing CD90, otherwise termed Thy-1, could regenerate the hematopoietic system in their irradiated recipient while the CD90- fraction were unable to do so (Baum et. al., 1992; Guenechea et. al., 2001). Finally, CD45RA was identified as an isoform of CD45, which is a pan-hematopoietic marker specific to humans that negatively regulates cytokine signaling of select classes (Chao et. al.; 2008). These cell surface markers serve as a powerful tool now in the hematopoietic field as it permits for easy isolation of a highly-enriched HSC population using fluorescence-activated cell sorting (FACS) and flow cytometry analysis.

In-vitro studies of HSCs is most reliant on the colony-forming unit (CFU) assay, which examines the self-renewal and differentiation potential of HSCs and progenitors. As previously mentioned, Dr. Beverly Pike and Dr. William Robinson contributed to pioneering this technique (Pike and Robinson, 1970), but it was in 1990 when Dr. Heather Sutherland had established the CFU assay for studying the proliferative capacity of HSCs

for sustained hematopoiesis. Their group termed these primitive cells as long-term culture-initiating cells (LTC-ICs) since they were capable of proliferating and producing hematopoietic cells in a colony for more than 6 weeks in an in-vitro culture on bone marrow feeder cells. Within each colony, Sutherland observed cells that resembled LTC-ICs with repopulation potential and many that were differentiated and lacked this capacity (Sutherland et. al., 1990). Colonies that can be generated in a CFU assay can be classified burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte (CFU-G), colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM), colony-forming unit-granulocyte, macrophage (CFU-GM), colony-forming unit-macrophage (CFU-M) and colony-forming unit-megakaryocyte (CFU-Mk) based on the colony morphology and differentiation pattern. The types of colonies and the numbers of colonies formed from a given CFU assay provide insight into the proliferative capacity, self-renewal ability and differentiation patterns of the output LTC-ICs.

In the hematopoietic field, in-vivo studies are the gold standard as they test the functionality of HSCs in recapitulating the entire hematopoietic system of its recipient. This can be quantified by engraftment, a measure of developmental potential of donor HSCs based on their output representation over time in the recipient. The engraftment level correlates well with the activity of transplanted HSCs and can be influenced by cell number, health and recipient mouse strain. An optimized mouse model is critical as human hematopoietic studies depend largely on xenogeneic reconstitution systems and certain mouse strains may or may not provide sufficient support for the survival and growth of

HSCs. As such, much effort has been placed on developing a mouse model that supports well the representative growth of human HSCs and their progenies in-vivo.

Progress for human HSC in-vivo studies advanced following the discovery of the severe combined immune-deficient (*Scid*) mouse model. This strain of mice lacked B- and T-cells thus, permitting the engraftment of human HSCs (Fulop and Phillips, 1990; Bosma et. al., 1983). Unfortunately, independent research groups found only establishment of long-lived lymphoid hematopoietic cells in the *Scid*-hu mice (Mosier et. al., 1988; McCune et. al., 1988). Myeloid progenitors did not engraft, likely due to the presence of recipient natural killer (NK) cells and lymphokine-activated killer (LAK) cells and the absence of appropriate human growth factors (Kamel-Reid and Dick, 1988). Moving forward, Dr. John Dick and his team began working with immune-deficient *Scid* and triple-deficient *bg/nu/xid* mice. These additional mutations rendered the mice athymic and therefore, reduced NK and LAK cells, leading to the generation of long-term myeloid progenitors in-vivo (Kamel-Reid and Dick, 1988; Lapidot et. al., 1992). However, *Scid* mice still had prominent levels of innate immune function and spontaneous emergence of B- and T-cells that interfered with the engraftment of human HSCs (Doulatov et. al., 2012). To improve upon this, Dr. Leonard Shultz crossed a *Scid* mouse with a non-obese diabetic (NOD) mouse to create the NOD-*Scid* mouse model that had defects in its innate immunity, which supported higher levels of human HSC engraftment. Despite the advantages of this mouse model, there were high incidences of thymic lymphoma that prevented long-term hematopoietic studies and NK cells continued to be present as a resistance for engraftment (Shultz et. al., 1995). Finally, the NOD-*Scid* mouse model evolved to include a deletion of the IL-2R common γ

chain (NSG), a critical component for interleukin signaling. This strain of mice had a complete loss of B-, T- and NK cells, allowing for a 5-fold increase in engraftment compared to its parent strain, NOD-*Scid* mice. The additional defect in cytokine signaling also prevented lymphomagenesis, resulting in possible long-term assessment of HSC-transplanted mice (Ito et. al., 2002; Shultz et. al., 2005). Additionally, in 2010, it has been noted that sex-specific factors in NSG mice may influence the engraftment of human HSCs. Female NSG mice showed a 6-fold increase in levels of engraftment compared to its male counterparts when transplanted with limiting doses of HSCs (Notta et. al., 2010). Therefore, the NSG strain for human hematopoietic studies is currently the favoured model. Overall, humanized mouse models are integral to hematopoietic studies and the effort placed into its development of better models can enable more sensitive detection, isolation and characterization of HSCs.

In summary, the combination of FACS, flow cytometry, in-vitro CFU assays and in-vivo mouse studies have played, and continue to play, a significant role in understanding the hematopoietic system.

3. The Cancer Stem Cell Model

3.1 Models of cancer: stochastic/clonal evolution vs. cancer stem cells

Malignancies of the body are traditionally thought to follow a stochastic/clonal evolution model, which postulates that every tumour cell has the potential to self-renew and therefore, has equal opportunity to seed and support tumour growth (Shackleton et. al.,

2009; Huntly and Gilliland, 2005; Bonnet and Dick, 1997). However, the probability of a specific cell to undergo such a process is quite low (Huntly and Gilliland, 2005). This model focuses heavily on genetic mechanisms as being the driving force of tumorigenesis. It states that malignancy arises from one dominant clone, which has undergone genetic mutations that confer a selective advantage to allow it to outcompete other clones. Since the tumour originates from one cell only, the stochastic model predicts that at a developed stage, the malignancy is homogeneous and has a unique, aneuploid karyotype (Nowell, 1976). With more recent understandings of cancer and the hematopoietic system, this traditional view of tumorigenesis has been revisited.

Upon discovery of the HSC and its defining properties, the cancer stem cell (CSC) model for tumorigenesis was developed. This model is a modern-day interpretation of a theory proposed independently by Dr. Rudolf Virchow and Dr. Julius Cohnheim in the late 19^{th} century (Huntly and Gilliland, 2005; Kreso and Dick, 2014). They noted that cancer retained features of embryological development and thus, was a result from the activation of dormant embryonic tissue remnants (Huntly and Gilliland, 2005). The current CSC model asserts that a tumour is comprised of a heterogeneous population of cells, organized in a hierarchy reflective of normal development and that within this, is a small fraction of cells that are exclusively responsible for the growth and propagation of the disease (Huntly and Gilliland, 2005; Kreso and Dick, 2014; Shackleton et. al., 2009). The cells with such potential have been termed cancer stem cells (CSCs) for their shared properties with normal stem cells and the fact that they are proposed to sit at the apex of a malignant, developmental hierarchy (Bjerkvig et. al., 2005; Vedi et. al., 2016; Bonnet and Dick, 1997).

The premise of the CSC model is based on the observed heterogeneity of a tumour, in terms of its cell morphology, genetic lesions, proliferative potential, reconstitution ability, and therapeutic response, and that non-genetic mechanisms are the source of the heterogeneity (Dick, 2008; Kreso and Dick, 2014; Shackleton et. al., 2009). In parallel to the genetic mutations, altered developmental pathways and epigenetic modifications contribute to the phenotypic and functional heterogeneity. Such modifications include DNA methylation, histone modifications, chromatin remodelling, and microRNA/non-coding RNA regulation (Kreso and Dick, 2014; Dick, 2008; Meacham and Morrison, 2013; Nguyen et. al., 2012). It is important that these epigenetic changes are largely irreversible as non-tumor-initiating cells cannot revert to a CSC state and propagate disease (Shackleton et. al., 2009).

3.2 The cancer stem cell

As aforementioned, the CSCs are the tumorigenic cells at the apex of a malignant hierarchy that have undergone irreversible epigenetic changes that form phenotypically and functionally diverse non-tumorigenic cells that compose the bulk of the tumour. These cells have several biological properties, such as resistance to therapy, evasion of cell death and quiescence, that distinguish them as intrinsically different from non-tumorigenic cells. CSCs function very similarly to normal stem cells. They sustain growth and spread of tumours by producing proliferative, but malignant progenitors that are capable of repopulating the distinct cell types within the tumour and most importantly, CSCs possess self-renewal capacity (Reya et. al., 2001; Kreso and Dick, 2014). Additionally, many of the pathways found to be associated in cancer are involved in the regulation of normal stem

cell development. Thus, the similarities between CSCs and stem cells have led to the idea that CSCs are derived from mutations in a stem cell population that have allowed for defiance in normal growth regulation (Reya et. al., 2001; Bjerkvig et. al., 2005; Passegue et. al., 2003). Further speculations have been made to support this idea, such as that stem cells have activated self-renewal machinery available for manipulation and whose activity persists for a longer period of time, allowing for greater opportunity to accumulate genetic lesions and epigenetic changes (Reya et. al., 2001; Passegue et. al., 2003). Despite this theory, a number of reports have highlighted that CSCs can be created by committed progenitors or differentiated cells that undergo a de-differentiation process (Turhan et. al., 1995; Blair et. al., 1997).

3.3 Leukemic stem cells in AML

The CSC model has gained recognition since its initial postulation as there is an increasing amount of experimental evidence. However, debate as to which model is most accurate remains in the scientific community. It appears that some solid tumours display no defined CSCs subset while other cancers such as leukemia, brain, breast and colon have identified the existence of CSCs and follow the CSC model of disease propagation (Shackleton et. al., 2009; Lapidot et. al., 1994; Bonnet and Dick, 1997; Singh et. al., 2004; Al-Hajj et. al., 2003; Dalerba et. al., 2007; O'Brien et. al., 2007).

AML, along with other leukemias, has been identified as a malignancy that generally follows the CSC model (Shackleton et. al., 2009; Deshpande et. al., 2006; Krivtsov et. al., 2006). The disease is viewed as aberrant hematopoietic tissue disseminated

by a population of leukemic stem cells (LSCs), which are hematopoietic tumour cells that have acquired self-renewal and high proliferative capacities through accumulated genetic and non-genetic mechanisms (Passegue et. al., 2003; Kreso and Dick, 2014). AML blast cells have limited proliferative potential and cannot sustain the tumour bulk (Reya et. al., 2001; Bonnet and Dick, 1997). LSCs are thought to originate from a transformed HSC or committed progenitor for a number of reasons including: 1) like HSCs, LSCs are located at the apex of a hierarchy and give rise to hematopoietic tissue comprised of heterogeneous blast cells, with varying phenotypic characteristics and proliferative potential, that reflect the normal hematopoietic developmental hierarchy (Goardon et. al., 2011; Vedi et. al., 2016; Bonnet and Dick, 1997); 2) majority of AML cells in patients lack CD34 expression and stemness (Goardon et. al., 2011); 3) the long lifespan of an HSC permits the accumulation of mutations and epigenetic changes needed for LSC transformation. It has been shown that older HSCs have a differentiation program biased towards the myeloid lineage, increased expression of leukemia-associated genes and decreased gene-expression contributing to DNA damage repair, genomic integrity and chromatin remodeling, thus predisposing old HSCs to transformation (Orkin and Zon, 2008). LSCs can also be derived from a committed progenitor that has reacquired the stem cell self-renewal program (Turhan et. al., 1995; Blair et. al., 1997). Regardless of origin, LSCs have gained a stem cell state with mutations that abnormally activate signaling mechanisms and transcriptional regulators that directly interfere with the hematopoietic differentiation program.

The first enrichment for an LSC population was carried out by the laboratory of Dr. John Dick in 1994, where they identified a cell population that possessed differentiative

and proliferative potential in AML (Lapidot et. al., 1994). This was also the first CSC to be identified and isolated as part of the CSC model. The Dick lab had fractionated AML samples into various populations based on hematopoietic cell surface markers, including a CD34+CD38- population, and conducted xenotransplantation (Lapidot et. al., 1994; Bonnet and Dick, 1997). Using the *Scid* mouse model, AML-initiating cells in the CD34+CD38- fraction were identified to home to the bone marrow, proliferate extensively and recapitulate disease. Dependent on the AML subtype transplanted, the bone marrow of xenografts displayed different blast types and were reflective of the initial disease (Lapidot et. al., 1994). The CD34- fractions of these AML samples were unable to engraft, despite being transplanted at higher frequencies (Lapidot et. al., 1994; Bonnet and Dick, 1997). Leukemic cell proliferation and colony formation were seen only in mice with CD34+CD38- for most AML FAB subtypes (Lapidot et. al., 1994). An exception is the FAB M3 subtype, in which cells capable of transferring disease in serial transplantations were identified in the CD34-CD38+ fraction due to a mutation not present in CD34+CD38- cells. This suggested that the LSCs of the FAB M3 subtype of AML resides in a more differentiated cell type (Bonnet and Dick, 1997).

Interestingly, the identified LSCs did not express CD90, a cell surface marker for HSCs (Seita and Weissman, 2010; Guenechea et. al., 2001), although LSCs are thought to be derived from an HSC (Chao et. al., 2008; Blair et. al., 1997). There are two plausible explanations to why CD90 expression is lost, 1) LSCs originate from HSCs but lose expression of CD90 during the process of transformation or 2) transformations to generate LSCs occur in downstream committed progenitor cells that no longer express CD90 (Chao

et. al., 2008). To the latter point, it has been proposed that LSCs emerged in this scenario from a late stage HSCs population that expressed Lin-CD34+CD38-CD90-CD45RA., where CD90 expression had subsided (Chao et. al., 2008; Jaiswal et. al., 2003; Cozzio et. al., 2003). Importantly, LSCs do not solely reside in the CD34+CD38- fraction (Goardon et. al., 2011; Cozzio et. al., 2003). Primary AML samples have shown LSC activity in both CD34+CD38- and CD34+CD38+ cell populations (Goardon et. al., 2011; Taussig et. al., 2008). LSCs have also varying degrees of proliferative capacity. Like for LT-HSCs and ST-HSCs, there are transient and stable LSCs observed upon xenotransplantation of AML cells. Some clones in the primary xenograft are able to persistent through serial transplants, while other clones do not. In addition, new clones have also been found in serially transplanted recipients that were not detectable in the primary xenograft (Hope et. al., 2004). The varying cell surface marker identifications of LSCs and proliferative capacities are highly supportive of the different origins of an LSC, the hierarchical organization of AML and show that a small subpopulation of LSCs have extensive self-renewal potential that aggressively drives AML growth and relapse.

To experimentally validate a population of cells as containing LSCs, recapitulation of stable leukemic disease upon xenotransplantation is a necessary assay. As leukemic progenitors exist in the aberrant AML hierarchy and represent the malignant counterparts of normal hematopoietic progenitors, the CFU assay can also be utilized to similarly measure leukemic progenitors. Together, these two assays are equivalent to those used to study the normal hematopoietic hierarchy and follow the same premises. The CFU-assay examines the activity of blast progenitor cells while the xenotransplantation tests the self-

renewal capacity of a distinct population of LSCs. Since self-renewal is critical for defining LSCs, the ability to generate a xenograft that is representative of the initial leukemia and to persist through serial passages in-vivo is mandatory for conclusion of the existence of a bona fide LSC.

3.4 Clinical implications of the existence of LSCs

Uncovering the existence of LSCs and defining AML as a disease which adheres to the CSC model has significantly impacted its perception and therapy options within the clinic. Traditional treatment of AML based on the stochastic model sought to eliminate as many malignant cells as possible since they were believed to all have tumorigenic potential (Shackleton et. al., 2009). Conventional chemotherapies are largely cytotoxic to the highly proliferative cell population, but spare the relatively quiescent cells that are defined as the LSCs (Saito et. al., 2010). These dormant LSCs can seed a new malignancy given enough time for expansion, therefore the failure to respond to therapy and AML relapse cases are now viewed as a result of surviving LSC that had repopulation potential rather than an acquisition of new mutations in healthy cells (Tehranchi et. al., 2010). The characterization of LSCs have led to new therapeutic targets in signaling pathways, epigenetic regulation, nuclear export and antigens that could have increased effectiveness in promoting remission in patients as it focuses on the cause of malignancy (Majeti et. al., 2007; Jamieson et. al., 2004; Dohner et. al., 2015).

Aside from improvements in therapeutic design, the CSC model has accelerated the prognostic field of AML and other cancers. The stemness of LSCs in an AML sample can

predict and influence the clinical outcome of a patient, and thus, recent works have sought a LSC-specific gene signature that could help determine patient prognosis. In 2011, Eppert et al. performed a global gene expression analysis on functionally validated LSC fractions from AML samples with a variety of karyotypic alterations to yield a LSC-related (LSC-R) signature that identified 42 genes whose expression is enriched in LSCs. Using gene set enrichment analysis (GSEA), it was found that there were 44 leading edge genes driving an HSC program in LSCs. The implications of the LSC-R signature and its stemness are improved predictions of patient survival as those patient's whose cells express more stem cell-related genes have a worse prognosis (Eppert et. al., 2011). In 2016, Ng et al. determined a 17-gene score for LSCs (LSC17) that was highly prognostic and predictive of therapy response in diverse AML subtypes. Patients with a high LSC17 score have poorer outcomes to current therapeutic strategies (Ng et. al., 2016). The deep understanding of LSC biology and gene expression profiles have offered scientists and clinicians a powerful tool to help identify new therapies to better predict and battle AML in patients.

Overall, the discovery that many cases of AML are driven by LSCs and in turn the understanding that other cancer types conform to the cancer stem cell model, has had an extensive impact in the clinic and has opened windows of research opportunity for better clinical treatment of AML and other cancers.

4. RNA-Binding Proteins and Musashi-2 in Hematopoiesis and Leukemia

4.1 RNA-binding proteins in hematopoiesis and disease

The maintenance of HSC function requires a complex regulatory network that consists of many different mechanisms. The most efficient and rapid mechanism for altering protein expression is post-transcriptional regulation of transcribed messenger RNAs (mRNAs) (Audic and Hartley, 2004). Post-transcriptional regulation includes mRNA processing, nucleo-cytoplasmic export, mRNA localization, mRNA stabilization and translational regulation (Audic and Hartley, 2004). The two regulatory events of high interest in this thesis are mRNA stability and translation. Both of these processes are controlled through intricate networks of mRNA and protein interactions in which specific mRNAs are recognized by RNA-binding proteins (RBPs).

RBPs are critical cell regulators and take part in all aspects of RNA biogenesis, stability, function, transport and cellular localization (Glisovic et. al., 2008). In eukaryotic cells, there are hundreds to thousands of RBPs, each with a unique binding activity and protein-protein interaction characteristics (Glisovic et. al., 2008; Audic and Hartley, 2004; Anantharaman et. al., 2002). RBPs can act directly to alter translational efficiency through mRNA target binding or indirectly by bridging proteins together on mRNAs (Audic and Hartley, 2004). They were first characterized following their discovery after gel electrophoresis of UV-crosslinked nuclear extracts and RNA affinity purification coupled with mass spectrometry (Dreyfuss et. al., 1984; Pinol-Roma et. al., 1988). Specific mRNA

targets of RBPs can be elucidated using RNA-immunoprecipitation (RIP) coupled with cDNA array hybridization (Tenenbaum et. al., 2000; McHugh et. al., 2014).

In many cases, RBPs have domains that recognize and act on the 3' untranslated region (UTR) of an mRNA, which contains specific regulatory sequences, to regulate translational activation/repression or stability/instability (Audic and Hartley, 2004). RBPs may contain one or more of such domains. One of the best characterized RNA-binding domains is the RNA recognition motif (RRM) (Glisovic et. al., 2008; Burd and Dreyfuss, 1994). This domain is composed of 90-100 amino acids and can recognize single stranded pre-mRNA, mRNA, pre-ribosomal RNA and small nuclear RNA (Sakakibara et. al., 1996; Burd and Dreyfuss, 1994). The identifying feature of the RRM is its consensus sequence, comprised of two short sequences, RNP1 and RNP2, alongside other mostly hydrophobic conserved amino acids interspersed throughout the motif (Sakakibara, 1996; Gerstberger et. al., 2014; Burd and Dreyfuss, 1994).

RBPs are of interest for study because it is now known that many oncogenic mRNAs are stabilized upon binding by specific RBPs, which lead to dysregulated protein production that predisposes cells to unrestrained division and growth (Audic and Hartley, 2004). In addition, numerous genes that are not yet defined as oncogenic are often overexpressed at the protein level during cellular transformation due to increased mRNA stabilization (Audic and Hartley, 2004). Immortalized cell lines and tumours also express elevated levels of mRNA-RBP complexes compared to normal tissue. Interestingly, certain tissues, whether normal or malignant, contain specialized RBPs with 90% identified in germline, brain, muscle, liver, and bone marrow cells (Gerstberger et. al., 2014). Focusing

on the bone marrow, the presence of unique RBPs in this tissue could suggest that within more restricted subsets of the hematopoietic system there might also be specific RBPs that play a significant role in HSCs and the regeneration of the blood system. Therefore, the study of RBPs and how they contribute to disease progression can open doors to new therapeutic strategies and targets. The RBP under investigation for this project is Musashi-2 (MSI2).

4.2 The Musashi family

Musashi-2 (MSI2) is a part of an evolutionarily conserved Musashi family, from drosophila to mammals. The mammalian Musashi family consists of two RBP homologues, MSI1 and MSI2, which targets developmental transcriptional factors and cell cycle regulators. Although, the focus of this project is on MSI2, much of the knowledge about MSI2 has been based on the work on MSI1.

First discovered by Nakamura et al. in 1994, MSI1 in drosophila (d-MSI) encodes for a RBP that is essential for neural development and asymmetric cell division in the adult sensory organ system (Nakamura et. al., 1994; Sakakibara et. al., 1996). d-MSI inhibits the expression of tramtrack (TTK), a translational repressor that is involved in neuronal fate and is elevated in mature glia cells (Imai et. al., 2001; Xiong and Montell, 1993). Based on homology to d-MSI, Sakakibara et al. named the novel murine neural RBP present in neural stem cells (NSCs) of the central nervous system (CNS) as MSI1 (Sakakibara et. al., 1996). NSCs and progenitors have high expression of MSI1, which is gradually downregulated

upon neural differentiation and only low-level expression profiles persist in adult tissues (Imai et. al., 2001; Sakakibara et. al., 1996).

The MSI1 protein is 362 amino acid residues in length and is 39 kDa (Sakakibara et. al., 1996). It has two conserved RRMs with motifs RNP-1 and RNP-2 in its N-terminal half and a nuclear export signal in its C-terminus (Imai et. al., 2001). This signal is consistent with the observed localization of MSI1 in the nucleus and cytoplasm. The RRMs of MSI1 are 60% identical to those in d-MSI, suggesting homologous roles and target mRNAs in neural development in the mammalian system (Sakakibara et. al., 1996).

The target mRNAs of MSI1 are postulated to be involved in maintaining the self-renewal activity of NSCs and/or preventing the terminal differentiation of neural cells (Sakakibara et. al., 1996). One such target that has been found is Numb. MSI1 appears to activate intracellular Notch signaling through the translational repression of the Numb mRNA by binding to its 3'UTR (Imai et. al., 2001). Numb functions as a cell fate determinant and is an antagonist to Notch, which is associated with an immature and undifferentiated cellular state. The activation of the Notch signaling pathway is a positive regulator of NSCs self-renewal and maintains the proliferative state of the cell by promoting cell cycle progression while inhibiting apoptosis (Nishimoto and Okano, 2010; Audic and Hartley, 2004). Dysregulation of Notch and Numb, potentially through the Musashi proteins, could result in a selective growth advantage in Notch-activated cells.

MSI1 has been implicated to work through the process of tumorigenesis in several adult tissues. Its expression is most elevated in tissues that are undergoing early cancer

development, but tapers as the cancer advances (Nishimoto and Okano, 2010; Bobryshev et. al., 2010). This advocates for the potential that CSC stemness could be maintained by MSI1. Interestingly, the levels of MSI1 correlate to the tumour's grade of malignancy and proliferative state (Audic and Hartley, 2004). For instance, in normal human adult tissues, low levels of MSI1 are present in the brain. However, in aggressive gliomas such as glioblastoma and primitive tumours such as medulloblastoma, there is elevated expression of MSI1 compared to non-neoplastic tissue (Toda et. al., 2001).

In addition to most neural tissues, MSI1 is also found to be expressed in the intestines, breast, hair follicles, pancreas and germ cells as well, however it is nearly undetectable in the hematopoietic system, where instead, MSI2 is present at significant levels.

4.3 Musashi-2 in hematopoiesis and leukemia

Many of the observations made with MSI1 in the developing CNS have also been seen with MSI2 in the hematopoietic context. MSI2 is the second homologue in the mammalian Musashi family and it is predicted that it may have arisen from gene duplication of MSI1 (De Andres-Aguayo et. al., 2012; Barbouti et. al., 2003; Sutherland et. al., 2013). MSI2 exhibits high sequence homology to MSI1, with more than 90% identity at the amino acid level within the RNA-binding domain (Nishimoto and Okano, 2010). Full-length MSI is comprised of 328 amino acid residues and can be identified at 37 kDa on a Western blot (Barbouti et. al., 2003). The MSI2 protein however has two isoforms, the full-length and a shorter splice variant, both of which have been shown to be essential for

the process of self-renewal in embryonic stem cells (Sutherland et. al., 2013; Wuebben et. al., 2012). MSI2 is expressed in neural progenitors, hair follicles, pancreas and hematopoietic cells (De Andres-Aguayo et. al., 2012; Szabat et. al., 2011; Sugiyama-Nakagiri et. al., 2006; Kharas et al., 2010; Hope et. al., 2010; De Andres-Aguayo et. al. 2011).

MSI2 is thought to regulate mRNA activation/repression through a mechanism that halts translation initiation and ultimately suppresses the production of its target mRNAs into functional protein. The definitive mechanism through which this is thought to happen was observed for MSI1 and due to the homology between the Musashi proteins, MSI2 is believed to exert its effects in a similar fashion. Briefly, upon completion of mRNA processing, translation initiation begins with the formation of the 43S pre-initiation complex, comprised of the 40S ribosomal subunit, the initiator transferase-RNA (tRNA) and eukaryotic initiation factor 2 (eIF2). Once the initiation complex is recruited to the 5' end and recognizes the AUG start codon, eIF5 triggers guanine tri-phosphate (GTP) hydrolysis of eIF2 to release associated initiation factors. The 60S ribosomal subunit then joins and forms the final 80S ribosome to begin the process of elongation (Colegrove-Otero et. al., 2008).

The recruitment of the 43S complex requires the action of an eIF4F complex that includes eIF4G, eIF4E and eIF4A. eIF4G is an essential scaffolding protein that provides the framework for the initiation complex. More importantly, eIF4G is a binding partner of the poly-A binding protein (PABP), which binds the 3'UTR of mRNAs and circularizes it upon interaction with eIF4G, a process which is essential for efficient translation of

mRNAs (Colegrove-Otero et. al., 2008; Okano et. al., 2005). MSI1 influences mRNA translation by targeting this circularization process during translation initiation. MSI1 has binding affinity for PABP, and when bound, it sterically hinders the interaction between PABP and eIF4G (Kawahara et. al., 2008; Sutherland et. al., 2013; Byers et. al., 2011). This ultimately results in a no interaction between PABP and eIF4G, a lack of mRNA circularization and decreased translational activity of target mRNA.

In the hematopoietic compartment, translational repression of specific mRNA targets by MSI2 is believed to be a mechanism that produces pro-self-renewal effects in the primitive compartment. Through an in-vivo RNA-interference functional genetics approach, Hope et al. evaluated the role of 20 different conserved fate determinants in mouse HSCs and identified three enhancers of HSC-derived reconstitution, including MSI2. It was observed that MSI2 shRNA-mediated depletion significantly impaired HSC repopulation in-vivo and promoted differentiation in-vitro. Comparison of target genes upon MSI2 knockdown and overexpression in HSCs showed changes in the expression of regulators implicated in self-renewal pathways and cell cycling. Therefore, it was proposed that MSI2 is a stem cell regulator of the self-renewal process in the hematopoietic system and is necessary for the maintenance of HSCs (Hope et. al., 2010).

In further support of MSI2 and its pertinent role in HSCs, its expression profile indicates that it is most elevated in the primitive hematopoietic populations including LT-HSC, ST-HSC and early MPPs. As these cells progress through differentiation, the expression levels of MSI2 diminish (De Andres-Aguayo et. al., 2011; Kharas et. al., 2010; Hope et. al., 2010).

This expression profile prompted interest into the potential role MSI2 may play in leukemia, a disease that conforms to the CSC theory and is thought to arise from dysregulation of self-renewal and differentiation decision-making. MSI2 was first observed in chronic myeloid leukemia (CML) by Barbouti et al. as a novel gene that encoded an RBP that was rearranged and fused with HoxA9, a prominent gene involved in the disease (Barbouti et. al., 2003). Further studies in the murine system, including those by Ito et al., have shown that elevated MSI2 is implicated in the progression of chronic phase CML to the more aggressive blast crisis stage and that MSI2 represses the Notch inhibitor, Numb, in order to increase disease primitiveness and severity. Indeed, expression of MSI2 was found to be most enriched in the primitive fraction of blast crisis CML while Numb expression was lower than in chronic phase CML. In-vivo mouse models with increased Numb expression or loss of MSI2 resulted in the development of a more differentiated phenotype and failed disease propagation (Ito et. al., 2010). Finally, in the clinical setting, upregulation of MSI2 is associated with poor prognosis in human CML patients in support of the above findings in the mouse system (Ito et. al., 2010; Thol et. al., 2013).

The significance of MSI2 in hematopoietic malignancies extends past its initial clinical relevance in CML. MSI2 is also present in AML, particularly in FAB M1, M2, M3 and M4 subtypes and in those with common AML mutations (Byers et. al., 2011). Similar to the clinical observations with CML, the overall survival of AML patients with high MSI2 expression levels is less than those with low MSI2 expression (Byers et. al., 2011; Thol et. al., 2013; Sutherland et. al., 2013). In-vitro studies with human AML cell lines showed decreased proliferation and increase apoptosis when MSI2 is depleted (Kharas et. al.,

2010). In addition, Kharas and his team showed that MSI2 can directly influence the mixed-lineage leukemia (MLL) self-renewal program in specific AML subtypes and retain efficient translation of relevant mRNAs. Furthermore, it has been shown that mice injected with mouse AML cells depleted of MSI2 lacked overt disease, had normal levels of myeloid cells and reduced spleen size when compared to a control cohort. When disease does manifest, the leukemic burden is substantially reduced with diminished blast infiltration in bone marrow and peripheral organs. This study and others mentioned support the preposition that MSI2 is functionally required for AML progression. It appears that this protein is necessary for LSC transformation from primitive murine HSCs and is critical for the maintenance of AML that originates from mouse committed myeloid progenitors (Park et al., 2015). Interestingly though, a study led by Thol et al. did not observe any correlation between Numb and MSI2 in AML that were implicated in CML. Therefore, they suggested that MSI2 may act on several other independent signaling pathways than the Musashi-Numb axis in AML (Thol et. al., 2013).

The discovery of MSI2, its function as a positive stem cell regulator through post-transcriptional regulation and the postulated role it may have to disease progression of AML have significant impact in the scientific and clinical communities. However, as virtually all of the above mentioned functional studies have been carried out in the mouse system, much is left unexplored and there are many unanswered questions as to the role of MSI2 in human hematopoietic malignancies and its mechanism of action within these settings.

5. The Aryl Hydrocarbon Receptor Signaling Pathway

The aryl hydrocarbon receptor (AHR) is a ligand-dependent, intracellular receptor that when activated, will initiate or repress the transcription of multiple genes involved in metabolism and cell physiology (Hankinson, 1995; Denison and Nagy, 2003; Barouki et. al., 2007). The AHR evolved about 450 million years ago and is evolutionarily conserved (Hankinson, 1995; Hahn et. al., 1997). It is present in simple *Drosophila melanogaster* and *Caenorhabditis elegans* to complex mammals such as mouse and human (Nebert et. al., 2004). Moreover, its expression is typically constitutive throughout development and in certain adult tissues (Barouki et. al., 2007; Abbott et. al. 1995).

5.1 Structural characteristics of the AHR

The AHR gene is located on the human chromosome 7, within bands p21 and p15 (Le Beau et. al., 1994). In 1988, Dr. Gary Perdew and colleagues described a partial purification of the AHR and the full protein was eventually cloned in 1992 (Perdew and Poland, 1988; Dolwick et. al. 1993). The AHR contains a basic helix-loop-helix (bHLH) motif near the N-terminus and a segment of homology to two drosophila regulatory proteins, PER and SIM, known as the PAS domain at the C-terminus which mediates dimerization of the receptor (Hankinson, 1995; Nambu et. al., 1991; Barouki et. al., 2007). The bHLH motif is common in transcription factors that bind specific DNA recognition sequences as a homodimer or a heterodimer. Both the murine and human AHR proteins are similar, although there are differences found within the C-terminus. Through sequence comparison, there is 60% conservation between the species. However, in the basic region,

HLH region and PAS domain of the murine and human AHR, there is 100%, 98% and 87% conservation (Beischlag et. al., 2008). Although the AHR belongs in a large group of bHLH family of transcription factors, it is unique because of the PAS domain and its ligand-dependent activation (Hankinson, 1995). Therefore, the AHR may also have a unique functionality as a transcription factor.

5.2 The AHR is a mediator of toxicological effects

For the past 30 years, the AHR signaling pathway has been historically studied as a mediator of toxicological effects caused by environmental toxins or xenobiotics such as halogenated aromatic hydrocarbons (HAHs) and polyaromatic hydrocarbons (PAHs) (Singh et. al., 2009; Hankinson, 1995; Nebert et. al., 2004). These are often by-products of combustion and are observed as potent carcinogens. The most potent xenobiotic currently known to induce AHR signaling is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), discovered in 1957 (Hankinson, 1995; Beischlag et. al., 2008). Its exposure has been linked to medical cases of chloracne, liver diseases, blood diseases, tumours and death (Beischlag et. al., 2008).

The activation of AHR by TCDD, or other exogenous ligands, induces the transcription of the cytochrome P450 (CYP) enzymes involved in drug metabolism (Casado et. al., 2010; Hankinson, 1995; Nebert et. al., 2004; Beischlag et. al., 2008; Jones et. al., 1985). The principal cytochromes are CYP1A1 and CYP1B1, two proteins which are assessed as readouts for AHR activity in many studies including this one (Hankinson, 1995; Nebert et. al., 2004). The CYP family of proteins are responsible for metabolically

activating and detoxifying the HAHs and PAHs present from combustion products. Many of their enzymatic substrates are also ligands for the AHR, creating a negative feedback loop (Mitchell and Elferink, 2009). The activation of the AHR pathway by TCDD can further induce other phase I (oxidation), phase II (conjugation) and phase III (excretion) metabolic enzymes to regulate the toxicity of xenobiotics within a cell (Beischlag et. al., 2008; Xu et. al., 2005).

5.3 Mechanism of the AHR signaling pathway

In the absence of a ligand, the AHR resides within the cytoplasm in a complex termed the aryl hydrocarbon receptor complex (AHRC) (Hankinson, 1995). Its constituents are two molecules of the heat-shock protein 90 (HSP90), hepatitis B X-associated protein 2 (XAP2) and a co-chaperone p23 (Denison and Nagy, 2003; Barouki et. al., 2007; Perdew, 1988; Kazlauskas et. al., 1999; Meyer et. al., 1998). The role of HSP90 is to suppress constitutive AHR activity since without it, the AHR cannot bind its ligand (Carver et. al., 1994; Whitelaw et. al., 1995). XAP2 is required for the localization of the AHR to the cytoplasm and p23 prevents spontaneous formation of an activated receptor conformation in the absence of a ligand (Petrulis et. al., 2000). Together, these proteins in the inactive AHRC allows for a conformation of the receptor that permits the maximal ligand binding and protein stabilization (Beischlag et. al., 2008).

In the presence of a ligand, such as TCDD, the AHR is bound and becomes activated. Its nuclear localization signal is exposed, leading to the translocation of the receptor into the nucleus (Denison and Nagy, 2003; Pollenz et. al., 1994). To this moment,

it is unclear whether dissociation of the AHR from the AHRC occurs prior to or following nuclear translocation. Once within the nucleus, the receptor associates with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Denison and Nagy, 2003; Probst et. al., 1993). The AHR binds to HSP90 and ARNT via the HLH and PAS domains, therefore these associations are mutually exclusive (Beischlag et. al., 2008; Fukunaga et. al., 1995; Perdew and Bradfield, 1996). The AHR-ARNT heterodimer forms a high affinity DNA-binding protein that recognizes specific sequences termed xenobiotic recognition elements (XREs) (Denison and Nagy, 2003; Denison et. al., 1988; Hankinson, 1995). There are functional XRE consensus sequences conserved in all mammalian species that are required for the transcription of gene targets inducible by the AHR. The central core of an XRE consensus sequence is 5'-CGTG-3' and any substitutions to this site will either eliminate or reduce the binding affinity of the AHR (Hankinson, 1995; Lusska et. al., 1993; Shen and Whitlock, 1992; Yao and Denison, 1992). Upon interacting with DNA, the AHR-ARNT heterodimer modifies chromatin conformation by bending, stretching or looping the promoter region of the target gene to allow access of regulatory proteins for transcription activation or repression (Hankinson, 1995; Elferink and Whitlock, 1990). Immediately following nuclear translocation, ubiquitination of the AHR is triggered for rapid protein degradation (Denison and Nagy, 2003; Mitchell and Elferink, 2009).

5.4 Dietary and endogenous ligands of the AHR

Ligands for the AHR are typically hydrophobic aromatic compounds that are planar or can become coplanar in shape (Hankinson, 1995). However, despite this structural similarity between ligands, the AHR has quite a promiscuous binding site. There are

numerous dietary and endogenous ligands of the AHR, with diet providing the most exposure to AHR ligands. A few examples include flavonoids, which are bioavailable in the human bloodstream at levels that inhibit and activate the AHR pathway, and tryptophan (Trp). Dependent on its concentration, these dietary ligands can exert either agonistic or antagonistic effects within a cell (Denison and Nagy, 2003).

Activation of the AHR signaling pathway is commonly mediated by the amino acid, Trp. With exposure to visible light or UV radiation, Trp produces a class of compounds by photolysis that competitively bind the AHR with high affinity. 6-formylindolo-[3,2-b] carbazole (FICZ) is a tryptophan metabolite that has high affinity for the AHR (Denison and Nagy, 2003; Rannug et. al., 1995; Smith et. al., 2013; Wincent et. al., 2012). It is an extremely potent endogenous ligand, with a K_D value of 0.07 nM, compared to 0.48 nM for TCDD, and upregulates the expression of AHR-inducible genes efficiently and transiently (Rannug et. al., 1995; Wincent et. al., 2012). FICZ is an ideal substrate for the CYP superfamily of enzymes and thus, can be rapidly metabolized in a negative feedback manner. Activation by FICZ has many physiological effects including alterations in circadian rhythms, adaptive responses to UV light, changes in cytokine production and regulation of inflammatory responses in autoimmune disease (Wincent et. al., 2012).

5.5 The physiological role of the AHR signaling pathway

Traditionally, proteins of the bHLH family are involved with several physiological processes, such as circadian rhythms, organ development, neurogenesis, metabolism and stress response to hypoxia (Barouki et. al., 2007). Thus, it has been suggested that the AHR

signaling pathway may have a role in normal cell physiology via activation by endogenous ligands. Compelling evidence for the AHR in cell physiology comes from animal studies with AHR-null mice. It has been observed that these mice lacking the AHR have abnormal phenotypes, including lowered viability, fertility and impaired organ development (Nebert et. al., 2004; Fernandez-Salguero et. al., 1995; Lahvis et. al., 2000). The genetic profile of AHR-null mice also showed changes in expression patterns of 392 genes (Barouki et. al., 2007; Tijet et. al., 2005). These results from independent studies have implicated a role for the AHR signaling pathway in regulating the development and maintenance of mammalian tissues, more specifically, cell proliferation, apoptosis and differentiation (Barouki et. al., 2007; Singh et. al., 2009; Smith et. al., 2013; Mitchell and Elferink, 2009). It has also been shown to contribute to autoimmune responses, inflammation, cell migration and most importantly for this project, cancer progression (Wincent et. al., 2012; Smith et. al., 2013).

The AHR signaling pathway has further been studied extensively for its role in the immune and hematopoietic systems. In the immune system, it is important for the development of Th17 cells, regulatory T-cell subsets and gut-associated T-cells (Casado et. al., 2010; Smith et. al., 2013). When the receptor is constitutively active or lacking, mouse studies show immune system toxicity and dysfunction (Casado et. al., 2010; Singh et. al., 2009; Fernandez-Salguero et. al., 1995). In addition to T-cell development, the AHR pathway influences B-cells as well. It has been noted that following TCDD exposure, the immature B-cell department is substantially decreased while mature B-cells are unaffected (Singh et. al., 2009). In AHR-null mice, the opposite result is observed. The immature B-cell progenitors increased in numbers by 2-fold (Singh et. al., 2011). This suggested that

immature progenitors or stem cells could be more differentially affected by AHR activation.

Further studies with a focus on hematopoiesis has unraveled the role of AHR in stem cells biology. Phenotypically defined HSCs show expression of the AHR mRNA and protein, as well as the associated machinery necessary for signaling. From studies with TCDD exposure, the number and functionality of HSCs and progenitors are shown to be modulated by AHR activation. Upon TCDD treatment, there are elevated numbers accompanied with altered gene expression in defined LT- and ST-HSCs, but not in MPPs, CLPs or CMPs. The exposure to dioxin biased lineage differentiation towards the myeloid lineage and affected the ability of the HSCs to fully reconstitute the hematopoietic system of irradiated mice (Singh et. al., 2009; Singh et. al., 2008). AHR-null mice have been used for studying hematopoiesis in the Gasiewicz lab. Aside from the developmental abnormalities previously mentioned, they found changes in the spleen and blood. Animals had enlarged spleens and a 1.5-fold increase in cellularity. The peripheral blood showed a doubling of white blood cells and a 20% decrease in red blood cells. Most interestingly though, these AHR-null mice had elevated numbers of HSCs and progenitors with extensive proliferative activity ex-vivo and low apoptotic activity despite a downregulation of genes such as Jag-1, KitL, Tal-1, Tlr-3 and Tlr-4 that are associated with HSC maintenance, growth, differentiation and trafficking within the bone marrow microenvironment (Singh et. al., 2011). As a result of this work, the Gasiewicz group has speculated that the AHR is a negative regulator of HSC proliferation as its downstream

signaling pathway is repressed during proliferation and self-renewal, but activated during periods of quiescence.

More recent studies in the literature have shown scientific evidence towards the hypothesis that AHR activation is necessary for proper maintenance of quiescence. Experimental evidence provided by Boitano et al. have shown ex-vivo HSCs expansion with AHR inhibition. Using StemReginin-1 (SR1), a synthetic antagonist of the AHR, researchers found that repression of signaling of the AHR pathway, as a readout by decreased CYP1B1 expression, promoted in-vitro HSC growth and expansion (Boitano et. al., 2010). Another independent group hypothesized that AHR activation by endogenous ligands can regulate stem cell growth and differentiation. They differentiated induced pluripotent stem cells (iPSCs) into hematopoietic progenitor cells (HPCs) and stimulated with FICZ. After 72 hours of treatment with 0.2μM FICZ, there was a significant increase in CYP1B1 expression in HPCs, decreased apoptosis and increased cell numbers. This study also determined that expression levels of CYP1B1 were dependent on the dose of FICZ and that prolonged exposure led the iPSCs-derived HPCs to express cell surface markers indicative of differentiation and a mature phenotype (Smith et. al., 2013). Similar to Boitano et al.'s findings, this study antagonized the initial hypothesis set forth by Gasiewicz and his colleagues. These opposing observations of AHR activation in the hematopoietic system is partially attributed the ligands studied by each group and highlights the fact that the effects of AHR in cell physiology is dependent on the cell type, the context in which the cells are studied (ie. in-vitro vs. in-vivo), tissue type and identity of ligand under investigation.

5.6 Sustained vs. transient activation of the AHR signaling pathway

Since the relevance of the AHR signaling pathway in normal cell physiology relies heavily on the ligand and its characteristics, it is important to consider the timing of activation imposed by different ligands. For example, TCDD causes a prolonged response within cells because it is partially resistant to metabolism and has a half-life of seven years in the human body (Beischlag et. al., 2008; Hankinson, 1995). On the other hand, FICZ elicits a transient response within cells and has a relatively short half-life. The AHR pathway has several mechanisms for preventing inappropriate, prolonged AHR signaling, one being AHR ubiquitination and proteasomal degradation following nuclear translocation (Mitchell and Elferink, 2009; Pollenz, 1996; Reick et. al., 1994). During transient activation, this process of ubiquitination is not observed and suggests that there is no degradation fate for the receptor protein (Mitchell and Elferink, 2009; Levine-Fridman et. al., 2004). Therefore, the premise is that sustained and transient AHR signaling have different effects on the transcriptome of a cell and culminates in distinct biological responses upon receptor activation. Tijet et al. showed that over 200 genes were differentially expressed in the gene profiles of AHR-null mice compared to wild-type. This proposed that endogenous signaling contributes to numerous physiological responses that include reproduction, growth and development. Treatment with exogenous ligands to activate the AHR pathway did not elicit a response from the same genes (Tijet, 2005). Thus, this finding supported the notion that exogenous and endogenous ligands have different effects within the cell and that endogenous ligands are likely the ones responsible for initiating or maintaining biological processes for proliferation.

5.7 The AHR signaling pathway in disease

The complexity of AHR function in cells lends support to the idea that dysregulation of the AHR signaling pathway by exogenous and/or endogenous ligands can be involved in disease propagation. As a transcription factor, altered levels of the receptor protein can affect its interactions with many other co-regulators of the epigenome (Casado et. al., 2010). Subsequently, resulting in a dysregulation of critical factors that influence the cellular programs. In the case of cancer and the CSC model, inappropriate or lack of signaling by AHR may result in differentiation blocks and accumulation of immature cell phenotypes.

Alternatively, aside from its ligand dependency, AHR expression levels may be altered and cause disease. The human AHR promoter has an abundance of CpG islands that are prone to methylation and in certain leukemic lines (ie. K562), cells do not express AHR and have elevated levels of promoter methylation (Barouki et. al., 2007; Mulero-Navarro et. al., 2006). The silencing of the AHR in such malignant cells suggest a possible tumour suppressor role in hematopoietic cells. In addition, findings with AHR-null mice further illustrate that there are prominent genetic changes within the hematopoietic that are associated with HSC hyperproliferation, leukemia and accelerated aging (Singh et. al., 2014).

Additionally, the crosstalk between multiple pathways are often implicated in disease. Similar to many signaling pathways, the AHR cooperates with other signaling molecules to sustain cell proliferation and survival (Puga et. al., 2009). This is likely taken

advantage of in multiple malignancies. For example, in breast cancer cell line MCF-7 cells, the AHR synergizes with NF-KB to transactivate the proto-oncogene, *c-myc* (Barouki et. al., 2007; Kim et. al. 2000). However, the AHR signaling pathway may also cause anti-proliferative effects. The receptor protein can directly form a physical interaction with the retinoblastoma (Rb) tumour suppressor protein. Together, they function as a G1 cell cycle checkpoint regulator and have anti-proliferative activity (Barouki et. al., 2007; Puga et. al.; 2000). In Jurkat T-cells, this crosstalk of pathways is observed as constitutively active AHR can arrest cells in the G1 phase and induce apoptosis (Barouki, 2007; Ito et. al., 2004).

Research regarding the AHR as an oncogenic agent has been extensive, however, the clear complexity of its regulation across different cellular contexts and its now well validated capacity to promote human HSC differentiation, warrant further study of its potential tumour suppressor role in hematopoietic malignancies. As such, this project aims to study the AHR signaling pathway in human AML.

6. MSI2 attenuates the AHR signaling pathway in the hematopoietic system

In the healthy human hematopoietic context, it has been shown that MSI2 overexpression in HSCs leads to greater potential of hematopoietic reconstitution, both in-vivo and ex-vivo. Specifically, this is due to MSI2 overexpression promoting HSC self-renewal at the expense of differentiation. To gain mechanistic insight into this phenomenon, Rentas et al. carried out RNA-sequencing (RNA-seq) on CD34+ HSCs that overexpressed MSI2 or had MSI2 knocked down, to capture transcriptional changes

induced by modulating MSI2 levels. Differentially expressed genes were examined and an enrichment for AHR gene targets (including CYP1B1) was found in those genes downregulated by MSI2 overexpression and upregulated by MSI2 knockdown. Similarly, experiments with SR1, an AHR antagonist, showed similar downregulation of genes compared with MSI2 overexpression. When SR1 was added to control cells and MSI2 overexpressing cells in culture, the effect of SR1 on MSI2 overexpression cells was minimal relative to control. This suggested that SR1 and MSI2 converged on a similar pathway to promote HSCs self-renewal. MSI2 overexpressing cells were also grown in culture with FICZ, an AHR agonist. It restored AHR activity and reduced the self-renewal promoting effects of MSI2 overexpression, thereby further supporting the notion that MSI2 exerts its effects by downregulating the AHR signaling pathway (Rentas et. al., 2016).

Finally, through cross-linking-immunoprecipitation sequencing (CLIP-seq) analysis, significant mRNA targets of MSI2 were found to be a part of electron transport, estrogen receptor signaling regulation and metabolism of small molecules, all processes that are transcriptionally regulated by the AHR. Among the top 2% of enriched targets were HSP90 and CYP1B1, both components of the AHR signaling pathway. The uncoupling effect of mRNA and protein expression for both of these genes upon MSI2 overexpression (ie. RNA levels stayed consistent or increased while protein levels significantly decreased) indicated their post-transcriptional regulation by MSI2. The outcome of protein level of repression of HSP90 and CYP1B1 in MSI2 overexpressing cells was therefore an attenuation of AHR signaling (Rentas et. al., 2016).

It is important to note that as HSP90, through ensuring AHRC integrity and ligand binding capacity is a positive regulator of AHR signaling. This its post-transcriptional repression by MSI2 overexpression ultimately yields significant downregulation of AHR-dependent transcription. It can be considered then that the mechanisms through which MSI2 inhibits the AHR signaling pathway in HSCs is at both the transcriptional (indirect) and translational (direct) levels. Ultimately HSC self-renewal enforcement by MSI2 does converge on the translational level of regulation as reduction of CYP1B1 protein (a final effector of AHR signaling) within HSCs can cause a differentiation block and promote self-renewal (Rentas et. al., 2016).

7. Summary of intent

In summation, HSCs are at the apex of the hematopoietic hierarchy, producing all cells of the human hematopoietic system while LSCs are their malignant counterparts. LSCs can self-renew, produce immature blast cells and drive disease progression. Both HSCs and LSCs are dependent on regulatory networks, and in the healthy setting, MSI2 plays a significant role in controlling self-renewal by attenuating the AHR signaling pathway.

Working from the current knowledge and open questions in the field, I hypothesize that the inhibitory effect on the AHR signaling pathway imposed by MSI2 in the normal, healthy HSC context is potentially exploited in the LSC compartment of AML and is more pronounced in this compartment because of enhanced MSI2 levels. I further propose that

this inhibition in a developmental and differentiation pathway is a key driver of the disease progression in AML.

The objective of this project is to determine if AML disease progression can be slowed and if AML blast cells can be induced to differentiate via the activation of the AHR signaling pathway in the LSC compartment through modulation of MSI2 levels or administration of FICZ in-vitro and in-vivo.

To accomplish this project objective, I have pursued three aims:

1) To determine the functional role of MSI2 in the LSCs compartment

This aim hopes to find evidence of MSI2's role as a positive regulator of self-renewal in LSCs. It is important to note that work with MSI2 has either been in the context of mouse HSCs/LSCs or human HSCs. Therefore, it is crucial to show that MSI2 has a role in human LSC biology. To achieve this aim, human AML samples will be fractionated into LSC+ and LSC- populations. The LSC+ population will be infected with lentivirus, packaged with shRNA targeting MSI2, and effects on LSC function will be assessed through in-vitro and in-vivo experiments.

2) To evaluate the status of AHR signaling and the relationship of this pathway with MSI2 expression in the AML hierarchy

The second aim of the project focuses on exploring a potential relationship between MSI2 and the AHR signaling pathway in AML, and more specifically within the LSC compartment. I predict that this relationship will reflect the one observed in HSCs by

Rentas et al., where MSI2 downregulates the protein production of HSP90 and protein and transcript levels of CYP1B1 to prevent the differentiation effects in LSCs caused by AHR signaling. To investigate this, a series of GSEA and correlative analyses will be conducted to examine expression of CYP1B1 and other AHR gene targets with MSI2 in primary AML datasets.

3) To induce differentiation or impair leukemic progression by promoting AHR signaling through manipulation of MSI2 expression or administration of an agonist

This aim works toward reducing disease progression of AML by activating the repressed AHR signaling pathway. MSI2 expression will be modulated in human AML cells to promote activation of the receptor, as a measured by CYP1B1 levels, and effects on leukemia will be examined. In-vitro and in-vivo studies will be carried out with FICZ, the potent AHR agonist, to evaluate if direct AHR activation can occur and the effects it has on LSCs function and disease progression.

The results from this project will hold significance in the field of AML and in clinical settings. This thesis ultimately aims to offer new understanding of the mechanisms underlying the disease with the hope of uncovering opportunities to define novel effective AML therapeutic targets.

CHAPTER 2: MATERIALS AND METHODS

Mice

NOD-*scid*-*IL2Rγc*$^{-/-}$ (NSG) mice (Jackson Laboratory) were bred and maintained in the Stem Cell Unit animal facility at McMaster University. All procedures received approval of the Animal Research Ethics Board at McMaster University.

Primary AML Patient Samples

All AML patient samples (Table 3) were obtained with informed consent and with the approval of the local human subject research ethics board at the University Health Network. Healthy mobilized peripheral blood (mPB) samples were obtained with informed consent and with the approval of the local human subject research ethics board at McMaster University.

Cell Culture

Primary AML samples were thawed in PBS + 10% FBS with 100µg/mL DNAse (BD Biosciences). These samples were grown in culture in IMDM media + 15% FBS + 50µM β-mercaptoethanol + 100ng/mL SCF + 10ng/mL IL-3 + 20ng/mL IL-6 + 10ng/mL TPO + 10ng/mL FLT3. AML cell lines such as HL60, NB4, MV-411, and THP-1 were grown in culture in IMDM or RPMI 1640 base media (Gibco) + 10% FBS.

Cell Counts

Cell samples were mixed in a 1:2 dilution with trypan blue staining solution (Gibco). Viable cells were counted using the Cellometer Vision (Nexcelom Biosciences). Low cell numbers were obtained from manual counting using a haemocytometer.

Cell Apoptosis Assay

Cells were washed, then stained with 5µL annexin V 350 (Thermo Fisher) and 1.25µL 7-AAD (BD Biosciences) in 100µL of 1X annexin V binding buffer (BioLegend) for 15 minutes at room temperature, in the dark. Cells were topped up with 200µL of 1X annexin V binding buffer before analysis on the LSRII (BD Biosciences).

Cell Cycle Assay

AML cell lines were cultured with 10µM BrdU (BD Biosciences) for 3 hours at 37°C. The cells were washed and fixed with 100µL cytofix/perm solution (BD Biosciences) for 10 minutes at 4°C. Samples were washed with 1mL 1X permwash solution (BD Biosciences) and stored overnight at 4°C. The following day, cells were treated with 100µL cytoperm+ solution (BD Biosciences) for 5 minutes at 4°C and washed with 1X permwash solution. Cells were subjected to a secondary fix and wash using 100µL cytofix/perm solution for 1 minute at room temperature before its treatment with 30µg/mL DNAse for 1 hour at 37°C. After one final wash with 1X permwash solution, cells were stained with 1:50 anti-BrdU APC antibody (BD Biosciences) and 1:50 Hoechst (BD Bioscience) for flow cytometry analysis.

Colony-Forming Unit (CFU) Assay

Cells were collected and plated in a methylcellulose-based media (ColonyGel), supplemented with DMSO (Sigma-Aldrich) or 750nM FICZ (R&D Systems). Primary colonies were scored on days 10-14. For secondary CFU assays, the methylcellulose media was diluted with PBS to allow for centrifugation. Cells were then plated in fresh methylcellulose media without supplementation of DMSO or FICZ. Secondary colonies were scored on days 10-14.

Cytospin Preparation and Morphology Stains

Human primary AML cells and AML cell lines grown in suspension were collected and cytospun with Cytospin3 (Thermo Shandon) at 500RPM for 5 minutes onto glass slides. After drying, slides were stained for morphology using the Kwik-Diff Stains (Thermo Fisher) and washed. Slides were then mounted with Histomount Mounting Solution (Thermo Fisher) and imaged with the ScanScope (Aperio) at 20X magnification.

Flow Cytometry Analysis

Cells were washed with 1mL PEF (PBS + 0.2% EDTA + 2% FBS) and blocked for mouse Fc and human IgG prior to staining for flow cytometry. The blocking cocktail consisted of 1:50 rat anti-mouse Fc block and 1:10 human IgG (Sigma-Aldrich) in 100µL PEF. The staining cocktail defining human engraftment was a master mix of PEF with 1:100 CD45-APC (BD Biosciences), 1:100 CD33-PE (BD Biosciences) and 1:100 CD34-FITC (BD Biosciences). The differentiation cocktail was a master mix of PEF with 1:100 CD14-FITC (BD Biosciences), 1:100 CD15-APC (BD Biosciences) and 1:100 CD11b-PE

(BD Biosciences). Each cocktail was added as 100µL to the cells for 20 minutes at 4°C. Samples were then washed and re-suspended in PEF for flow cytometry analysis on the LSRII (BD Biosciences).

Cell Lysate and Western Blotting

Whole cell lysates were prepared by lysing cells with a small volume of 1X RIPA buffer (50mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50mM Tris pH 8.0, 1mM EDTA and Halt Protease Inhibitor Cocktail (Thermo Fisher) for 10 minutes on ice and then centrifuged at maximum speed for 10 minutes at 4°C. The supernatant was collected and quantified using the Bradford assay (Bio-Rad). Protein samples were normalized to 1µg/µL in 1X NuPAGE LDS sample buffer (Thermo Fisher) with β-mercaptoethanol (Sigma-Aldrich) as a reducing agent and boiled for 5 minutes at 95°C prior to electrophoresis.

Protein samples of 15-20µg were loaded into individual wells of a 4.5% stacking and 10% resolving acrylamide gel and ran at 100V for 1-1.5 hours. The protein was transferred onto PVDF membranes (Li-COR Biosciences) using a wet-transfer system at 200mA for 2 hours. The membranes were blocked with 0.5% BSA in 1X TBS-T (TBS + Tween 20) for 30 minutes at room temperature prior to an overnight incubation with primary antibodies at 4°C. The following primary antibodies were used at a 1:1000 dilution in 0.5% BSA blocking buffer for each western blot: rabbit-anti-Msi2 (Abcam), rabbit-anti-CYP1B1 (Abcam), rabbit-anti-α-tubulin (Cell Signaling Technology) and mouse-anti-β-actin (Cell Signaling Technology). The following day, membranes were washed 3X for 5 minutes each with 1X TBS-T and incubated with secondary antibodies for 1 hour at room

temperature. The following secondary antibodies were all used at 1:10 000 dilution in 1X TBS-T: IRDye 680 goat-anti-rabbit (Li-COR Biosciences) and IRDye 800 goat-anti-mouse (Li-COR Biosciences). Membranes were then washed 3X with 1X TBS-T for 5 minutes each and imaged with the Odyssey Classic Imager (Li-COR Biosciences).

Immunocytochemistry Staining

The immunocytochemistry staining of cells was completed by Dr. Stefan Rentas. Cells were collected and fixed with 100µL 4% PFA for 10 minutes at room temperature. Samples were washed and re-suspended in PBS, up to a maximum of three days in storage at 4°C, and then cytospun onto glass slides at 800RPM for 5 minutes. Cell spots on the slides were incubated with 200µL perm buffer (0.2% Triton + PBS) for 20 minutes at room temperature and washed in PBS. Slides were then incubated with 50µL blocking buffer (10% goat or donkey serum + 0.1% saponin + PBS) for 30 minutes at room temperature, and then washed. Cell spots were stained with primary rabbit-anti-CYP1B1 (Abcam) antibody at a 1:30 dilution for 1 hour at room temperature. After a wash with PBS, cell spots were incubated with secondary antibodies at a 1:2500 dilution for 1 hour at room temperature, in the dark. Slides were then mounted with 20µl of thawed Prolong Gold mounting media and DAPI (Invitrogen) and imaged on the Operetta (Perkin Elmer).

RNA Extraction and RT-qPCR

For qRT-PCR analysis, total cellular RNA was isolated with Trizol LS reagent (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was completed using the qScript cDNA Synthesis Kit (Quanta Biosciences). qRT-PCR was done in

triplicate with PerfeCTa qPCR SuperMix Low ROX (Quanta Biosciences) with gene specific probes (Universal Probe Library, Roche) and primers (Table 4). The mRNA content of samples compared by qRT-PCR were normalized based on the amplification of GAPDH.

Lentivirus Production

Short hairpins for knocking down MSI2 in human were designed and validated by Dr. Nicholas Holzapfel. The hairpin sequences targeting a RFP control (shControl) and MSI2 (shMSI2) were cloned into the H1GIP vector (Sch. 2), expressing GFP. HEK293FT cells were cultured with Geneticin (Gibco) prior to its transfer into T150 flasks (Corning). At 80-90% confluency, cells were transfected with 23µg H1GIP vector targeting RFP or MSI2, 9µg pMDG.2 and 13µg psPAX2 using Lipofectamine LTX (Invitrogen). Transfection efficiency was determined with green fluorescence using the EVOS microscope (Thermo Fisher). After 48-60 hours, the supernatant was collected, filtered and spun at 25000RPM for 2 hours at 4°C to concentrate the lentivirus. The viral titer was determined by flow cytometry with serial dilutions in HeLa cells.

Knockdown of MSI2 in AML

For in-vitro studies, primary AML samples were transduced overnight with lentivirus targeting RFP or MSI2 at an MOI of 30. AML cell lines were transduced with lentivirus targeting RFP or MSI2 (Table 5) at an MOI of 10 for 48 hours. All samples were sorted for green fluorescence.

For in-vivo studies, primary AML samples were sorted for the CD34+ population and transduced overnight with lentivirus targeting RFP or MSI2 at an MOI of 50. NSG mice were irradiated 24 hours prior to transplantation. Each mouse received a dose of $1x10^5$ CD34+ AML cells via intrafemoral injection. Excess cells were left in culture for 3 days to determine the transduction efficiency by flow cytometry. After 8 weeks following xenotransplantation, peripheral blood from mice was collected via tail-vein to assess engraftment levels. Mice were sacrificed after 12 weeks and bone marrow were processed for flow analysis of CD45+ CD33+ human engraftment (Sch. 4).

FICZ-treated Xenografts

Prior to xenotransplantation, primary AML samples were treated in-vitro overnight with DMSO or 750nM FICZ. Mice were irradiated 24 hours before transplant and given a sample-dependent dose of cells, ranging from 1 to 5 million cells, via intrafemoral injection. After 7 days, post-transplant, mice were weighed weekly and given intraperitoneal injections of DMSO or 100-500µg/kg of FICZ for 4 weeks. FICZ was dissolved in DMSO, then diluted in 30% Captisol (Captisol) and PBS for a total volume of 1mL for intraperitoneal injections. Mice were sacrificed by cervical dislocation and the bone marrow and spleen were collected for analysis of CD45+ CD33+ human engraftment.

Processing of Bone Marrow and Spleen

Bone marrow and spleen were harvested from the mice upon cervical dislocation and kept in IMDM + 3% FBS on ice. The right femur, or the injected femur, was separated from the rest of the bones. Sample tissues were crushed twice with a mortar and pestle, and

filtered through a 40µm cell strainer before centrifugation at 1500RPM. Red blood cells in samples were lysed using ammonium chloride (Stem Cell Technologies) for 10 minutes at 4°C. Cells were centrifuged and re-suspended in PEF for downstream analyses.

Suspension Cultures with FICZ

AML cell lines were seeded at a density of $1x10^5$ cells/mL in media supplemented with DMSO or varying concentrations of FICZ.

For the HL60 experiment, the range of FICZ doses were 200nM, 300nM, 500nM and 750nM. All cells were replated with freshly supplemented media after collection for analysis. At day 10, FICZ was removed and cells were replated with DMSO only.

For the remaining AML cell lines experiments, the range of FICZ doses were 1µM, 3µM and 5µM. Cells were replated at a density of $1x10^5$ cells/mL in freshly supplemented media after each day of analysis.

Suspension Cultures with RA

NB4 cells were cultured at a density of $1x10^5$ cells/mL in media varying in doses of RA (125nM-1µM) (Sigma-Aldrich) to generate a dose-response curve. Cells were replated at the same density of $1x10^5$ cells/mL in newly supplemented media after each analysis.

Suspension Cultures with FICZ and RA

NB4 cells were treated in-vitro with FICZ and RA using two different approaches. Cells were plated at a density of $1x10^5$ cells/mL in media supplemented with 1) DMSO or

3μM FICZ and 100-125nM RA for the duration of the experiment and 2) DMSO or 125nM RA for 48 hours, then 3μM FICZ for the duration of the experiment. Cells were replated at the same density of $1x10^5$ cells/mL in freshly supplemented media after collection for analysis.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software version 6.0). Unpaired student t-tests were performed with $p<0.05$ as the limit for statistical significant.

GSEA and gene correlation analysis were completed by Dr. Stefan Rentas with data provided by Dr. John Dick's laboratory.

CHAPTER 3: RESULTS

MSI2 has a functional role in LSCs and AML disease propagation

The role of MSI2 in HSCs has been thoroughly well studied in both murine and human systems, however, evidence of MSI2 expression and its function in LSCs are lacking in the field. Therefore, experiments were designed to assess the expression and importance of MSI2 in LSCs. Optimization of experiments and the results presented regarding MSI2 knock-down (KD) in primary LSC+ populations in Fig. 1 were completed by Dr. Nicholas Holzapfel. His collection of unpublished work is foundational to this project and therefore, will be discussed.

Although MSI2 expression is upregulated in AML, its expression in a human LSCs population has yet to be determined. With expression data from 78 primary patient samples generated by Dr. John E. Dick's laboratory and intracellular flow cytometry staining for MSI2 protein, it was observed that MSI2 mRNA transcript (Fig. 1A) and protein (Fig. 1B) levels are significantly elevated within the CD34+ LSC-enriched (LSC+) fraction compared to the LSC- (CD34-) fraction. Intracellular flow cytometry analysis revealed that 80% of CD34+ primitive cells express MSI2, while only 50% of CD34- differentiated cells are positive for MSI2 staining (Fig. 1B; right). Distribution patterns within the two cell fractions from intracellular flow cytometry further showed a directional expression profile for the CD34+ fraction and a scattered expression profile for the CD34- fraction.

After concluding that MSI2 transcript and protein expression are elevated in LSCs, its function was to be assayed through KD in-vitro and in-vivo studies. Dr. Nicholas

Holzapfel generated a lentiviral construct in the H1GIP vector backbone containing short-hairpin RNAs (shRNAs) that targeted RFP (shControl) or MSI2 (shMSI2), and showed successful reduction of MSI2 protein in the AML FAB M3 NB4 cell line by western blot (Fig. 1C).

The validated shRNAs were used to infect primary AML samples in colony-forming unit (CFU) assays to examine the effects of MSI2 KD on progenitor activity. In the four primary AML samples tested, shControl and shMSI2 showed no difference in their colony formation ability (Fig. 1D). Next, in-vivo studies were carried out to establish potential effects MSI2 KD may have on LSCs and their ability to survive in a living system. Primary AML samples were sorted for the CD34+ population and infected overnight with lentiviral constructs prior to intrafemoral transplantation in NSG mice. After 12-weeks, mice were sacrificed and analyzed by flow cytometry for levels of engraftment (Fig. 1E). The human myeloid cell surface markers, CD45 and CD33 respectively, exhibited significantly higher levels of engraftment in the injected femurs of mice with shControl LSCs than mice with shMSI2 LSCs across the majority of the primary AML samples used (Fig. 1F). The observed lowered engraftment of LSCs infected with shMSI2 in the injected femur was reflected in a similar lowered engraftment trend in the remaining bone marrow (Fig. 1F).

The lentiviral constructs contained an additional green fluorescent protein (GFP) as a marker for successful transduction. For one AML sample where infection was at an intermediate level and could permit an output/input analysis of GFP levels within the graft as compared to the original GFP level in the transplant cell sample, GFP fluorescence was

visualized in infected LSCs with shControl and shMSI2 at 12-weeks post-transplantation. A significant decrease in GFP+ cells was observed in the human grafts within mice transplanted with shMSI2 LSCs in both the injected femur and whole bone marrow for primary AML sample 90191 (Fig. 1G).

Cumulatively, these results showed that MSI2 is preferentially expressed in the LSC population advocating for functional role for MSI2 in AML within this specific cell fraction. The MSI2 KD experiments provided important evidence that MSI2 is important for the ability of LSCs to engraft and survive within an in-vivo system to propagate AML.

MSI2 expression correlates with decreased AHR signaling in LSCs

The AHR signaling pathway has a function in hematopoiesis and the development of hematopoietic diseases with certainty. However, the literature has contrasting data and no understanding of the role AHR signaling pathway may play in human AML. This project hypothesizes that AHR signaling is inhibited by MSI2 expression and thus, contributes to disease propagation. To provide support for this idea, bioinformatic analyses on primary patient samples and AML databases were completed to examine the expression of the AHR and its pathway components in AML and more specifically, LSCs. The analyses and results presented were performed by Dr. Stefan Rentas.

To determine if the AHR signaling pathway is inhibited in LSCs, it is important to validate the expression of the AHR within this cell population to ensure competency for pathway activation and signaling. Expression data from 78 primary AML samples showed

no difference in AHR transcript levels between the LSC+ and LSC- cell fractions (Fig. 2A). However, when transcript levels of CYP1B1 were analyzed as a measure for AHR signaling activity, the LSC+ population had decreased CYP1B1 mRNA transcript expression (Fig. 2C).

Upon the confirmation that AHR is expressed in LSCs and that its pathway activity is reduced, a correlative analysis between MSI2 expression and CYP1B1 expression was carried out. The expression data from primary AML samples showed that in both the LSC+ and LSC- populations, CYP1B1 expression correlated negatively with MSI2 (Fig. 2B and 2D). In other words, AML samples with high MSI2 expression had low CYP1B1 (Fig. 2D).

In addition to CYP1B1 expression levels, expression of potential AHR gene targets, as predicted by iRegulon, from fractionated AML samples in the Microarray Innovation in Leukemia (MILE) study were investigated. The MILE study assessed the gene expression profiles of 16 acute and chronic leukemia subclasses, MDS and a non-malignant control group from untreated patients at the time of diagnosis using commercially available whole-genome microarrays (Haferlach et. al., 2010). Gene set enrichment analysis (GSEA) comparing the LSC+ population to the LSC- population resulted in a negative normalized enrichment score (NES) of -1.349 (Fig. 2E) which indicates that the expression of AHR target genes in the LSC+ cell fraction is indeed lower than in LSC- cells.

Further analysis of downregulated AHR gene targets from the MILE study revealed leading-edge genes that were also reduced in expression in LSCs, other unfractionated AML subtypes and CML relative to healthy bone marrow (Fig. 2F). The LSC population

showed a low expression profile for all the leading-edge genes while other unfractionated AML subtypes had decreased expression for certain gene targets. CYP1B1 expression is downregulated across all leukemic subtypes except myelodysplastic syndrome (MDS), and is most prominent in the AML t(15;17) FAB M3 subtype.

Next, an analysis was performed wherein AHR targets, as validated by chromatin immunoprecipitation sequencing (ChIP-seq), were evaluated for their expression in LSCs, unfractionated AML, CML, and MDS relative to healthy bone marrow to seek additional evidence of compromised AHR signaling in AML (Fig. 2G). Global downregulation of validated direct AHR target genes was observed in LSCs, all unfractionated AML subtypes, and CML. Within AML samples specifically, the LSC population had the lowest number of target genes downregulated consistent with the concept that AHR signaling is restricted in this compartment while in bulk disease, the AML t(15;17) subtype had the highest number of target genes affected and MDS samples showed the least number of downregulated targets.

Additional GSEA was completed for multiple unfractionated AML subtypes, CML and MDS in comparison with healthy bone marrow (Fig. 3A). Most unfractionated AML subtypes, with the exception of AML with complex karyotype, and CML yielded a negative NES value and downregulation of AHR target genes. In contrast, samples of MDS produced a positive NES and showed upregulation of AHR target genes relative to healthy bone marrow intriguing as MDS is often a precursor to leukemia and thus, is closest in cellular profile to normal bone marrow.

To expand the ChIP-seq data analysis and as most unfractionated AML subtypes showed downregulation of AHR gene targets, analysis for overlap of downregulated gene targets in AML subtypes compared with healthy bone marrow was completed (Fig. 3B). Each solid grey circle represents the leukemia in question. The LSC population, denoted by a green circle, is relative to a non-LSCs population. High overlaps of downregulated gene targets were observed between a single subtype and healthy bone marrow. The greatest overlap of downregulated gene targets between various subtypes is between AML with normal karyotype, AML t(11q23) subtype, AML inv(16) subtype, AML t(15;17) subtype and AML t(8;21) subtype. Many other comparison analyses between subtypes yielded low overlapping gene targets. Drawing attention to the LSC population, there are 15 overlapping AHR gene targets detected by ChIP-seq between the LSC+ fraction and LSC- fraction. However, when these two fractions are compared with other AML subtypes, there was only small numbers of genes that overlapped.

In summation, these bioinformatic analyses demonstrate that the expression pattern of CYP1B1 and numerous other AHR gene targets support that AHR signaling activity is decreased in multiple AML subtypes, in CML and most interestingly, in the LSC population. These downregulated target genes of AHR also vary between and may be unique to AML subtypes and cell populations. Intriguingly these data also suggest that there may be a unique cell-context specific downregulation of specific AHR targets in LSCs.

MSI2 KD promotes the activation of the AHR signaling pathway in AML

With MSI2 expression and function explored and the speculated downregulation of AHR signaling activity specifically in LSCs, the investigation proceeded to focus on the relationship between MSI2 and AHR in the leukemic context. To study this possible inhibitory phenomenon of MSI2 on AHR signaling, the level of MSI2 protein was modulated to assess the effects on the AHR pathway in LSCs and on disease progression with in-vitro and in-vivo experiments.

In-vitro suspension cell cultures of AML cell lines were infected with the validated shControl and shMSI2 lentivirus (Fig. 1C) and AHR signaling measured using CYP1B1 expression as a well-validated proxy. After seven-days post-transduction, CYP1B1 protein was qualitatively and quantitatively determined by western blot and band fluorescence intensity respectively in NB4 (Fig. 4A) and THP-1 (Fig. 4B) cells. Both cell lines infected with shMSI2 showed an upregulation of CYP1B1 protein, represented by elevated signal in the protein bands at 75kDa. Fluorescence intensity of these species on the western blot were quantified relative to the loading control and revealing that in both NB4 and THP-1 cells, the fluorescence intensity was approximately twice as high in shMSI2 compared to shControl settings.

Primary AML samples were infected similarly with shControl and shMSI2 lentivirus in overnight in-vitro suspension cultures to achieve effective MSI2 KD after which AHR signaling activity was measured by evaluation of CYP1B1 protein levels. Due to limitations in the numbers of cells available from primary AML cells,

immunofluorescence was carried out in place of western blots for protein expression. Here, a clear increase in fluorescence intensity for CYP1B1 staining was observed in AML cells treated with shMSI2, confirming a significant increase of CYP1B1 in both primary AML samples infected with shMSI2 relative to shControl (Fig. 4C and 4D).

Finally, in-vivo studies carried out with primary AML sample 100348 wherein MSI2 KD was achieved via lentiviral infection followed by the xenotransplantation of transduced cells (Fig. 1) allowed for determination of AHR signaling by immunofluorescence in collected bone marrow cells at the transplantation endpoint. Levels of CYP1B1 protein had indeed significantly increased qualitatively and quantitatively by multiple folds in these grafts as a result of MSI2 repression (Fig. 4E).

The presented results derived from these MSI2 KD experiments in AML cell lines and primary samples support the inhibitory nature of MSI2 on the AHR signaling pathway. It can be concluded that as occurs in likewise to HSCs, MSI2 attenuates AHR signaling in the leukemic context at least in part by post-transcriptional regulation of CYP1B1.

Stimulation of in-vitro AML cultures with AHR agonist impairs leukemic proliferation

After obtaining evidence that supported AHR signaling inhibition imposed by MSI2 in LSCs, it became imperative to interrogate the effects of activating AHR to evaluate the effects of this stimulation on LSCs biology and AML progression, both in-vitro and in-

vivo. We relied on administration of the well characterized and potent agonist of AHR, FICZ, to address this.

Various AML cell lines (HL60, MV411 and NB4) were first treated with FICZ, with varying doses over a time-course. As toxicity was not previously determined, the experimental doses used were within a range of observed tolerance. The HL60 cell line was the most responsive to FICZ treatment, and showed significantly decreased cell proliferation in a dose-dependent manner relative to the DMSO control by day three of the time-course (Fig. 5A). However, the impairment in cell growth did not reach the extent of the positive control, retinoic acid (RA). Additional cell physiological processes, such as cell cycling and apoptosis, were assessed. HL60 cells treated with FICZ had a lower percentage of cells entering the S phase of the cell cycle (Fig. 5B). This effect was also observed to be dependent on FICZ concentration. Flow cytometric analysis of treated HL60 cells for Annexin V positivity, an early apoptotic marker, did not exhibit an increase in apoptosis but rather a significant decrease relative to DMSO control (Fig. 5B).

Following up on these observations, the reversibility of AHR signaling activation by FICZ on HL60 cells was evaluated. Previously FICZ-treated HL60 cells were cultured in medium supplemented with DMSO over a time-course. The effects of FICZ on cell proliferation is irreversible as FICZ-treated HL60 cells were unable to recover cell numbers (Fig. 5C). Low-dose FICZ treatment of HL60 cells showed some recovery, but at a slower rate relative to DMSO controls.

MV411 and NB4 cells were additional AML cell lines treated with FICZ in-vitro. These cells were unresponsive to the low-dose FICZ used with the HL60 cell line. Thus, higher doses in the micromolar ranges were attempted. MV411 cells treated with high-dose FICZ showed defective cell proliferation relative to DMSO control after day four of the time-course (Fig. 5D). NB4 cells were observed to have insignificant decrease in cell proliferation after treatment with high-dose FICZ compared to DMSO control (Fig. 5E).

Since NB4 cells remained unresponsive to 5μM of FICZ, these cells were further investigated to determine the reason for their resistance to AHR signaling. I first completed a dose-response curve with RA treatment alone and show that RA can induce detrimental effects in NB4 cells at 1μM concentration down to a concentration as low as 125nM (Fig. 6A). When treated with low-dose RA and high-dose FICZ simultaneously, NB4 cells were found to have significantly decreased cell proliferation relative to DMSO and FICZ controls (Fig. 5F).

The possible synergistic effect between RA and FICZ was further explored with NB4 cells in-vitro experiments. Morphological stains of NB4 cells treated with low-dose RA and high-dose FICZ in combination had pronounced changes in cell appearance compared to RA- and FICZ-independent treatments (Fig. 6B). These changes were consistent with an enhanced differentiation state of the myeloid cells as FICZ and RA cells exhibit much smaller nuclei and were larger in size. Moreover, NB4 cells became apoptotic when treated with RA and FICZ, with observed increases in Annexin V staining by flow cytometry analysis (Fig. 6C). Higher doses of RA with FICZ induced greater apoptotic effects.

The findings of synergy with RA and FICZ treatment of NB4 cells led us to investigate RA necessity for AHR activation by FICZ within this specific cell lines. Experimental strategies were employed that aimed to test if RA was essential to initiate the response to FICZ and the ability of FICZ to maintain the effects of RA in NB4 cells. Cultures were treated with RA for 48-hours, then media was changed for media supplemented with DMSO (RA-DMSO) or high-dose FICZ (RA-FICZ) over a time-course. Impaired cell proliferation was observed in both RA-DMSO and RA-FICZ conditions (Fig. 6D). However, unlike RA-DMSO cells, RA-FICZ treated NB4 cells demonstrated limited recovery and remained significantly low in terms of cell numbers relative to control cells. Morphological evaluation revealed alterations to cell appearances in RA-FICZ NB4 cells that are absent in control conditions, such as multiple vacuoles and irregular cell shapes (Fig. 6E). Flow cytometry analysis unveiled significantly greater expression of apoptotic markers, 7AAD and Annexin V, in NB4 cells treated with RA and FICZ (Fig. 6F and 6G) relative to DMSO and RA-DMSO conditions.

Together, this data has demonstrated AHR signaling activation by FICZ in AML cell lines in-vitro, leads to detrimental effects including decreased cell proliferation and enhanced cell death and can synergize with effects induced by existing pro-differentiative therapeutic compounds such as RA.

Primary human AML cells respond to AHR activation by potent agonist in-vitro

To examine the anti-leukemic effects of enforcing AHR signaling beyond the context of immortalized AML cell lines, I next explored the effects of FICZ addition to primary AML samples in-vitro. CFU-assays were conducted with optimal cellular density for each AML sample in methylcellulose supplemented with DMSO or FICZ. At the two-weeks time point, colony scores revealed a decreasing trend in colony formation across all primary AML samples, except AML 596, when treated with FICZ (Fig. 7A). Primary AML samples 90191 and 100348 reached statistical significance. In addition, FICZ-treated AML samples often formed colonies that appeared sparse, irregularly-shaped and smaller relative to the control colonies that were dense, round and large. Primary AML sample 100348 and 596 were capable of forming secondary colonies after serial replating (Fig. 7B) though in both cases, replating efficiency appeared dampened.

In order to more conclusively attribute the observed defects in colony formation to AHR signaling initiated by FICZ, analyses of CYP1B1 mRNA transcripts and protein were performed on overnight DMSO or FICZ suspension cultures of primary AML samples. Relative to DMSO control, all samples showed an upregulation of CYP1B1 mRNA transcript and no changes in expression of AHR (Fig. 7C). Increased CYP1B1 protein level, as indicated by western blot, was seen in primary AML sample 90191 after overnight treatment with FICZ (Fig. 7D), emulating results from MSI2 KD (Fig. 4).

To summarize the results from these in-vitro experiments with primary AML samples, treatment with FICZ decreases colony formation and increases CYP1B1 mRNA and protein expression.

Activation of AHR by FICZ in-vivo impairs AML progression in mouse models

Results from the in-vitro primary AML studies provided rationale for investigating the effects of AHR activation by FICZ on LSC function and AML progression in an in-vivo model system. Primary human AML samples were treated for 16-hours in-vitro with DMSO or FICZ, and were then injected intrafemorally into NSG mice. One-week post-transplant, NSG mice were given DMSO or FICZ intraperitoneally for four-weeks before sacrifice (Sch. 5). Of note, both the dose of FICZ and its frequency of administration were chosen based on those that had previously been utilized in mice and shown to exhibit no detrimental effects on their physiology (Duarte et. al., 2013; Wheeler et. al., 2014)

At the transplant endpoint, flow cytometry analysis of human myeloid markers, CD45 and CD33, revealed the levels of engraftment in each mouse per condition for each primary AML samples (Fig. 8A, 8C-8E). Two independent in-vivo experiments with primary AML sample 90191 showed lower engraftment in NSG mice treated with 100µg/kg FICZ compared to the DMSO cohort (Fig. 8A). Of note, in the second experiment where the 90191 sample was transplanted at a lower cell dose to achieve a reduced level of leukemic engraftment, AHR agonism elicited a more pronounced graft reduction relative to control-treated mice suggesting that higher doses of FICZ may need to be administered

when leukemic is very elevated. In addition to engraftment levels, primary AML sample 90191 was stained for CD34+ expression. Detection of CD34+ in FICZ-treated mice were lower than the DMSO control animals (Fig. 8B). Importantly, this impairment in the ability of primary 90191 AML cells to engraft upon FICZ treatment in NSG mice was observed for four other primary samples tested in-vivo with 250µg/kg FICZ (Fig. 8C-8E). The plotted engraftment levels were representative of an adjusted average for the injected femur and bone marrow (contralateral femur, tibias and pelvis). Some primary AML samples did not elicit a response to the in-vivo 100µg/kg FICZ treatment (Fig. 8F).

To attempt to understand why some primary AML samples responded (responders) and some did not (non-responders) at the FICZ doses tested, mRNA transcripts of CYP1B1 and AHR of harvested cells from treated xenografts were measured for a subset of samples (Fig. 8G). Relative to DMSO control, AHR transcripts were expressed at slightly higher levels in the responders than non-responders. However, the most notable difference was in CYP1B1 transcript expression. Responders to FICZ in-vivo had significantly elevated levels of CYP1B1 mRNA expression with respect to the DMSO cohort than non-responders.

In-vitro pre-treatment of primary AML samples with FICZ may contribute to lower engraftment levels and lead to an overemphasis on the effects of FICZ in-vivo. Therefore, to address this, mRNA expression of CYP1B1 prior to transplant (after in-vitro FICZ treatment) and post-transplant (after in-vivo FICZ treatment) were analyzed (Fig. 8H). Responders to FICZ, as represented by light grey bars, exhibited increased levels of CYP1B1 and AHR transcripts post-transplant while non-responders failed to demonstrate

this upregulation which suggests that a deficiency in in-vivo enhancement of AHR signaling, potentially due to insufficient amount/frequency of FICZ administration, that contributes to sample non-responsiveness. This finding also supports the concept that in-vivo AHR agonism is key to the anti-leukemic effects observed for responding samples.

Finally, secondary xenotransplants were completed with harvested primary AML sample 90191 cells to determine effects on long-term self-renewing LSCs in-vivo. NSG mice were injected intrafemorally with two different doses of human AML-engrafted murine bone marrow harvested. No further intraperitoneal injections with DMSO or FICZ were given to secondary animals. Results revealed that only the high-dose of injected cells from DMSO-treated primary mice were able to produce grafts comparable in size to that in the primary mice. The levels of engraftment showed a pronounced effect previously observed in primary xenotransplants in that DMSO-treated human AML cells produced a larger graft in NSG mice than FICZ-treated AML cells (Fig. 8I) suggesting that long-term LSCs were indeed significantly repressed within the primary treated mice.

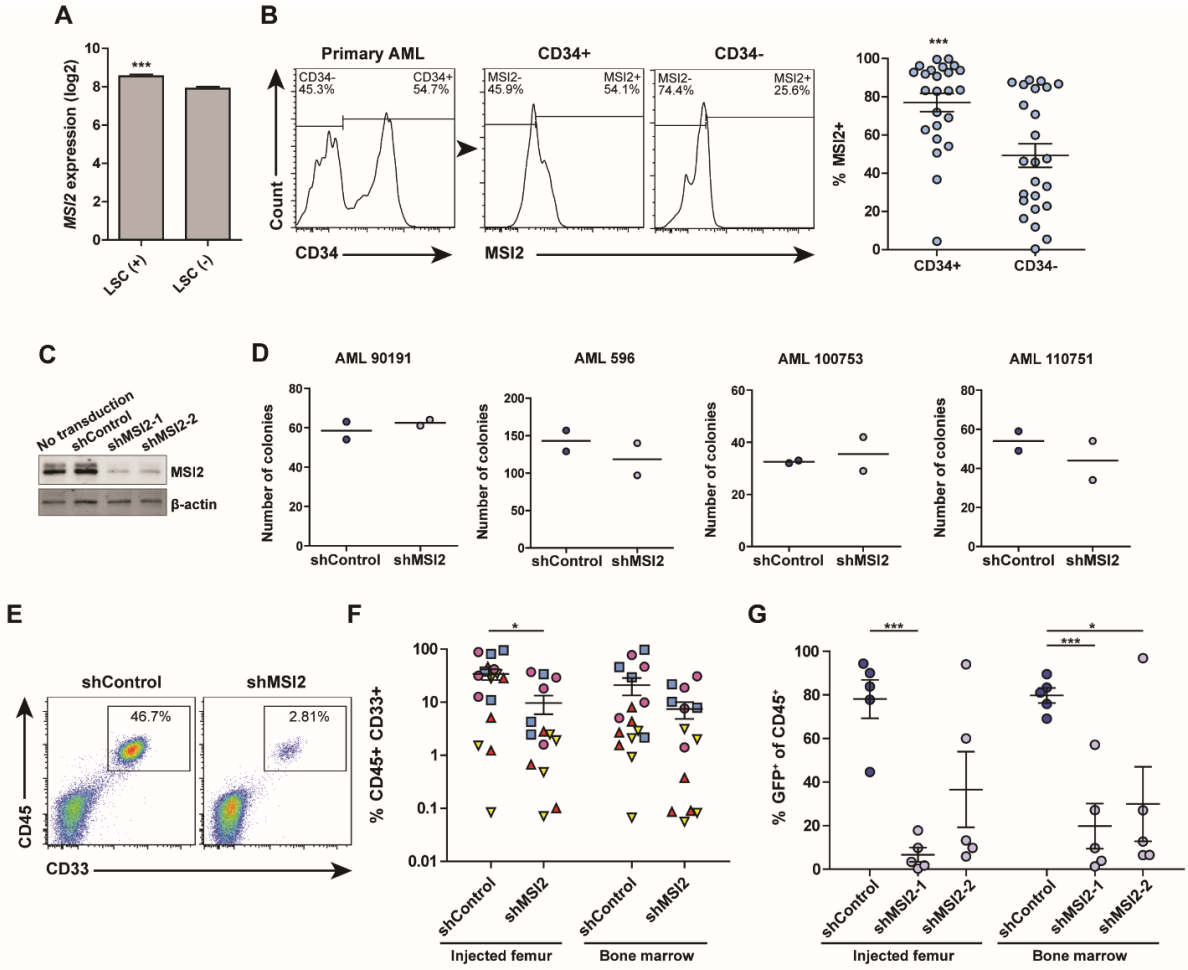

Figure 1: MSI2 is preferentially expressed in AML LSCs and supports LSCs activity in-vivo. A) MSI2 transcript levels from primary AML samples fractionated into LSC+ and LSC- populations (n=138 LSC+ population, n=89 LSC- population from 78 AML patient samples). B) Left: gating strategy for the quantification of MSI2 protein by intracellular flow cytometry in CD34+ LSCs enriched in primary AML patient samples. Right: Summary of MSI2 protein expression via intracellular flow cytometry in CD34+ and CD34- populations (n=24 AML patient samples). C) Western blot validating decreased protein levels in NB4 cells infected with two independent lentiviral shRNAs targeting

MSI2. D) CFU-assays of CD34+ sorted primary AML samples after lentiviral knockdown of MSI2 or non-targeting control hairpin (n=4 AML patient sample transductions, plated in duplicate). E) Representative flow plots of human AML bone marrow engraftment in NSG mice 12-weeks post-transduction with shControl or shMSI2. F) Summary of primary AML engraftment after 12-weeks post-transduction in injected femur and bone marrow (pelvis, contralateral femur, tibias) of NSG mice. Shapes and colours denote experiments (n=16 shControl AML transplanted mice, n=14 shMSI2 AML transplanted mice, from 4 independent patient AML samples). G) Levels of GFP+ present in human graft of NSG mice transplanted after 12-weeks post-transduction. Patient AML cells were transduced with shControl and two unique hairpins targeting MSI2 (n=5 mice per condition). Data shown as mean ±SEM. *$p<0.05$, ***$p<0.001$.

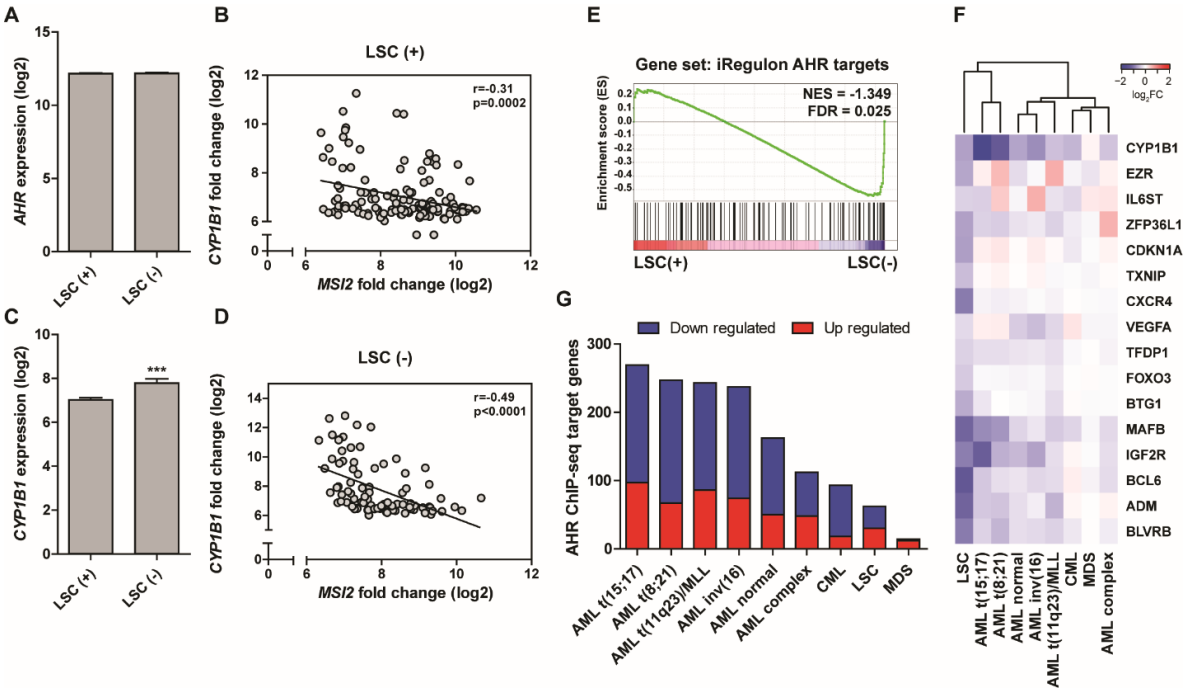

Figure 2: AHR target gene, CYP1B1, is downregulated in AML and the AHR signaling pathway is attenuated in LSCs. Transcript levels in LSC+ and LSC- populations for (A) AHR and (C) CYP1B1 (n=138 LSC+ populations, n=89 LSC- populations from 78 AML patient samples). Correlation of MSI2 and CYP1B1 transcript expression in distinct (B) LSC+ populations (n=138) and (D) LSC- populations (n=89). E) GSEA comparing LSC+ to LSC- populations for AHR target genes. F) Heatmap of leading edge genes driving negative enrichment score of AHR target genes in LSC+ population, along with their expression across multiple unfractionated leukemia subtypes relative to healthy bone marrow. G) Enrichment of AHR Ch-IP-seq targets with significantly up- and downregulated genes from unfractionated leukemia subtypes relative to healthy bone marrow. Data shown as mean ±SEM. ***p<0.001.

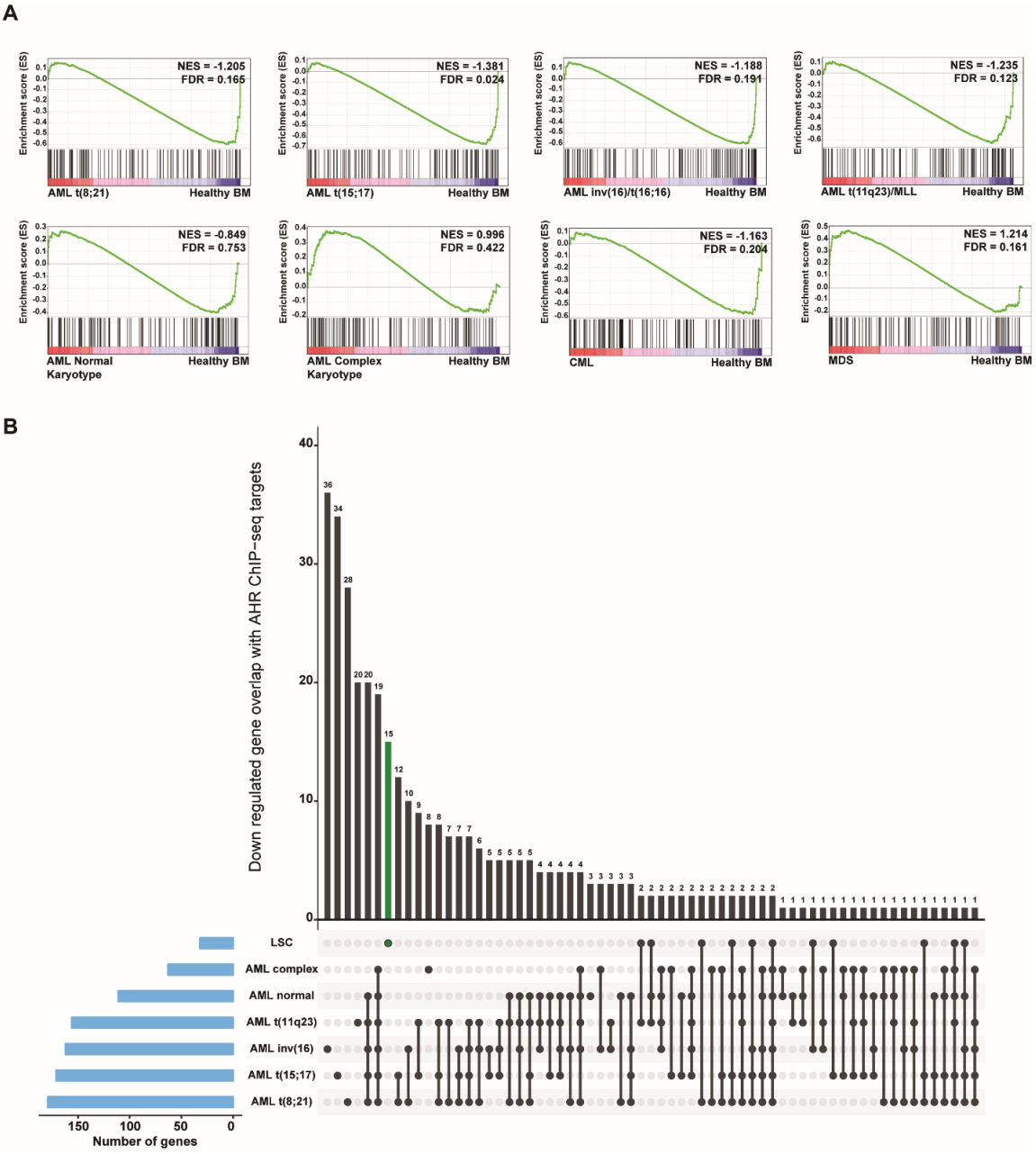

Figure 3: Decrease in global gene targets of the AHR signaling pathway in LSCs and multiple AML subtypes. A) GSEA for iRegulon AHR targets in AML. AML subtypes and healthy bone marrow gene expression data derived from the Microarray Innovations in Leukemia (MILE) study were compared and analyzed by GSEA with the iRegulon AHR

target gene list. B) Plot shows intersections between lists of significantly down regulated AHR-target genes in AML-subtypes compared to healthy bone marrow and LSCs compared non-LSCs. Blue bars represent total number of downregulated AHR-targets per condition. Numbers above columns indicate the number of genes that intersect with each condition diagramed below. Green highlights the LSC specific AHR-target genes.

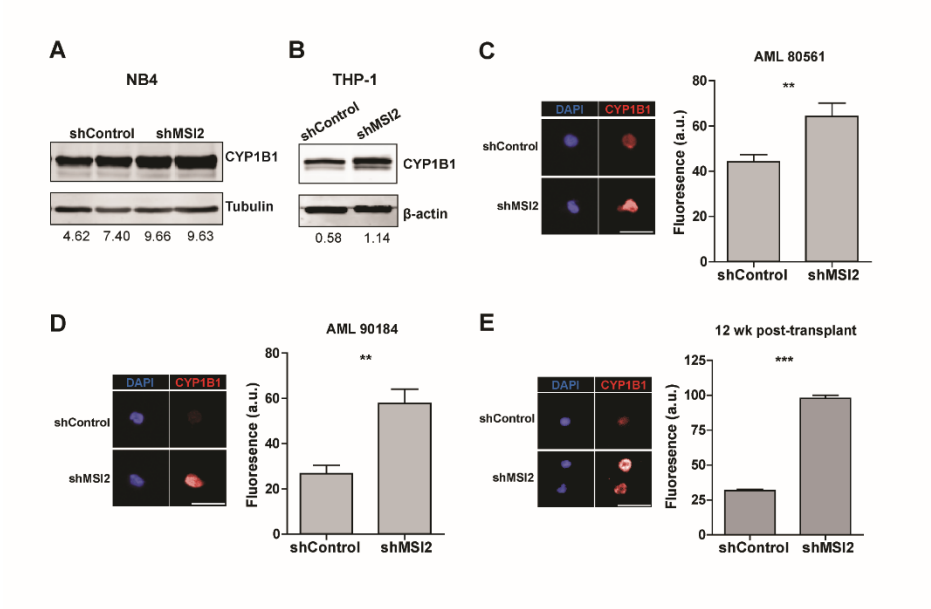

Figure 4: Downregulation of MSI2 promotes expression of CYP1B1 in AML. CYP1B1 protein levels by western blot after lentiviral knockdown of MSI2 in (A) NB4 and (B) THP-1 leukemia cell lines. Values represent the fold change from the relative intensity of CYP1B1 to loading control. CYP1B1 protein levels by immunocytochemistry and imaging after lentiviral-mediated knockdown of MSI2 in primary AML samples (C) 80561 (n=217 shControl and n=137 shMSI2 cells) and (D) 90184 (n=76 shControl and n=162 shMSI2 cells). (E) CYP1B1 protein levels by immunocytochemistry from bone marrow of 12-weeks engrafted mice transplanted with shControl or shMSI2 treated AML. Analysis performed on flow-sorted engrafted AML cells from two mice per condition (n=665 shControl and shMSI2 cells). Scale bar is 25 µm. Data shown as mean ±SEM. **p<0.01, ***p<0.001.

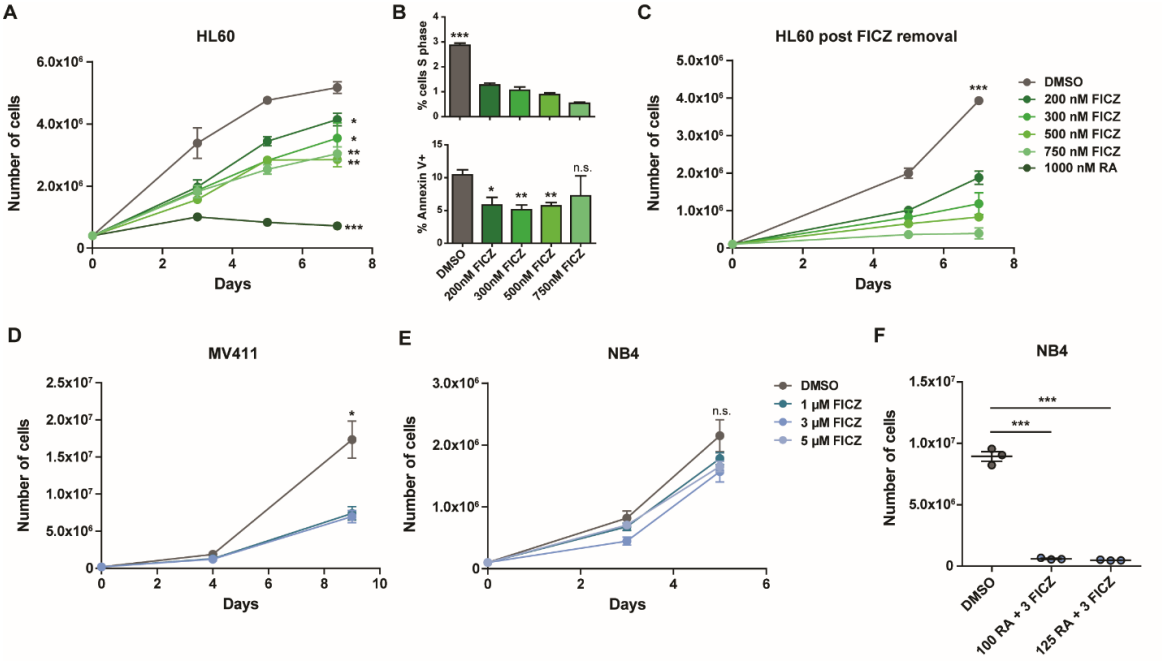

Figure 5: Activation of AHR by potent agonist negatively affects proliferation of AML cell lines. A) Time course of in-vitro proliferation of HL60 cells treated with varying doses of AHR agonist FICZ, retinoic acid (RA) or DMSO vehicle (n=3). B) Top: Cell cycle analysis by BrdU incorporation after seven days of culture with FICZ. Bottom: Cell apoptosis by Annexin V flow cytometry analysis after seven days of culture with FICZ. C) Time course of in-vitro proliferation of HL60 cells after removal of FICZ. D) MV411 and E) NB4 cell proliferation with varying doses of FICZ (n=3). F) NB4 cell output after 10 days of in-vitro culture with high-dose FICZ and low-dose RA (n=3). Data shown as mean ±SEM. *p<0.05, **p<0.01, ***p<0.001.

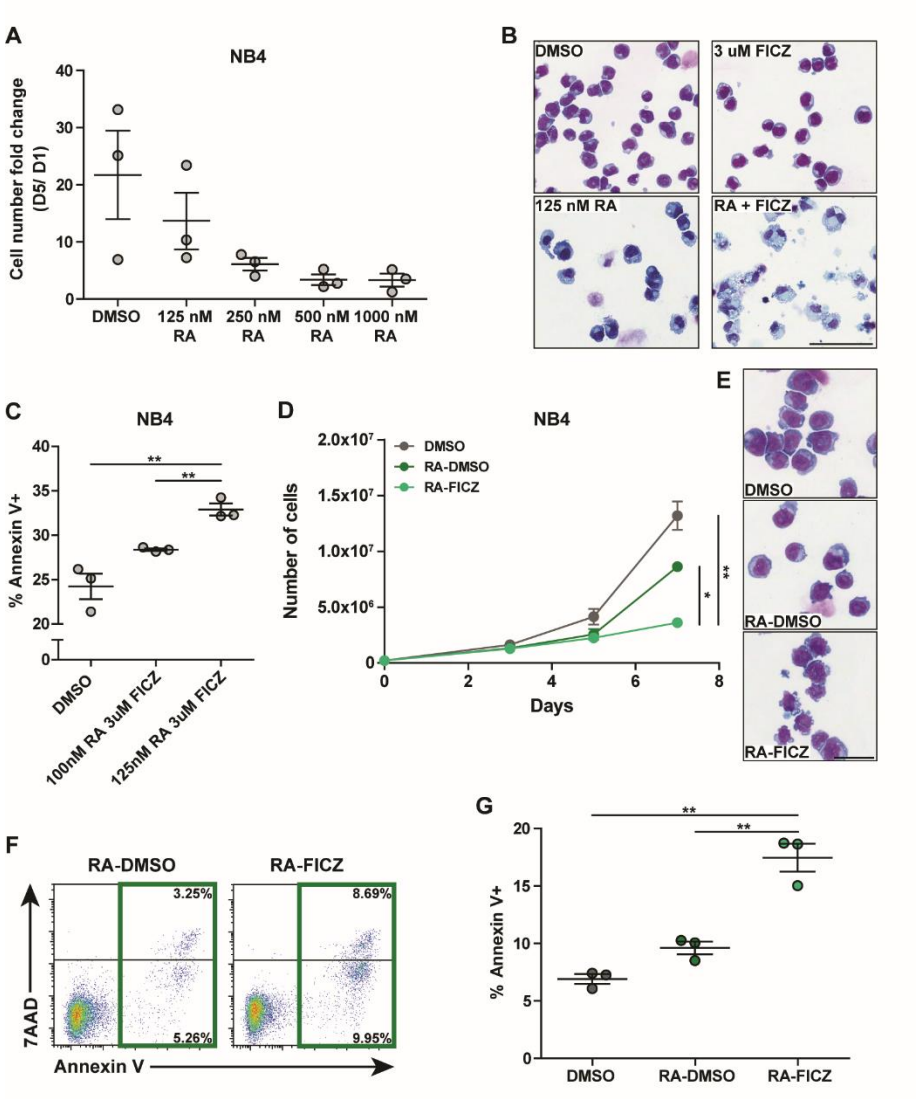

Figure 6: AHR signaling synergizes with retinoic acid to produce anti-leukemic effects. A) Fold change in cell growth after five days in culture with decreasing concentrations of RA (n=3). B) Giemsa-Wright stains of NB4 cells showing differentiated cell morphology with RA+FICZ in a seven-day culture. C) Early apoptosis of NB4 cells treated with low-dose RA and FICZ together. D)Time course of NB4 cell growth after initial stimulation with RA or DMSO vehicle, followed by treatment with DMSO (RA-

DMSO) or FICZ (RA-FICZ) (n=3). E) Differentiated morphology of NB4 cells with RA-FICZ treatment for seven days. F) Representative flow plots of early apoptosis by Annexin V staining in NB4 cells treated with RA-DMSO and RA-FICZ for seven days (n=3). G) Early apoptosis of NB4 cells treated with RA-DMSO and RA-FICZ for seven days (n=3). Scale bar is 25 μm. Data shown as mean ±SEM. *$p<0.05$, **$p<0.01$.

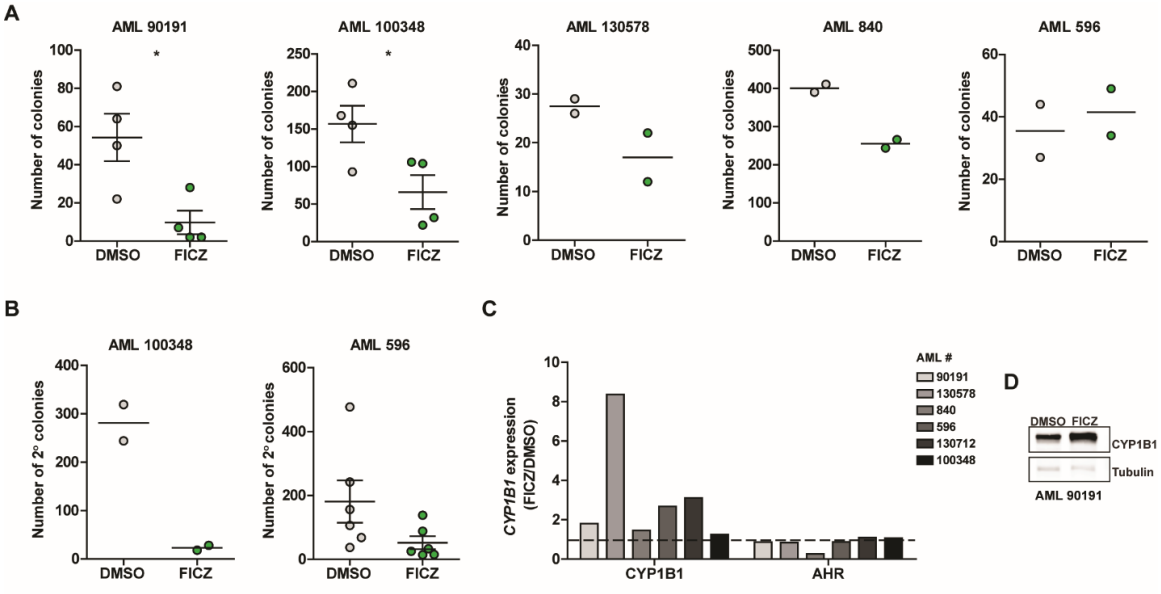

Figure 7: AHR pathway activation impairs clonogenicity in primary AML samples. A) Resultant colony numbers from primary AML CFU-assays after FICZ or DMSO vehicle treatment. Each plot represents replicate or pairs of replicate colony outputs from different patient AML samples. B) Secondary AML-CFU potential with FICZ treatment. C) Patient AML CYP1B1 and AHR transcript levels after in-vitro FICZ stimulation relative to DMSO vehicle. D) CYP1B1 protein level by western blot in a patient AML sample after FICZ exposure. Data shown as mean ±SEM. *$p<0.05$.

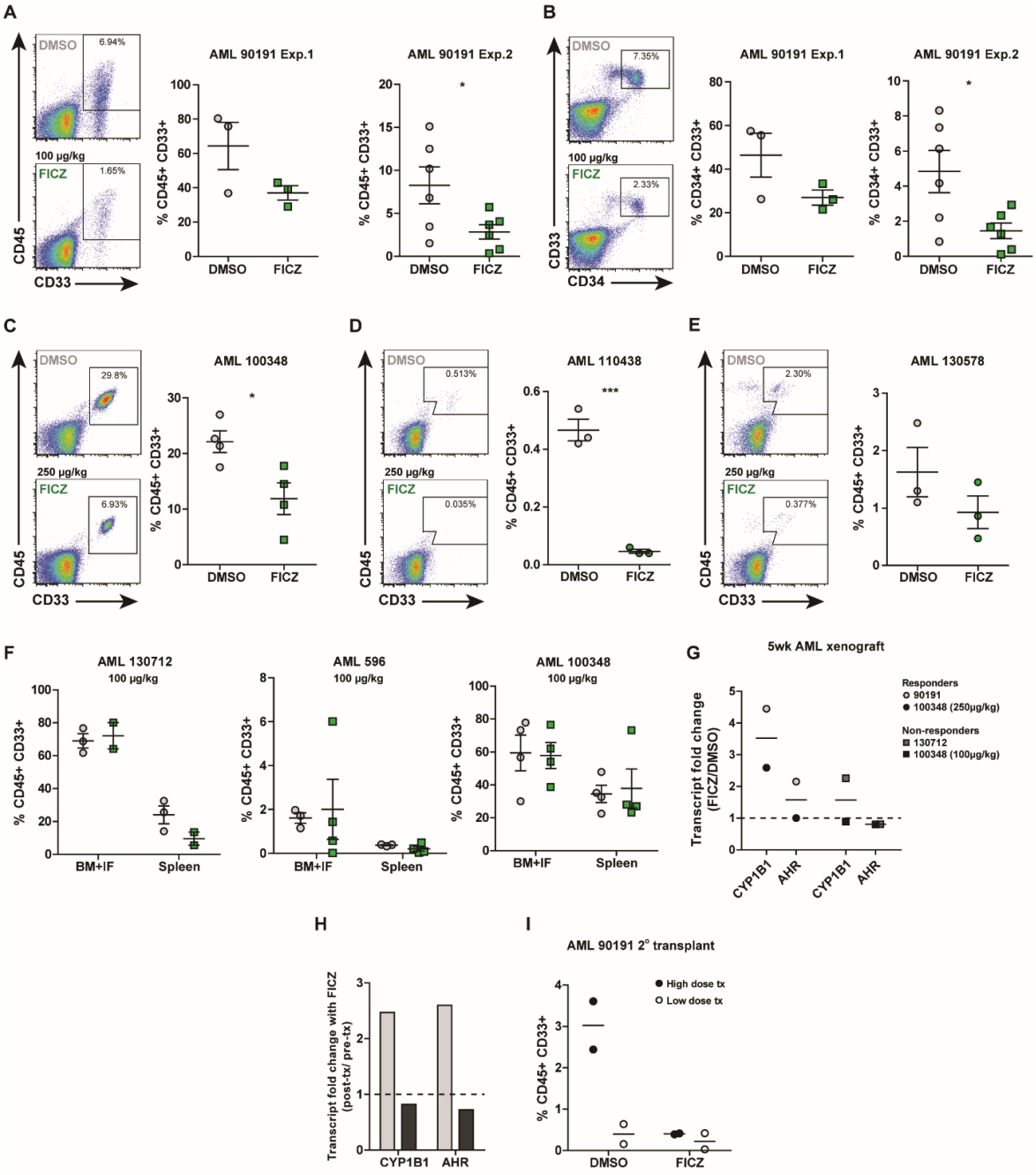

Figure 8: In-vivo AHR pathway activation decreases reconstitution capacity of primary AML xenografts. A) Left: Representative flow plots of patient AML engraftment in NSG mouse bone marrow after in-vivo treatment with DMSO vehicle or 100 µg/kg

FICZ. Right: Summary of engraftment levels from two in-vivo FICZ experiments with patient AML 90191 (Exp. 1, n=3 and Exp. 2, n=6 per condition). B) Left: Representative flow plots of LSCs in patient AML engraftment in NSG mouse bone marrow after in-vivo treatment with DMSO vehicle or 100 µg/kg FICZ. Right: Summary of LSC+ levels from two in-vivo FICZ experiments with patient AML 90191. C) Patient AML 100348 (n=4 mice per condition), D) patient AML 110438 (n=3 mice per condition) and E) patient AML 130578 (n=3 mice per condition) Left: Representative flow plots of patient AML engraftment in NSG mouse bone marrow after in-vivo treatment with DMSO vehicle or 250 µg/kg FICZ. Right: Summary of human AML engraftment levels from in-vivo FICZ treatments with each patient AML sample. F) Summary of NSG bone marrow and spleen engraftment from three independent experiments with different AML patient samples after in-vivo FICZ treatment. Each symbol represents a mouse. G) CYP1B1 and AHR transcript levels in FICZ responding and non-responding AML patient samples. Each point is representative of two mice. H) Average CYP1B1 expression after in-vivo treatment compared to the average CYP1B1 expression prior transplantation in responding and non-responding AML patient samples. I) Human AML engraftment levels after secondary transplantation with FICZ or DMSO vehicle treated human-mouse chimeric bone marrow. Two mice were transplanted per condition at high and low cell doses. Data shown as mean ±SEM. * $p<0.05$, *** $p<0.001$.

CHAPTER 4: DISCUSSION

This project was initiated with the goal of deepening our understanding of MSI2 and its mechanism of action on the AHR signaling pathway within LSCs and how it may influence the progression of AML. We sought to investigate the two important factors in LSCs independently, as well as collectively, in the leukemic context.

MSI2 expression and function within the context of human LSCs

With the presented work by Dr. Nicholas Holzapfel, MSI2 was found to have a significant part in the proper functioning of LSCs. His results showed an upregulation of MSI2 mRNA and protein within the LSC+ population and that mirrors the reported data by Kharas et. al. (2010) in mouse leukemia and evidence of MSI2 expression in HSCs. This is indicative that MSI2 is a positive regulator of self-renewal within the LSCs context and its elevation in expression contributes to the self-renewal abilities of LSCs. Interestingly, the LSC+ population has a more uniform expression of MSI2 with most CD34+ cells having high expression of MSI2. The LSC- population has a relatively more scattered expression profile, showing an expression gradient amongst the differentiating cells. This is congruent with our current understanding of MSI2 in HSCs, where cells undergo a loss of MSI2 expression as they mature (Kharas et. al., 2010).

Surprising results came from the CFU assays of primary LSCs with MSI2 KD, in which no differences were observed from the colony formation between shControl and shMSI2. HSCs with MSI2 overexpression showed an increase in colony formation (Rentas

et. al., 2016), thus the opposite effect was anticipated. The lack of difference in colony numbers suggests that MSI2 in AML may play a more important role in LSCs and have less influence on progenitor activity.

Evidence of MSI2's significance in LSCs is shown with the in-vivo KD experiments. An important and defining function of LSCs is their ability to engraft within a recipient host and recapitulate the initial disease. When sorted LSCs from primary AML samples were infected with a shRNA targeting MSI2, the majority of samples had a decline in engraftment levels and number of viable cells. This indicates that the presence of MSI2 and its function is essential for the survival of LSCs within an in-vivo system and their ability to propagate disease effectively. A caveat to this experiment is the measurement of GFP fluorescence as a reflection of cells expressing MSI2 shRNA. The GFP encoded within the vector backboneused for these experiments has been shown for some samples to be silenced within mice. Nonetheless, as in all cases, AML samples were infected at an efficiency of >70%, later silencing of the GFP transduction marker within MSI2 KD human grafts (where MSI2 KD was validated to have occurred in infected cells prior to transplantation) that were significantly smaller than control RFP KD grafts is less of a concern. Indeed to circumvent this issue, we did analyze CD45+ CD33+ engraftment levels (Fig. 1F) and found that leukemic shMSI2 grafts were significantly decreased in size relative to control grafts. Unfortunately, this does not distinguish the true extent of MSI2 KD's effects in LSCs because some shMSI2 grafts may have remained elevated due to outgrowth of more competitive untransduced cells. For one of the tested primary AML samples green fluorescence was retained in-vivo. Comparing the results of Fig. 1F to Fig.

1G, the level of human GFP+ cells within the injected femur graft is significantly lower compared to shControl, a trend not observed to a similar extent in the analysis of grafts with CD45 and CD33 only. Although our results are supportive that MSI2 knockdown does impair leukemic engraftment the full effects of MSI2 KD in LSCs may have been underestimated for some samples because of vector silencing.

The AHR signaling pathway expression and activity in AML

It is important to note that in the field of AHR signaling, much contradictory evidence has been provided in the literature regarding its role in development and leukemogenesis (Boitano et. al., 2010; Rentas et. al., 2016; Singh et. al., 2009; Casado et. al. 2011). The stimulation with different ligands and varied cell types used in independent studies could account for such differences. We decided to embark on the analysis of AHR expression and signaling activity with bioinformatic strategies using published AML databases and large patient collections from collaborators where in samples have not been subjected to any manipulation of the pathway. To begin, expression of the AHR itself was observed to be at similar levels between the LSC+ and LSC- cell fractions, and thus is present and thus presumably available for signaling functions in either population. Expression of CYP1B1 provides a canonical measurement of the AHR signaling activity occurring within differentiating cells (Soucek et. al., 2005). In the LSC+ population, because of the statistically significant lowered expression of CYP1B1 transcript, AHR signaling activity is interpreted to be downregulated relative to the LSC- population. Due

to the role of AHR in cell differentiation, we believe its signaling activity being heightened in LSC- cell fractions is consistent with its role in reducing the stemness of AML cells.

Correlations between MSI2 and CYP1B1 expression within the two fractionated cell populations show an inverse relationship. High MSI2 levels can predict low CYP1B1 expression and vice versa. This supports the idea that elevated MSI2 in LSCs potentially inhibit the AHR signaling pathway to decrease levels of CYP1B1 and differentiation effects. It is striking that the correlation is greater in the LSC- population, but this may be due to the distribution of data. The LSC+ population appears to be more diverse relative to the LSC- population, thus potentially reducing the R^2 value of the correlation. Reasons for such diversity in expression levels of MSI2 and CYP1B1 include different genetic backgrounds, stage of differentiation block, driver mutations, AML subtypes and so forth. Additionally, blast cells are phenotypically more homogenous in contrast to LSCs (Brent Ferrell Jr. et. al., 2016).

In addition to CYP1B1 expression, other potential AHR target genes, as predicted from iRegulon using the consensus XRE sequences, were also analyzed in human AML samples. GSEA compared the expression of such target genes within the LSC+ and LSC- settings, and the resultant NES was a negative value. As the NES is an additive score, a negative result indicates a general downregulation of the queried genes, because the NES is composed of more negative values (under-expression) than positive values (over-expression). Thus, this analysis implies that AHR signaling activity is repressed within the LSC+ population and it is downregulating the global AHR network, affecting cell

physiology from all aspects of proliferation, apoptosis, cell cycling and differentiation that may contribute to AML.

Leading-edge genes of the GSEA are genes that contribute the greatest amount to the NES. A list of 16 leading-edge genes that are predominantly downregulated components of the AHR signaling pathway have been identified in the LSC+ cell fraction, across multiple unfractionated AML subtypes, in CML and in MDS. These particular gene targets of the AHR can be classified into various physiological processes associated with the pathway, such as metabolism, signal transduction, cell cycling, proliferation, differentiation and apoptosis. Certain genes such as CYP1B1 and MAFB have decreased expression in all AML subtypes while other targets are downregulated in select leukemias. It is intriguing that both CYP1B1 and MAFB are involved in hematopoietic cell differentiation. CYP1B1 functions primarily to metabolize compounds in phase I oxidation and in cellular differentiation (Soucek et. al. 2005; Jones et. al., 1985). MAFB is a transcriptonal repressor responsible for regulating lineage-specific differentiation by repressing a subset of erythroid genes in myeloid cells (Sieweke et. al., 1996). Other notable genes identified as relevant contributors to the negative NES are ZFP36L1, CDKN1A and FOXO3. The gene ZFP36L1 encodes for an mRNA decay activator protein that destabilizes mRNA transcripts by poly-A tail removal as a means to attenuate translation. ZFP36L1 like MSI2 binds 3'UTR regions, however they are mechanistically different with regards to the poly-A tail (Hodson et. al., 2010). MSI2 binds to its targets wherein the poly-A tail is intact, while ZFP36L1 removes it. This raises questions as to why MSI2's repression of the AHR signaling pathway ultimately results in significant decrease of ZFP36L1 expression.

It is very possible that they attenuate the translation of transcripts with opposing effects as indeed ZFP36L1 is known to act on genes involved in self-renewal to promote the cellular differentiation (Ball et. al. 2014; Hodson et. al., 2010). CDKN1A, also known as p21, is a leading-edge gene downregulated predominantly in the LSC+ fraction only. It codes for cyclin dependent kinase inhibitor-1 and participates in cellular responses to DNA damage (Davies et. al., 2015; Ducoux et. al., 2001). The repression of CDKN1A in LSCs can be a factor in the propagation of damaged DNA to daughter blast cells in AML. Lastly, FOXO3 is a transcriptional activator that triggers apoptosis in the absence of survival factors and prevents the translation of MYC, a key oncogene dysregulated in many leukemias (Lehtinen et. al., 2006; Kress et. al., 2011; Brondfield et. al., 2015). Without the expression of FOXO3, cells that should be eliminated have an opportunity to survive and possibly transform into leukemic cells. The mentioned genes (CYP1B1, MAFB, ZFP36L1, CDKN1A and FOXO3) above and those reflected in the list of leading-edge genes are all part of physiological processes that when dysregulated due to AHR inhibition, can contribute to the the defining, malignant cellular properties of LSCs.

The examined leukemic cell types included LSC+ cell fractions, bulk AML t(15;17), AML t(8;21), AML with normal karyotype, AML inv(16), AML t(11q23)/MLL, AML with complex karyotype, CML and MDS. The LSC+ populations were FAC-sorted as phenotypically defined, CD34+ expressing cells. AML t(15;17) is classified as acute promyelocytic leukemia (APL) with a chromosomal translocation that fuses the promyelocytic leukemia (PML) gene with the retinoic acid receptor-alpha (RAR-α) protein, ultimately leading to modified subcellular localization and differentiation block (Lavau and

Dejean, 1994). The blasts are sensitive to RA, which is currently a therapeutic treatment in the clinic for APL. AML t(8;21) is a frequently observed chromosomal abnormality in AML that results in fusion proteins between AML1 and another gene that affects proliferation, differentiation and viability of leukemic blast cells (Miyoshi et. al., 1993; Reikvam et. al., 2011). The AML inv(16) subtype contains a translocation with AML1 that causes interferences in its transcriptional regulation (Lutterbach et. al., 1999). Finally, AML t(11q23)/MLL is a very aggressive subtype of leukemia with poor overall survival in patients. This genetic abnormality affects the histone methyltransferase critical for controlling gene expression during embryonic development and hematopoiesis (Chen et. al., 2013). There are also AML subtypes classified to have a normal or complex karyotype. With such fundamental diversity in the disease, the significance of the AHR signaling pathway and its target genes may be different from subtype to subtype. Thus, the number and identification of genes that are downregulated or upregulated varies. The LSC+ population is the only group of leukemic cells that consistently have decreased expression of all leading-edge AHR target genes relative to bone marrow. This could be explained as the subtypes of AML examined are unfractionated and thus, their gene expression profiles are diluted by differentiated blast cells that lack the highly elevated levels of MSI2 present in LSCs that are inhibiting the AHR signaling pathway.

Specifically considering the AML t(15;17) subtype, we found it has the lowest basal CYP1B1 expression and a majority of AHR targets repressed. The GSEA results report the most negative NES value, suggesting this subtype of AML at the bulk level experiences extensive inhibition of the AHR signaling pathway. Importantly, the ChIP-seq data

emulates the GSEA results. These observations made regarding the results of AML t(15;17) are useful for interpreting further results in this project because many of the AML cell lines in use are classified as AML t(15;17) cells.

Unexpectedly, the LSC+ fraction was not the population with the most downregulated targets. This could presumably be due to the control populations or that LSCs are strongly affected by the AHR inhibition on select few genes because of the processes its targets partake in and/or because other cellular pathways serve to upregulated certain AHR targets not involved in LSC control. Further examinations of the AHR ChIP-seq targets of LSCs that do not coincide with those in the bulk cells of other tumour subtypes support the concept that the LSC population is quite unique in its downregulation of AHR gene targets.

Another interesting AML subtype with peculiar results is AML with complex karyotype. This category implies a mixed genetic background and many genetic lesions underlying the leukemia. Thus, the mild effects of AHR inhibition witnessed is most likely because the AHR signaling pathway has a smaller responsibility in disease propagation and maintenance relative to other subtypes.

Additional leukemias that were investigated with bioinformatics were CML, a slow progressing disease with less aggression until blast-crisis, and MDS, a precursor to leukemic disease. In both hematopoietic malignancies, the inhibition effect on the AHR signaling pathway is relatively mild. Fractionation of CML into chronic phase and accelerated blast-crisis phase would provide insight on AHR inhibition and leukemic

aggression. The data currently stands to show that in the more aggressive AML compared to CML and MDS, AHR signaling activity is decreased.

There are some considerations of this informatics analysis that must be taken into account when assessing their broader clinical relevance. First, the LSC+ gene expression profile is a composite that is derived from many AML samples. Because of this, it is not possible to ascertain from this dataset whether these AHR target gene expression profiles are representative of LSCs from all AML subtypes or are biased towards a selective subset(s). Second, the AML subtypes under review were unfractionated, means that their expression signature derives from the dominant homogenous blast population. Because of this, the unfractionated samples are useful for elucidating AHR signaling repression within a whole cancer. Third, while it was not performed in this analysis, expression profile mining of segregated phases of CML could shed insight on the level of AHR activity and disease aggression.

Repression of the AHR signaling pathway by MSI2 in AML

The inhibition of AHR signaling by MSI2 in HSCs holds true in AML and the LSC compartment, as established through the host of in-vitro and in-vivo assays performed wherein removal of MSI2 protein via lentiviral KD activated the AHR signaling pathway and increased CYP1B1 protein levels in both contexts.

Immortalized cell lines are great in-vitro models since primary AML cells do not survive well in culture. THP-1 and NB4 cells were lines that with MSI2 KD elicited the

response of increased AHR signaling as illustrated by the western blots fluorescence intensities showing CYP1B1 protein increase. It was noticed that THP-1 cells had a more significant increase in CYP1B1 protein levels, and thus greater AHR activation, than NB4 cells. This could provide an interesting paradigm for sample response prediction. Certain AML samples and subtypes for example could very well have determinants that allow them to be more susceptible to AHR activation. The THP-1 cell line is classified as FAB M5 (acute monocytic leukemia) (Bosshart and Heinzelmann, 2016) and NB4 cells are FAB M3 (APL) (Lanotte et. al., 1991). Thus, THP-1 cells are of an AML subtype that is characterized by the accumulation of blasts that represent relatively more differentiated myeloid cells. As such, it is tempting to predict that the higher basal level of AHR activity in THP-1 cells as a result of decreased MSI2 expression could lend these cells to achieving a higher level of AHR signaling with FICZ activation (ie. overcoming MSI2's inhibition would not be as difficult in these more differentiated cells). To truly make this claim about the observed results however, data regarding cell phenotypes and expression levels prior to KD must be collected for comparison.

When MSI2 KD was propagated in-vitro with primary AML samples for a brief period of time, the results from immunofluorescence were similar to the work with cell lines. Immunofluorescence was the chosen technique for this assay with primary AML samples because of limited cell numbers. Similar to western blotting, a higher intensity equated to greater abundance of CYP1B1 protein in the cell with MSI2 KD. From the short-term in-vitro experiments, I showed that after MSI2 KD, AHR signaling occurs almost immediately to increase levels of CYP1B1 protein. Immunofluorescence was also carried

out on an AML sample that had been infected with shMSI2 and xenotransplanted into mice for a 12-weeks incubation period without any form of treatment. These results revealed elevated levels of CYP1B1 in LSC-derived grafts with MSI2 KD compared to shControl and in-vitro experiments. This is an indication that the activation of AHR signaling by modulating MSI2 levels is not a transient effect, but an immediate and prolonged response. Additionally, the immunofluorescence results combined with engraftment analysis link AHR signaling activity to LSCs function via MSI2's mRNA target network as it was observed that following MSI2 KD, LSCs exhibit lower engraftment, reduced viability and increased AHR signaling. Therefore, it is possible to deduce that activity of the AHR signaling pathway contributes to the failure of LSCs to engraft effectively and its apoptotic events in-vivo. The presence of MSI2 is necessary to prevent AHR activation and to support the function of LSCs in disease propagation.

Anti-leukemic effects on AML samples by potent agonist, FICZ

After the unraveling of effects of AHR signaling in AML, the potential for the pathway to serve as a therapeutic target was apparent especially as this strategy would be realistically more straightfoward than attempting to modulate MSI2 levels in patients through other means. Thus, to activate the AHR signaling pathway, a potent agonist such as FICZ was used to overcome the differentiation block imposed by MSI2. The benefits of using FICZ are 1) it is endogenous and naturally-occurring in the human body, 2) no

cytotoxic effects have been reported in literature and 3) it is extremely potent and has high affinity for the AHR.

Literature of FICZ in similar experimental designs were limited, therefore the selected concentrations were arbitrary speculations. However, it was known that 200nM FICZ is a sufficient dose for HSC treatment from Rentas et. al. (2016). The range of concentrations elicited dose-dependent responses in the HL60 cell line, but not others. The exposure to FICZ impacted proliferation, apoptosis, cell cycling and differentiation as a consequence of AHR signaling, which takes part in all four physiological processes.

As mentioned, the study of the AHR pathway tends to always reveal contradictory evidence in the literature. From our experiments, there were contrasting results to published studies. The culturing of HL60 cells in medium supplemented with FICZ did not increase the percentage of cells entering the cell cycle as many have reported. The significant decrease in cycling cells may be caused by the upregulation of AHR signaling activity and possible increased expression of CDKN1A, which halts cell cycling if DNA damage is found. Furthermore, FICZ-treated cells were not found to have decreased cell death, suggesting that AHR activation in HL60 cells did not induce an apoptotic response despite the declining proliferation. Therefore, exposure to FICZ likely caused cell cycle arrest inthese particular leukemic cells.

Unlike TCDD, FICZ activates the AHR signaling pathway transiently. However, in the time-course experiment, FICZ was consistently added into the cell culture and so perhaps, the prolonged exposure may have induced permanent effects. Upon removal of

FICZ from culture, and only supplementing with the DMSO vehicle, HL60 cells continued to show dose-dependent impairment in proliferation relative to controls. Hence, prolonged exposure to FICZ can leave long-term effects on leukemic cells.

Aside from the HL60 cells, the effects of FICZ activation of the AHR signaling pathway was queried in MV411 and NB4 cells. Concentrations used previously with HL60 cells did not create responses in these other cell lines. At higher doses, FICZ caused defects in the proliferation of MV411 cells, however these effects were not dose-dependent. NB4 cells remained unresponsive. These results exemplify that subtypes of AML have different sensitivities and responses to FICZ and AHR activation. HL60 cells are FAB M2, which are acute myeloblastic leukemia cells with maturation, carrying the t(15;17) translocation, other cytogenetic abnormalities and *c-myc* amplification (Dalton et. al., 1988). It is plausible that this cell line was responsive because of its relative maturation and reduced basal inhibition of the AHR signaling pathway. Additionally, the increase in AHR signaling upregulated FOXO3, a repressor of MYC expression and its oncogenic activities. The MV411 cell line is FAB M5 (acute monocytic leukemia) and similarly to HL60 cells, its level of maturation may have been the underlying feature that allowed for more successful AHR activation with FICZ. NB4 cells are of the FAB M3 classification for APL, with the translocation t(15;17) and a signature fusion PML-RARα protein (Lanotte et. al., 1991). A theory for the lack of response to FICZ in NB4 cells is that altered cellular localization of the RAR has impacted the ability of NB4 cells to activate AHR signaling because of crosstalk between pathways (Bunaciu and Yen, 2012; Puga et. al., 2009). Morphological studies of NB4 cells provide qualitative evidence of synergy between the AHR and RAR

pathways since cells treated with RA and FICZ appear more differentiated and apoptotic than independent treatments. Unlike HL60 cells, NB4 cells had increased staining for apoptotic markers. It correlated with higher doses of RA, not FICZ, implying that 1) FICZ induced different effects depending on the cell type and concentrations or 2) FICZ does not influence the apoptotic response in AML.

On the idea of a synergistic relationship between the two pathways in NB4 cells, it was thought that RA may be used as a priming agent to initialize the signaling cascades while FICZ sustained or exacerbated the effects. NB4 cells treated with RA-FICZ failed to recover at a rate that matched the control, RA-DMSO cells and had slowed proliferation, poor cellular shape and increased apoptosis. The failure to recover its proliferation like RA-DMSO cells suggested that FICZ sustained the effects of RA. The increased apoptosis experienced by RA-FICZ implies that FICZ does have apoptotic effects on leukemic cells and enhances the effects of RA. Therefore, it can be concluded from these experimental results that NB4 cells can elicit a response to FICZ after an initial, temporary exposure to RA.

Importantly, adverse effects were observed in primary human AML samples when treated with FICZ. Lowered colony formation and smaller colonies arose from FICZ treatment, proposing defective progenitor activity due to AHR activation in-vitro. Primary AML sample 596 did not respond to FICZ initially. However, the sample showed decreased colony forming potential in the secondary CFU-assay. Thus, this signified delayed AHR signaling or that only the self-renewal activity of progenitor populations from this sample were susceptible to FICZ. Interestingly, MSI2 KD in primary samples did not result in a

difference in colony formation while FICZ treatment did, however both are means of activating the AHR signaling pathway in-vitro. There is currently no explanation for this discrepancy between results, but the AHR pathway is known to elicit different responses to cell physiological process depending on the type and timing of activation.

mRNA levels of CYP1B1 transcripts were evaluated as evidence that FICZ at a specific dose was able to activate the AHR signaling pathway in the bulk AML samples. The transcript levels of AHR were also measured to confirm that effects were not due an increase in receptor expression, but because of increased activity from FICZ stimulation. CYP1B1 expression did not correlate with the extent of effects on the samples. For instance, AML samples 90191 and 100348 showed the greatest differences between the control and treatment cohort, but a relatively small increase in CYP1B1 mRNA expression. On the other hand, AML sample 130578 did not have a pronounced effect on colony formation after FICZ treatment, but revealed an 8-fold increase in mRNA transcripts of CYP1B1. AML sample 596 also spiked an increase in CYP1B1 despite no changes in colony formation. All these results suggest that CYP1B1 expression is indicative of active AHR signaling, but do not predict the response or level of effects in AML. Some samples are sensitive and slight increase in AHR signaling activity yield anti-leukemic effects while other samples require greater stimulation of the pathway to achieve similar outcomes.

In-vivo studies were able to show that intraperitoneal injections of FICZ activated AHR signaling in engrafted huamn AML cells and provided important proof-of-principle experiments. The initial treatment of samples with DMSO or FICZ in an overnight culture prior to transplants assures that all clones have been equally exposed, especially the LSCs

that have potential to propagate the new tumour (Klco et. al., 2014). Since engraftment levels were lowered in cohorts treated with FICZ, it was an indication that activation of the AHR signaling pathway with an agonist impaired the function of LSCs. Additionally, a decrease in CD34+ cells was observed, suggesting that this population was targeted for apoptosis or differentiation. Consistently, there was lowered engraftment in the whole bone marrow relative to injected femur, implying an additional defect in LSCs homing or migration mechanism after AHR activation (Casado et. al., 2011; Wang et. al., 2013).

Some AML samples engrafted in-vivo and did not respond to AHR activation. These samples did not show a difference between injected femur or whole bone marrow, providing additional support for the role of AHR in cellular homing and migration. Similar to the in-vitro experiments with AML cell lines, these primary AML samples lacked response to FICZ treatment potentially due to 1) multiple genetic lesions, 2) increased aggression and/or resistance and 3) therapeutic concentrations of FICZ not achieved. The concentration of FICZ chosen for in-vivo injections stemmed from the literature on anti-inflammatory diseases and therefore, may be not therapeutic for leukemia. In AML sample 100348, there was no response when the treatment was 100µg/kg FICZ. Once the dose increased to 250µg/kg, the human AML engraftment levels were significantly lowered in the FICZ cohort of mice than controls. Thus, effective FICZ concentrations vary between AML samples in order to activate AHR signaling. Furthermore, in non-responding AML samples, the AHR signaling pathway was inactive as shown by subtle changes in CYP1B1 mRNA transcripts, 5-weeks post-transplant. Meanwhile, responding samples, AML 90191

and 100348 treated with 250µg/kg FICZ, had CYP1B1 transcripts increased significantly relative to control, indicating active AHR signaling in-vivo.

As briefly touched upon above, the in-vitro treatment prior to transplant assures complete exposure of all clones to FICZ, but it also raises questions about the in-vivo activation of the AHR signaling pathway by FICZ intraperitoneal injections. Likely, the cells have activated AHR pathways entering the in-vivo system, so how can we validate that the impaired functions of LSCs is a result of the in-vivo activation by FICZ, and not the in-vitro treatment? Sample mRNA data from before and after transplantation were collected and assessment of this data showed a significant upregulation of CYP1B1 in-vivo relative to in-vitro, in AML samples that respond positively to the treatment. As a single FICZ exposure is a transient phenomenon, the observed elevation in CYP1B1 expression after 5-weeks is very likely to have been caused by the intraperitoneal injections of FICZ activating the AHR signaling pathway. Non-responders did not show the same expression patterns despite the identical injection protocol with FICZ, suggesting that the dose given in-vivo was insufficient in turning on the AHR pathway in-vivo.

Lastly, gathered evidence showed impairment in long-term reconstitution of AML disease in mice through secondary xenotransplants. Different cell doses were transplanted into mice to test the limiting number of cells required to seed a new graft. Only the high dose whole bone marrow cells contained sufficient human AML cells to grow comparable grafts between the control and treatment groups. Analysis showed long-term implications as the ability of human AML cells to engraft in secondary murine recipients diminished after activation of the AHR pathway via FICZ in-vivo. This opens potential doors to

treatment for relapse patients as AHR activition weakens LSCs that may be contributing to the returning disease.

CHAPTER 5: CONCLUSION

Overall, this project aimed to probe MSI2 and AHR function in LSCs and to in addition, explore the potential for an inhibitory relationship between the two genes driving the AML disease progression in-vivo. We were able to observe MSI2 expression and functionality within the LSCs population, which also showed a correlation with CYP1B1 downregulated expression, signifying decreased AHR activity. The differentiation block imposed by MSI2 could be relieved by either modulating MSI2 expression or by FICZ treatment, both in-vitro and in-vivo, leading to impaired function of LSCs and decreased leukemic burden within transplanted animals.

This project provided answers that we sought, but also opened up opportunities for new questions to be asked. Understanding the predictors (ie. genetics, proteonomics, disease burden, etc.) of response to FICZ treatment and AHR signaling activation is crucial for translating these findings into the clinic. Also, comparing AML of different origins (ie. de novo, secondary or relapse) and its response to FICZ exposure. Further topics of exploration can focus on the adversity of AHR activation via FICZ in LSCs, such as possibility of increased mutations that confer a selective advantage and in HSCs/other tissues. To address the effects of FICZ on normal tissues and HSCs to validate its safety and specificity, current in-vivo studies are currently being initiated in the laboratory.

Finally, to conclude, this project has been a pleasure to work on the findings have the potential to make significant impact in the scientific and clinical communities. AML is a disease impacting more than 1% of the world's population and advancements in therapies

have been scarce in the past four decades. Further understanding of the disease and its underlying biology is critically needed. Through uncovering MSI2 and its mechanisms, we have opened windows of opportunities for new therapeutic target discovery in a relevant population of cells that drive the disease. The AHR signaling pathway, a target for MSI2, serves as a strategic anti-leukemic target for AML because it is a promiscuous receptor that activates a wide array of genes involved in physiological processes often hijacked in cancer. Our demonstration that the AHR signaling pathway can be activated in AML to significantly reduce the leukemic burden provides a foundation and inspiration for further advancement and refinement of this strategy towards achieving better patient outcomes for those inflicted with AML.

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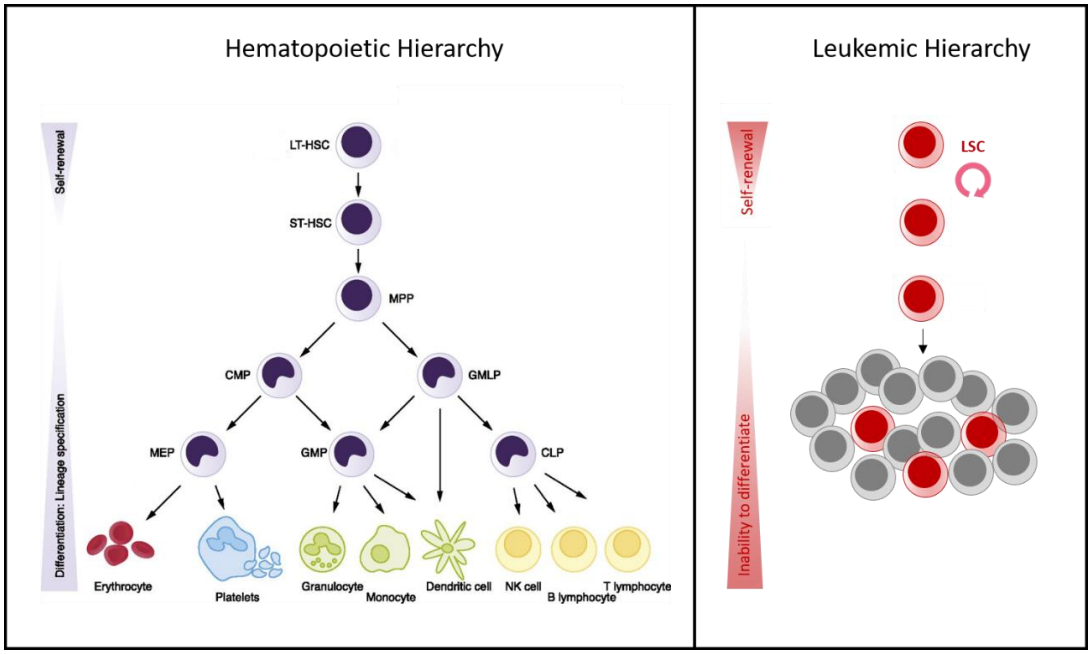
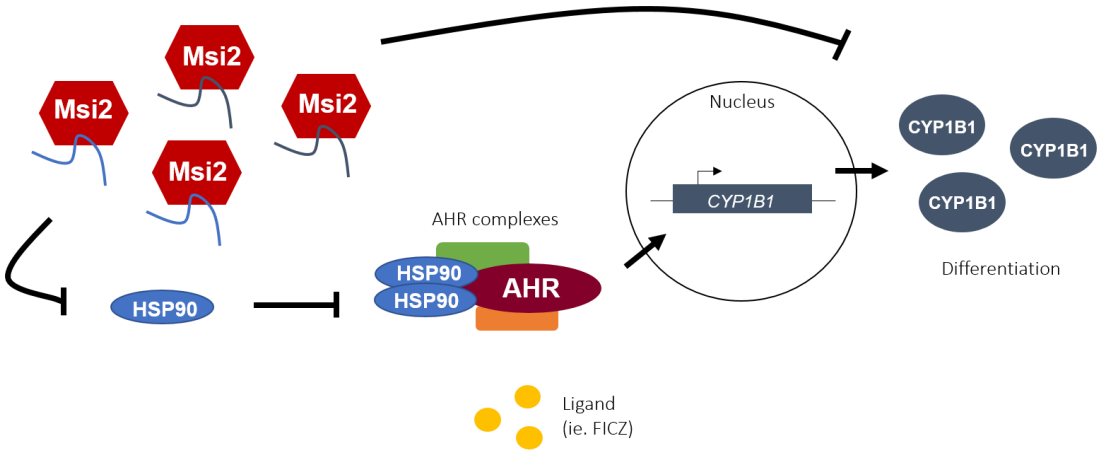
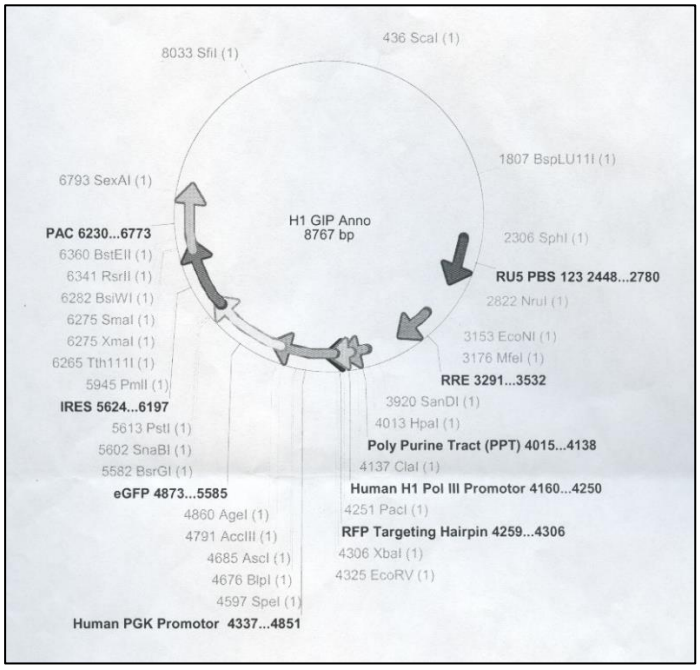
APPENDIX



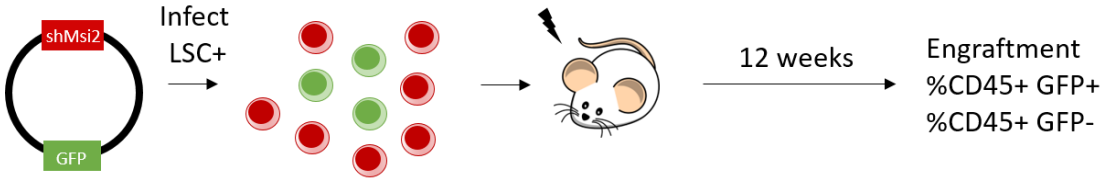
Schematic 1: Hematopoietic hierarchy vs. leukemic hierarchy, adapted from Blank (2015).



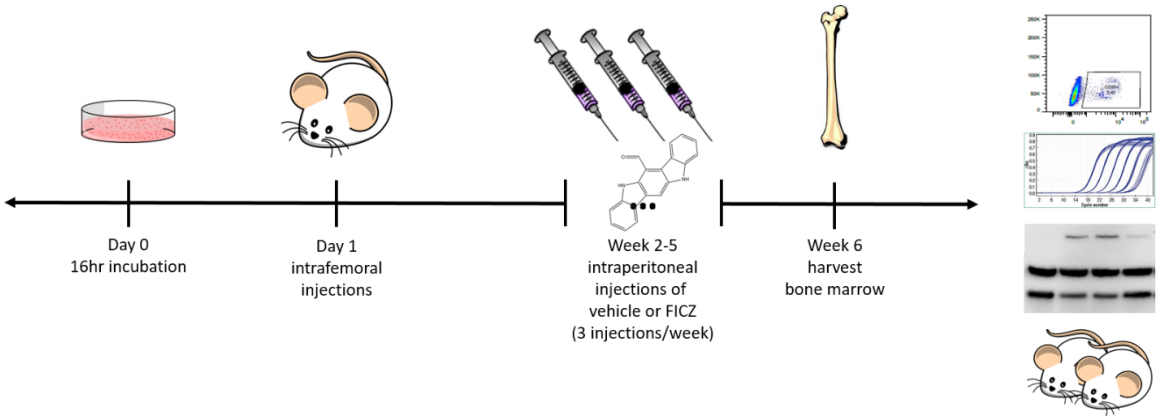
Schematic 2: Inhibition of AHR signaling by MSI2, as proposed in Rentas et. al. (2016).



Schematic 3: shRNA vector backbone, H1GIP.



Schematic 4: In-vivo experimental design for MSI2 KD in LSCs



Schematic 5: In-vivo experimental design for FICZ treatment of AML xenografts

FAB Subtype	Description
M0	Undifferentiated
M1	Myeloblastic without maturation
M2	Myeloblastic with maturation
M3	Promyelocytic
M4	Myelomonocytic
$M4_{Eo}$	Myelomonocytic with bone-marrow eosinophilia
M5	Monocytic
M6	Erythroleukemia
M7	Megakaryoblastic

Table 1: French-American-British Classification of AML (Tenen et. al., 2003)

Categories	
Acute myeloid leukemia with recurrent genetic abnormalities	AML with t(8;21)(q22;q22) AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22) APL with t(15;17)(q22;q12) AML with t(9;11)(p22;q23) AML with t(6;9)(p23;q34) AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) AML (megakaryoblastic) with t(1;22)(p13;q13)
Acute myeloid leukemia with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
Acute myeloid leukemia, not otherwise specific (NOS)	AML with minimal differentiation AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monoblastic/monocytic leukemia Acute erythroid leukemia Acute megakaryoblastic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferation related to Down syndrome	Transient abnormal myelopoiesis Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm	
Acute leukemias of ambiguous lineage	Acute undifferentiated leukemia Mixed phenotype acute leukemia with t(9;22)(q34;q11.2) Mixed phenotype acute leukemia with t(v;11q23) Mixed phenotype acute leukemia, B/myeloid, NOS Mixed phenotype acute leukemia, T/myeloid, NOS

Table 2: World Health Organization Classification of AML (Dohner et. al., 2010)

AML	Sex	Age	De Novo vs. Secondary	Sample Type	FAB Subtype	Karyotype
90191	M	32.7	De novo	Diagnosis	M1	46, XY, ider(7)(q10)del(7)(q21)[20]
596	M	64	De novo	Diagnosis	M1	46, XY[21]
100753	F	59.3	De novo	Diagnosis	M4Eo	46, XX, inv(16)(p13.1q22)[9]/46, XX[4]
110751	M	72.5	Secondary	Diagnosis	M5a	46, XY[20]
80561	M	56	De novo	Diagnosis	M4	46, XY[20]
90184	M	43	Secondary	Diagnosis	Unclassified	46, XX[20]
100348	F	66	Secondary	Diagnosis	ND	46, XX[17]
130578	M	62	De novo	Diagnosis	M4	46, XY[20]
840	M	78	De novo	Diagnosis	M1	46, XY[20]
130712	F	55	Secondary	Diagnosis	Unclassified	46, XX, t(9;11)(p22;q23)[10]
110438	M	72	Secondary	Diagnosis	ND	45, XY[20]
100091	M	43	De novo	Relapse	M4	46, XY[21]

Table 3: Clinical data of primary AML samples

Gene	Probe	Forward Primer	Reverse Primer
GAPDH	#60	5’ AGCCACATCGCTCAGACAC 3’	5’ GCCCAATACGACCAAATCC 3’
MSI2	#26	5’ GGCAGCAAGAGGATCAGG 3’	5’ CCGTAGAGATCGGCGACA 3’
AHR	#33	5’ AGCCGGTGCAGAAAACAG 3’	5’ CTATGCCGCTTGGAAGGAT 3’
CYP1B1	#20	5’ ACGTACCGGCCACTATCACT 3’	5’ CTCGAGTCTGCACATCAGGA 3’

Table 4: UPL probes and primers for qRT-PCR

Hairpin	Target Sequence
shControl	5’ GTGGGAGCGCGTGATGAACGGATCCGTTCATCACGCGCTCCCACTTT 3’
shMSI2	5’ TAAGAGAGATCCCACTACGAAAGGATCCTTTCGTAGTGGGATCTCTCTTTTT 3’

Table 5: Short hairpin sequences for MSI2 knockdown