STEM CELL-SPECIFIC INTERNALIZING IMMUNOTOXIN AS A THERAPY FOR MYELODYSPLASTIC SYNDROMES

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STEM CELL-SPECIFIC INTERNALIZING IMMUNOTOXIN AS A THERAPY FOR MYELODYSPLASTIC SYNDROMES

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The myelodysplastic syndromes (MDS) are a group of clonal hematopoietic disorders characterized by ineffective hematopoiesis resulting in cytopenias and an increased risk of transformation to acute myeloid leukemia. Currently, the only curative therapy for MDS is an allogeneic hematopoietic stem cell transplantation (HSCT); however, old age and other comorbidities limit tolerability of conditioning regimens for HSCT, offsetting its benefits for patients. Among a wide range of genetic and epigenetic aberrations associated with MDS pathogenesis, dysregulation of ribosome biogenesis has been implicated. Using the Vav-cre-Asxl1-Tet2 mouse model of MDS, we show that delivering a ribosomal inactivating protein, saporin, via the internalization of the CD117 receptor, results in the depletion of normal and malignant stem cells with minimal effects on bone marrow cellularity. Furthermore, we show that the depletion of malignant cells allows for sufficient engraftment of healthy stem cells into recipient mice. Together, these studies demonstrate that CD117-saporin treatment leads to the depletion of malignant stem cells and can serve as a conditioning regimen for HSCT opening a potential therapeutic window for MDS treatment.

BIOGRAPHICAL SKETCH

Sarah Qamar enrolled in the Life Sciences program at New York Institute of Technology in 2005. She completed her degree in December 2008 and after graduating, she worked in the laboratory of Jidong Lui at Memorial Sloan Kettering Cancer Center where she studied the role of mRNA granules in the miRNA pathway. She later went to work with Julia Kaltschmidt also at Memorial Sloan Kettering Cancer Center where she studied the role of transcription factor Satb2 in the specificity of interneuron connections. It was during her time there that she decided to peruse a PhD at Weill Cornell Medicine.

Sarah enrolled in the BCMB program at Weill Cornell Medicine in Fall 2011. In Summer of 2012, Sarah joined the laboratory of Christopher Y. Park where she studied the role of the aging hematopoietic system in the development of myelodysplastic syndromes and the use of an immunotoxin to deplete disease-causing stem cells. In addition to her work in the lab, Sarah has pursued her passion for teaching by volunteering with science education programs that serve underprivileged NYC students and community programs serving the elderly population. Sarah is also an adjunct at Marymount Manhattan College where she teaches heredity.

To my parents – for your tremendous sacrifices.

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LIST OF ABBREVIATIONS

AML: Acute Myeloid Leukemia

BM: Bone Marrow

CFU: Colony Forming Unit

CMP: Common myeloid progenitor

CSC: Cancer stem cell

HSC: Hematopoietic stem cell

HSCT: Hematopoietic stem cell transplant

GMP: Granulocyte macrophage progenitor

KD: Knockdown

LSC: Leukemia stem cell

MDS: Myelodysplastic syndromes

MEP: Megakaryocyte erythroid progenitor

MNC: Mononuclear cell

PB: Peripheral blood

RBC: Red blood cell

WBC: White blood cell

CHAPTER ONE
INTRODUCTION

CLINICAL FEATURES OF THE MYELODYSPLASTIC SYNDROMES

The myelodysplastic syndromes are a collection of chronic malignancies with unique clinical manifestations and prognoses. Identification and classification of MDS and other blood malignancies was only made possible after accurate enumeration and visual characterization of blood and bone marrow cells were made possible. Early tools, such as the light microscope, hemocytometers, and tissue staining methods, provided insights into the connection between disease and blood cells by providing quantitative and qualitative measures to correlate with disease symptoms. More recently, advent of flow cytometry and sequencing technologies have provided a deeper molecular understanding of disease biology.

Early observations found a link between anemia, BM failure, and leukemia resulting in MDS being described as a preleukemic condition. It wasn't until a study in 1970 showing patients with high blast counts at the time of diagnosis progressed to leukemia more so than patients with lower blast counts that raised the possibility for MDS being an independent malignancy (Najean and Pecking 2008). It was also noted that some patients with preleukemia never developed leukemia and the term myelodysplasia was proposed (William B. Fisher, MD; Steven A. Armentrout, MD; Russell Weisman Jr. 1973). It wasn't for another decade that a definitive classification was published and accepted. In 1982, the French American British (FAB) Group reported five subtypes of MDS diagnosed by blast percentage and ring sideroblasts (J. M. Bennett D. Catovsky M. T. Daniel G. Flandrin D. A. G. Galton H. R. Gralnick C.

Sultan 1982). Since then, FAB and the World Health Organization (WHO) have been updating the recommendations for diagnosis.

Epidemiology

It wasn't until 2001 that MDS cases became reportable to the National Cancer Institute's Survey, Epidemiology and End Results (SEER) cancer database. From the data collected, it is estimated that MDS occurs in 4.9 individuals per 100,000, 13.5 per 100,000 in patients 65 years and older (Cogle 2015). With a median age of 76 years at diagnosis, age is a major risk factor in the development of the disease **(Figure 1.1)** (Ma et al. 2007; Xiaomei 2012). Despite advances in understanding disease pathogenesis, no significant improvement in survival rates have been observed in the past decade (Mayer et al. 2017). Furthermore, as awareness of reporting criteria increases and as the population ages, the number of MDS patients is likely to increase in the coming decades making the need for new therapies even more critical.

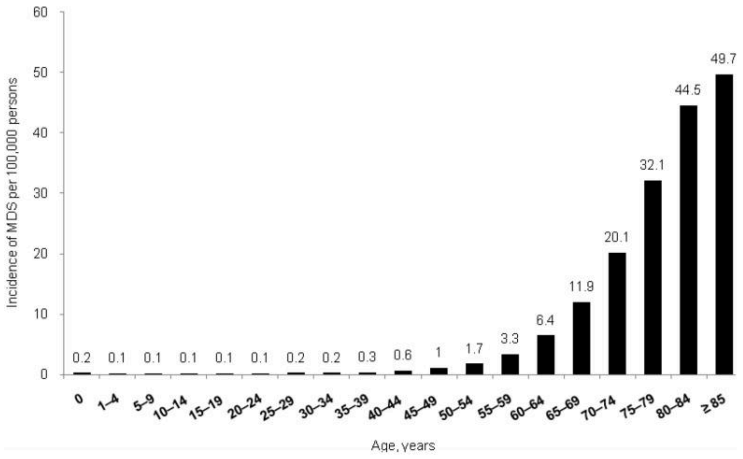


Figure 1.1. Incidence Rate of Myelodysplastic Syndromes (MDS) in Different Age Groups in the United States (2001 to 2008). *Adapted from Xiaomei, 2012.*

Diagnosis & Classification

MDS is first suspected after an abnormal complete blood count (CBC) or a blood smear show cytopenias. Once nonmalignant causes of the cytopenias are excluded, the BM is assessed for cellularity, dysplasia, presence of blasts, and cytogenic and mutational profiles are analyzed and based on the current WHO recommendations, the disease is subcategorized as MDS with single lineage dysplasia, MDS with multilineage dysplasia, MDS with ring sideroblasts with single lineage dysplasia, MDS with ring sideroblasts with multilineage dysplasia, MDS with isolated del5q, MDS with excess blasts 1, MDS with excess blasts 2, or unclassified MDS **(Table 1.1)** (Arber et al. 2016). In addition to these subtypes, MDS can be classified as primary or secondary. Primary MDS, or de novo MDS, is the more common type and no clear risk factors are known. Secondary MDS, also known as therapy-related MDS (t-MDS), generally occurs as a result of DNA damage from chemotherapy or radiation. (Cutler, 2014)

According to the International Prognostic Scoring System (IPSS-R) prognostic factors for MDS include cytopenia, blast percentage, cytogenetic abnormalities, fibrosis, and more recently, genetic mutations flow cytometry analysis of BM. Using these prognostic factors MDS is categorized into high-risk and low-risk.
Lower-risk (IPSS score ≤1.0) MDS patients primarily suffer from BM hypercellularity and peripheral cytopenia resulting from a significantly increased rate of apoptosis among BM hematopoietic cells. In contrast, BM hematopoietic cells in higher-risk MDS patients demonstrate resistance to apoptosis, and some of these patients subsequently develop AML (Greenberg 1998; Y. Yoshida, Anzai, and Kawabata 1995).

Table 1.1: WHO Classification of MDS Subtypes

↵* Cytopenias defined as: hemoglobin, <10 g/dL; platelet count, <100 × 10^9/L; and absolute neutrophil count, <1.8 × 10^9/L. Rarely, MDS may present with mild anemia or thrombocytopenia above these levels. PB monocytes must be <1 × 10^9/L

↵† If SF3B1 mutation is present.

↵‡ One percent PB blasts must be recorded on at least 2 separate occasions.

↵§ Cases with ≥15% ring sideroblasts by definition have significant erythroid dysplasia, and are classified as MDS-RS-SLD.

Name	Dysplastic lineages	Cytopenias*	Ring Sideroblasts	BM and PB blasts	Cytogenetics
MDS with single lineage dysplasia	1	1 or 2	<15%/<5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with multilineage dysplasia	2 or 3	1-3	<15%/<5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with ring sideroblasts					
MDS-RS with single lineage dysplasia	1	1 or 2	≥15%/≥5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS-RS with multilineage dysplasia	2 or 3	1-3	≥15%/≥5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with isolated del(5q)	1-3	1-2	None or any	BM <5%, PB <1%, no Auer rods	del(5q) alone or with 1 additional abnormality except −7 or del(7q)
MDS with excess blasts					
MDS-EB-1	0-3	1-3	None or any	BM 5%-9% or PB 2%-4%, no Auer rods	Any
MDS-EB-2	0-3	1-3	None or any	BM 10%-19% or PB 5%-19% or Auer rods	Any
MDS, unclassifiable					
with 1% blood blasts	1-3	1-3	None or any	BM <5%, PB = 1%,‡ no Auer rods	Any
with single lineage dysplasia and pancytopenia	1	3	None or any	BM <5%, PB <1%, no Auer rods	Any
based on defining cytogenetic abnormality	0	1-3	<15%§	BM <5%, PB <1%, no Auer rods	MDS-defining abnormality
Refractory cytopenia of Childhood	1-3	1-3	None	BM <5%, PB <2%	Any

As high-risk patients exhibit short survival and are more likely to progress to AML, more aggressive therapy, including hematopoietic stem cell transplantation (HSCT) is recommended with the aim to modify the course of the disease. Currently, HSCT is the only curative therapy for MDS; however, old age and other comorbidities limit tolerability of conditioning regimens for HSCT, offsetting its benefits for patients. Other therapies for high-risk patients include hypomethylating agents, azacytidine and decitabine (Cutler 2014). Low-risk MDS patients are treated with growth factors (EPO stimulating agents, G-CSF) and immunomodularory agents (antithymocyte, globulin, or lenalinomide) (Pierre Fenaux and Lionel Ad `es 2013). Over the last few years, better understanding of the disease biology has let to novel targets for therapy. Luspatercept, is a fusion protein that blocks transforming growth factor beta (TGF-B) alleviating the block in erythropoiesis in low-risk MDS patients (Platzbecker et al. 2017). Currently, phase III trials are evaluating efficacy, and if successful, Luspatercept will become the third drug to be approved by the FDA for MDS (Giagounidis 2018).

THE CELL OF ORIGIN

Identification Murine HSCs

MDS is a thought to be a clonal disorder originating in HSCs (Sperling, Gibson, and Ebert 2017; Woll et al. 2014). To better understand the pathogenesis of MDS, it is important to understand the defining features of HSCs.

19th century scientist, Franz Ernst Christian Neumann, was the first to propose the theory that all cell lineages in the blood originate from one cell. Early evidence of the HSCs came in a surprising finding when a group studying methods of recovery from

radiation injury observed that transplanting normal mouse BM protected recipient mice from lethal dose of radiation giving them a new source of blood cell lineages (Jacobson et al. 1951,Ford et al. 1956). More direct evidence of HSCs was proposed when Till and McCulloch performed transplantation of BM cells into irradiated mice and used x-ray induced lineage tracking to observe colony formation in the spleen. They showed that the number of cells transplanted was proportional to the number of colonies observed (Becker, McCulloch, and Till 1963; Till and McCulloch 1961). Moreover, these colonies distinct from one another, indicating multilineage potential of the transplanted cells (Becker et al. 1963). In 1957, E. Donnall Thomas performed the first successful stem cell transplantation demonstrating that injecting BM cells can lead to long-term repopulation of blood cells (E. Donnall Thomas, M.D.†, Harry L. Lochte, Jr., M.D.‡, Wan Ching Lu, Ph.D.§, and Joseph W. Ferrebee 1957). Since then, such transplants were being performed however, the true identity of the HSC was still unknown. It was these early studies that inspired the decades long exploration isolation and characterization of HSCs.

Prospective separation of HSCs based on differential expression of cell surface markers became possible with the advent of fluorescence-based cell sorting making possible the enrichment of the HSC population. The Weissman group designed a strategy to purify HSCs using known cell surface markers for positive and negative selection. Using $B220^{-}$, $Gr\text{-}1^{-}$, $Mac\text{-}1^{-}$, $CD4^{-}$, $CD8^{-}$, $CD90^{lo}$, and $Sca\text{-}1^{+}$, isolation of a multipotent HSC was made possible (GJ Spangrude, S Heimfeld 1988). In the following years, many groups identified additional cell surface markers by single-cell transplants to further define the hematopoietic hierarchy and to further purify the HSC population **(Figure 1.2)** (Benveniste et al. 2003; Ema et al. 2005; Kent et al. 2008; Kiel et al. 2005; Morrison and Weissman 1994; Osawa et al. 1996). From these

studies, a purified murine HSC population can be obtained using Lin$^-$, Sca-1$^+$, cKit$^+$, CD150$^+$, CD34$^-$, and CD48$^-$ combination staining.

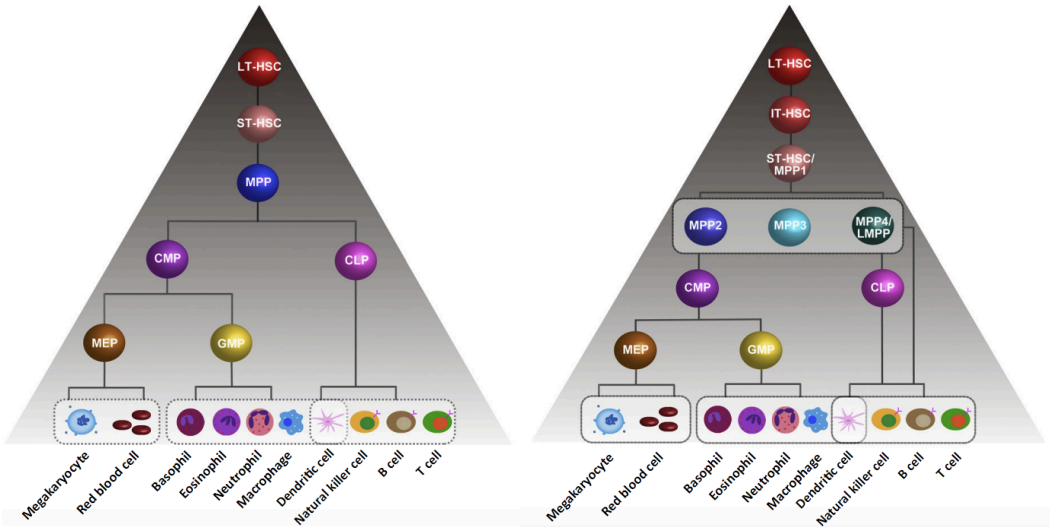


Figure 1.2. The Classical and Revised Roadmaps of Hematopoietic Hierarchy. (A) In the classical hierarchy roadmap, LT-HSCs and ST-HSCs are both multipotent but sit at different hierarchical levels owing to their distinct self-renewal abilities. The HSCs give rise to the MPPs with an accompanying reduction of self-renewal ability. Next there emerges a myeloid/lymphoid lineage segregation downstream of MPP. CLPs can produce lymphocytes. CMPs diverges into MEPs and GMPs. MEPs yield megakaryocytes and erythrocytes. GMPs generate granulocytes, macrophages, and dendritic cells. (B) In the revised hierarchy roadmap, LT-HSCs, IT-HSCs, and ST-HSCs/MPP1 cells are all multipotent but differ in their self-renewal ability. In the branching trees, the HSCs differentiate into MPPs, which consist of MPP2, MPP3, and MPP4/LMPP subpopulations. MPP2 and MPP3 cells mainly give rise to CMPs, whereas MPP4/LMPP cells predominantly generate lymphoid lineages. Next, CMPs can produce mature hematopoietic cells via MEP and GMP stages. CLPs can yield mature lymphocytes, which also can derive from MPP4/LMPP cells directly. Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; IT-HSC, intermediate-term hematopoietic stem cell; LMPP, lymphoid-primed multipotent progenitor; LT-HSC, long-term hematopoietic stem cell; MEP, megakaryocyte/erythrocyte progenitor; MPP, multipotent progenitor; ST-HSC, short-term hematopoietic stem cell. *Reprinted from Zhang et al., 2018*

Human HSC populations

The advent of in vitro clonal assays and xenotransplatation models allowed for progress to be made in purifying the human HSCs population. An in vitro colony forming assay was established by growing human BM cells on agar for several weeks (Gartner and Kaplan 1980; Sutherland et al. 1989) allowing the ability to grow colonies from candidate HSC populations. Long-term repopulating assays were made possible by the breakthrough in humanized mouse models. Severe combined immune-deficient (SCID) mice lacking T and B cells were used to engraft human BM cells (Kamel-Reid and Dick 1988; Lapidot et al. 1992). Success in this method provided a quantitative assay to measure long-term, multilineage potential of HSCs and gave a platform for the purification of human HSCs. The first cell surface marker found to enrich human HSCs was CD34 (Civin et al. 1984). Further studies established CD90, CD38, and CD45RA as additional markers to purify HSCs (Baum et al. 1992; Bhatia et al. 1997; Conneally et al. 1997; Lansdorp, Sutherland, and Eaves 1990; Majeti, Park, and Weissman 2007; Murray et al. 1995). These studies not only isolated HSC-rich populations, but also created an understanding of the hierarchy of the human hematopoietic system.

Characteristics of HSCs

Mature blood cells are generally short-lived and have to be replenished frequently to maintain normal peripheral blood counts. Residing in specialized niches in the BM, HSCs serve as a source of new blood cells by giving rise to terminally differentiated cells throughout the lifespan of an individual. This is made possible because HSCs maintain a balance of two key properties: differentiation and self-renewal. The ability to differentiate allows the HSCs to give rise to mature cell types including

erythrocytes, monocytes, granulocytes, megakaryocytes, T and B lymphocytes, natural killer, and dendritic cells. This multipotent ability is the result of a hierarchical structure in which HSCs residing at the top, progressively differentiating into multipotent progenitors, followed by committed progenitors with restricted lineage potential which eventually give rise to mature cells. Another defining characteristic of HSCs is their ability to self-renew which allows the cells to persist for long durations. HSCs undergo asymmetric cell division, giving rise to one differentiated daughter cell and one quiescent stem cell. While the differentiated daughter cells lose their ability to self-renew, the new quiescent stem cell maintains a long-term lineage reconstitution potential with each division (Seita and Weissman 2010). The balance between differentiation and self-renewal is key in maintaining a healthy supply of blood cells. Aberrations in these properties can cause hematologic disorders in which patient exhibit a loss of HSC pool or altered differentiation potential.

Early studies provided evidence that at any given time about 75% of HSCs exist in a quiescent state, 5% in the S/G2/M phase, and 20% in the G1 phase. More recent BrdU incorporation studies estimated that mouse HSCs divide approximately once in every 57 days (Cheshier et al. 1999). More stringent studies using inducible Histone H2B-GFP fusion protein to label cell division demonstrated that a more quiescent pool of HSCs divided only every 145 days (Foudi et al. 2008; Wilson et al. 2007).

Prospective separation of HSCs has allowed for a better understanding of HSC function in normal and malignant states and have also shed light on the alterations within the hematopoietic system associated during the aging process. Early studies investigating the frequencies of HSCs in mice showed that in the aged bone marrow, the number of phenotypic HSCs (lineage-cKit+sca+CD34-) is increased (Morrison et

al. 1996; Sudo et al. 2000). This increase in frequency is accompanied by an altered reconstitution potential as evident by decreased RBC and B cell output, as well as a myeloid bias (Chambers et al. 2007; Linton and Dorshkind 2004; Miller and Allman 2003; Rossi et al. 2005).

Aging is also associated with an increase in incidence of myeloid malignancies including MDS (Lichtman and Rowe 2004). Several early mutations, including DNMT3A, ASXL1, IDH1/2, and TET2, are found in MDS are also found in individuals without any overt sign of a hematologic malignancy (Busque et al. 2012; Jaiswal et al. 2014; Kwok et al. 2015). The presence of such mutations has been described as clonal hematopoiesis of indeterminate potential (CHIP). Studies of healthy samples from participants between the ages of 50 and 60 have shown that 95% of the individuals harbor mutations in DNMT3A and TET2, common preleukemic mutations. These mutations were found within both the myeloid and lymphoid compartments indicating that the mutation arose within the HSPC compartment (Young et al. 2016). While these mutations are correlated with an increased risk of hematologic malignancies, the risk is rather small, 0.5% per year overall, and despite the presence of clonal mutations, a majority of the people did not have hematologic malignancies (Jaiswal et al. 2014). Interestingly, looking at the mutational history of a founding clone in AML, many of the mutations were random events that occurred in the HSPC prior to the acquisition of the founding mutation (Welch et al. 2012). This suggests that accumulation of mutations in HSPCs may be a prior step in disease initiation.

Characteristics of MDS Stem Cells

The long-term sustenance of cancer can be attributed to the cancer stem cell (CSC). In a study by Furth and Kahn, a single mouse leukemia cell was shown to have tumor-initiating capacity in a recipient mouse (Furth, Kahn, and Breedis 1937). The CSC theory proposes that among the bulk cancer cells, a few cells act as stem cells by self-renewing and differentiating thereby sustaining the cancer. These cells result from the acquisition of genetic and epigenetic mutations and are capable of propagating the cancer (Clevers 2011).

While early studies suggested the existence of cancer stem cells (CSC) in hematologic malignancies, Dick and colleagues became the first to show direct evidence of the existence of a leukemia-initiating cell, or leukemic stem cell (LSC). In their studies, they showed that a subpopulation of leukemic cells expressing surface markers similar to normal hematopoietic stem/progenitor cells (Lineage- CD34+CD38-) were able to initiate leukemia in SCID mice and that this population did not consist of committed progenitor cells, but rather a more primitive cell population (Bhatia et al. 1997; Bonnet and Dick 1997; Lapidot et al. 1994; Larochelle et al. 1996). It has long been thought that MDS is a stem cell disease, however direct evidence was not provided until recent years (Pang et al. 2013; Tehranchi et al. 2010; Will et al. 2012). Early attempts to identify stem cell in MDS using xenograft models had been unsuccessful as the efforts to establish MDS in SCID mice resulted in poor grafts and did not develop signs of MDS (Benito et al. 2003; Grisendi et al. 2005; Kerbauy et al. 2004; Nilsson et al. 2000; Thanopoulou et al. 2004). However, global gene expression profile studies of CD34+CD38-CD90+ HSCs from del5q patients demonstrated that MDS HSCs had a closer identity to normal HSCs than to normal progenitors suggesting that normal HSCs may be the origin of the disease (Nilsson et al. 2007). Additionally,

fluorescence in situ hybridization (FISH) studies of del5q patient HSCs and highly purified HSC populations from -7/7q- demonstrated that nearly all HSCs and a majority of the myeloid cells had the cytogenetic the defect (Nilsson et al. 2000; Will et al. 2012). Together, these studies provided evidence that the origin of MDS lies in HSCs.

In addition to the cytogenetic abnormalities within MDS stem cells, quantitative changes have also been reported. Rigorous flow cytometry analysis of MDS patient samples showed phenotypic expansion of HSCs across subtypes, and an even greater expansion of the long-term HSCs (LT-HSCs), defined by Lin-,CD34+CD38-,CD90+ of high-risk cases (Pang et al. 2013; Will et al. 2012; Woll et al. 2014). High-risk patients were also shown to have expansion in the granulocyte-macrophage progenitors (GMP) (Pang et al. 2013). Across all subtypes of MDS, a decrease in megakaryocyte-erythroid progenitors (MEP) was observed suggesting apoptosis or a possible block in differentiation from common myeloid progenitors (CMP) to MEP, likely resulting in the anemia and thrombocytopenia observed in MDS patients (Will et al. 2012).

MOLECULAR ABERRATIONS IN MDS

Mutations Acquired in HSCs Lead to MDS

Recent findings focusing on the molecular underpinnings in MDS stem cells revealed genetic and epigenetic alterations. The use of whole-genome and whole-exome sequencing to study limited number of cells has enabled researchers to identify many recurrent mutations in MDS (Bejar et al. 2011; Haferlach et al. 2014; Papaemmanuil et al. 2013). Mutations have been characterized in DNA methylation regulators

(DNMT3A, TET2, IDH1, IDH2), chromatin modification (ASXL1, EZH2), spliceosome machinery (SF3B1, SRSF2, U2AF1, U2AF2, ZRSR2), transcription factors (RUNX1, GATA2), signal transduction (NRAS, JACK2, KRAS, CBL), and cell cycle regulator (TP53) (Ernst et al. 2010; Gelsi-Boyer et al. 2009; Graubert et al. 2011; Ley et al. 2010; Mardis et al. 2009; Yoshida et al. 2011). Among these mutations are early mutations, such as TET2, DNMT3A, ASXL1, TP53, SF3B1, and JAK2, which are responsible for the clonal expansion of HSCs and precede the development of MDS (Sperling et al. 2017).

Large scale studies of MDS patients have identified more than ten genes to be significantly correlated with prognoses, including SF3B1, SRSF2, ASXL1, RUNX1, TP53, BCOR, EZH2, and U2AF1. Additionally, the number of driver mutations at the time of diagnosis predicted the median leukemia free survival (Papaemmanuil et al. 2013). Mutations in TP53, EZH2, ETV6, RUNX1, and ASXL1 were shown to be associated poor survival (Bejar et al. 2011) and high probability of relapse. Furthermore, patients with ASXL1, RUNX1, and TP53 were shown to have higher relapse rate and decreased overall survival after HSCT (Della Porta et al. 2016).

Interestingly, early mutations associated with MDS are not exclusive to an overtly malignant state. Several studies have shown DNMT3A, ASXL1, SF3B1, and TET2 mutations are present in individuals without any clinical signs of hematologic malignancies (Busque et al. 2012; Genovese et al. 2014; Jaiswal et al. 2014). The presence of such mutations is described as clonal hematopoiesis of indeterminate potential (CHIP). CHIP is diagnosed in patients with a somatic mutation with a mutant allele fraction of at least 2% and no evidence of a hematologic malignancy (Steensma et al. 2015).

Mouse Models of MDS

Mouse models are invaluable and powerful tool in the study of cancers. While success has been achieved in creating xenografts of several hematologic malignancies, MDS cells have proven to be challenging to establish grafts with. However, as molecular aberrations of MDS begins to be more understood, developing genetically engineered models of the disease has been made possible. In 2002, the hematopathology subcommittee of the Mouse Models of Human Cancers Consortium developed a set of guidelines describing criteria for mouse models of hematologic malignancies including MDS. The criteria of MDS included three points: First, the PB should have either neutropenia, thrombocytopenia (in the absence of leukocytosis or erythrocytosis), or anemia (in the absence of leukocytosis or thrombocytosis). Second, maturation defect in non-lymphoid cells should have dysplasia in granulocytes, erythrocytes, or megakaryocytes, or have at least 20% blasts in the BM. Finally, the disorder should not meet the criteria for another non-lymphoid leukemia(Kogan et al. 2002).

One of the more studied models of MDS is the gene fusion model, NUP98-HOXD13. Using a vav promoter to drive the expression in hematopoietic tissues, mice develop anemia and neutropenia and, in some cases, macrocytosis and thrombocytopenia. The BM is either normal or hypercellular and multilineage dysplasia is observed and eventually the mice develop AML (Choi et al. 2009). This model has been used to identify several secondary mutation, including, Nras and Kras, that lead to AML (Slape et al. 2008).

An SRSF2 model was created using a hematopoietic specific Mx1-cre promotor to induce the Srsf2P95H mutation, found in 20-30% of MDS cases (Papaemmanuil et al.

2013; Yoshida et al. 2011). SRSF2 has an RNA motif to promotes spliceosome assembly to allow exon inclusion. Under the Mx1-cre, hematopoietic specific promoter, the Srsf2P95H/+ mice developed anemia with macrocytic erythrocytes, leucopenia, and multilineage dysplasia in the BM (Kim et al. 2015). SRSF2 was also shown to be is essential for HSC survival as the mutant mice exhibited decreasing HSCs. The mutant form altered its RNA splicing function, affecting genes involved in cancer development and apoptosis, contributing to MDS development (Komeno et al. 2015).

ASXL1 mutations are reported in 15%-20% of MDS patients (Boultwood et al. 2010; Gelsi-Boyer et al. 2009) The conditional deletion of Asxl1 in hematopoietic cells develop anemia, accompanied by an increase in erythroid precursors, consistent with an impaired differentiation phenotype. Leukopenia was evident by reduction in B cells, neutrophils, and monocytes. In vitro colony cultures also demonstrated impaired progenitor function observed by reduction in CMP, MEP, and GMP colony output. HSCs also exhibited defects in self-renewal capacity upon transplantation which is not a characteristic of MDS stem cells. A compound Asxl1-Tet2 loss restored the self-renewal defect induced by the Asxl1 loss, allowing the MDS stem cells to gain a competitive advantage (Abdel-Wahab et al. 2013).

TRANSLATION

Translation Levels in Hematopoietic Stem Cells

Maintenance of tissues is largely dependent upon the function of adult stem cells which exhibit tight regulation of gene expression to regulate fate commitment. Among the regulatory processes, translational control has been shown to be important for the

maintenance of stem cells across different species (Insco et al. 2013; Sampath et al. 2008; Zhang, Qiao; Shalaby, Nevine; Buszczak 2014). Moreover, several adult stem cells including hematopoietic (Signer et al. 2014, 2016), neural (Llorens-Bobadilla et al. 2015), skeletal muscle (Zismanov et al. 2016), hair follicle (Blanco et al. 2016), and drosophila germline stem cells (Sanchez et al. 2016) exhibit lower protein translation rates than more differentiated cells.

Maintenance of cellular protein, including synthesis, folding, targeting, and degradation, is one of the most regulated and energy consuming cellular processes (Buttgereit and Brandt 1995). Approximately 25-30% of the oxygen consumption coupled to ATP synthesis is used by protein synthesis (Brown 1997) a majority of which is spent on the biogenesis of components required for protein synthesis, notably the production, transport, and assembly of ribosomal subunits (Schmidt 1999; Strunk and Karbstein 2009; Warner 1999). Over the past few decades, progress in the structure and function of ribosomes and ribosomal proteins have provided insights into protein synthesis (Sonenberg and Hinnebusch 2007).

Ribosomes are essential components of the cellular machinery catalyzing the synthesis of proteins. Eukaryotes have 80S ribosomes consisting of a 40S and a 60S subunit both of which are made up of ribosomal RNA (rRNA) and approximately 80 ribosomal proteins. 60S, the larger subunit, is made up of approximately 50 ribosomal proteins (Rpl), and 3 rRNA molecules; 28S, 5.8S, and 5S. The smaller 40S subunit consists of about 33 ribosomal proteins (Rps) and an 18S rRNA (Strunk and Karbstein 2009). The biogenesis and assembly of ribosomal subunits is a highly regulated process taking place in the nucleolus, nucleus, and the cytoplasm. rRNA (28S, 5.8S, 5S, and 18S) are synthesized in the nucleolus and nucleolus. Ribosomal proteins are produced in the cytoplasm, transported to the nucleolus to assemble with rRNA

molecules, and then shuttled back to the cytoplasm where they mature. Protein translation requires the 40S and 60S ribosomal subunits, messenger RNA (mRNA), transfer RNA (tRNA), and initiation and elongation factors. Eukaryotic initiation factor (eIF) 2 recruits tRNA to the 40S subunit where the 5' end of an mRNA is recruited by eIF3 and eIF4 and scanned for the start codon. Finally, the 60S subunit is assembled to form the final 80S complex and elongation of the amino acid chain is initiated (Jackson, Hellen, and Pestova 2010).

Studies investigating regulation of protein synthesis in the hematopoietic system have shown that HSCs exhibit lower translation rates than restricted progenitors and mature hematopoietic cells (Signer et al. 2014, 2016). These reduced levels of translation were shown to be associated with the translational repressor, eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). In a hypophosphorylated state 4E-BP1 binds to eIF4, preventing recruitment of the 40S subunit to mRNA (Merrick, WC; Hershey 1996). HSCs were shown to have high levels of hypophosphorylated 4E-PB1/2 and 4E-BP1/2 deficient mice had increased rates of translation and impaired HSC function showing that regulation of translation is a vital to HSC function (Signer et al. 2016).

Defects in Ribosomal Proteins Lead to Impaired HSC Function

Ribosomal proteins are integral to the protein translation machinery and HSC function. Rpl24, a component of the 60S subunit, deficient mice ($Rpl24^{bst/+}$) exhibit overall decreased rates of protein synthesis. When compared to WT HSCs, HSCs from Rpl24 deficient mice had lower translation rates, decreased proliferation, and reduced reconstitution potential in transplant settings. Moreover, HSC exhaustion in Pten deficient mice, caused by increase translation rates, was rescued in the $Rpl24^{bst/+}$ background (Signer et al. 2014). In another study, deletion of notchless (Nle), essential

for 60S subunit maturation, results in the entry of quiescent HSCs into cell cycle and ultimately leads to their exhaustion. Interestingly, no increase in cell death pathways were observed leading to the possibility that the defects in ribosome biogenesis may lead to premature differentiation of HSCs (Le Bouteiller et al. 2013), an mechanism that has been demonstrated in HSCs and several other stem cells as a response to stress (Inomata et al. 2009; Li, Mangmang, Yunlong He, Wendy Dubois, Xiaolin Wu, Jianxin Shi 2013; Wang et al. 2012). Additionally, Runx1 deficient HSCs having impaired ribosome biogenesis have been shown to have decreased rates of apoptosis under homeostasis and genotoxic stress conditions (Cai et al. 2011, 2015). Together these studies demonstrate the delicate balance of protein synthesis required for proper HSC function.

Ribosomal Defects in Hematologic Malignancies

Defects in ribosomal biogenesis have long been implicated in congenital BM failure syndromes including diamond-blackfan anemia (DBF), Shwachman-Diamond syndrome (SDS), and x-linked dyskeratosis congenital (DKC) (Boocock et al. 2003; Draptchinskaia et al. 1999; Heiss et al. 1998). DBA, clinically characterized by anemia, macrocytosis, reticulocytopenia, and a block in erythropoiesis, is associated with mutations in several ribosomal proteins, including RPS19 and RPS24 which led to decrease 40S subunits (Choesmel V, Bacqueville D, Rouquette J, Noaillac-Depeyre J and Cretien A, Leblanc T, Tchernia G, Da Costa L 2007; Choesmel V, Fribourg S, Aguissa-Toure AH, Pinaud N, Legrand P and Gleizes PE 2008; Draptchinskaia et al. 1999). DBA was the first disease to be linked with defects in ribosomal proteins and became the first of a group of disorders to be categorized as ribosomopathies. A common feature of many of these syndromes is the increased risk of developing cancers including MDS (Blanche P. Alter, Neelam Giri 2009; Donadieu et al. 2005).

Del5q syndrome is the most common chromosomal abnormality in MDS patients and is classified as having low risk of transformation to AML and long survival (Van Den Berghe et al. 1974; Jacqueline Boultwood, Sian Lewis 1994). Ribosome biogenesis defected in MDS were first shown in studies of del5q patients showing haplo-insufficiency of two important ribosomal genes, RBM22 and RPS14 (Boultwood et al. 2007). RPS14 deficiency, causes defects in 18S RNA processing and lead to reduced levels of the 40 S subunit. Using RNAi to deplete RPS14, it was shown that haplo-insufficiency of RPS14 was played a causative role in development of the disease (Ebert et al. 2013).

Evidence of defects in ribosomal proteins in non-del(51) MDS patients suggested that ribosome biogenesis defects may not be limited to del5q (Czibere et al. 2009; Davendra P. Sohal, Andrea Pellagati, Li Zhou, Yongkai Mo, Joanna B. Opalinska, Christina Alencar, Christoph Heuck, Amittha Wickrema, Ellen Friedman, John Greally, Benjamin L. Ebert, Jonathan Warner 2008; McGowan et al. 2011). In a large scale study, CD34+ cells from 72 non-del5q MDS patients and 11 del5q MDS patients were screened and it was found that 72% of the non-del5q patients had decrease in RPS14 expression (Czibere et al. 2009). In another study, 97 MDS patients, 54 low-risk and 43 high-risk, RPL23, inhibitor of apoptosis, was overexpressed in high-risk patients and possibly caused disease progression (Qi et al. 2017).

Therapeutic Strategies

The dysregulation of protein synthesis implicated in many other cancers has provided a window for therapeutic intervention. Inhibiting ribosome biogenesis and translation

initiation by targeting RNA Pol I, EIF4A, EIF4e, EIF2S1, mTOR, and dual PI3K-mTOR have been explored.

Aberrant regulation of the mTOR pathway has been implicated in several hematologic malignancies including AML, T-cell acute lymphoblastic leukemia (T-ALL, chronic myelogenous leukemia (CML), and high-risk MDS (Altman and Platanias 2008; Teachey, Grupp, and Brown 2010). mTOR, hyperactive in many cancers, forms two complexes, mTORC1 and mTORC2 and drives anabolic pathways, including protein synthesis and ribosome production. Rapamycin, an mTORC1 inhibitor, has been proposed as a therapy for cancer. Rapamycin, which specifically targets mTORC1, has been clinically used since 2000 to prevent organ transplant rejection because of its immunosuppressant functions (MacDonald 2001).

Cycloheximide (CHX) has been shown to block the elongation phase of eukaryotic translation. It binds the ribosome and inhibits eEF2-mediated translocation (Hardesty 1971). CHX allows one complete translocation cycle to proceed before halting any further elongation (Pestova and Hellen 2003). CHX has been shown to have antiproliferative effects in leukemia cell lines (Myasnikov et al. 2016), however, since CHX has teratogenesis and reproductive side-effects it is not recommended to be used in vivo.

Homoharringtonine is a translation elongation inhibitor and acts by binding to the 80S ribosome and inhibits protein synthesis. Hamoharringtonine has been approved by the FDA as a therapy for CML (Alvandi et al. 2014) and is in trials for AML (Alvandi et al. 2014).

Several inhibitors of translation have been implicated in treating hematologic disease, however they have not shown to have specific effects on CSCs. As ribosomal defects are pronounced in MDS and other bone marrow failure diseases, targeting ribosomes further may serve as a powerful therapy for depleting MDS stem cells. In this manuscript, we describe using the Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ and Mx-cre- Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ to assess the role for the aging stem cell in MDS pathogenesis and the targeting of the 60S ribosomal subunit in the Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ mouse model of MDS stem cells using an immunotoxin, saporin.

CHAPTER TWO
THE ROLE OF AGING STEM CELLS IN MDS PATHOGENESIS

INTRODUCTION

Aging is associated with changes in the hematopoietic system due to alterations in HSCs including increased HSC frequency, reduced red blood cells and lymphocytes, an increase in myeloid cells (Chambers et al. 2007; Jaiswal et al. 2014; Linton and Dorshkind 2004; Miller and Allman 2003; Morrison et al. 1996; Pang et al. 2011; Rossi et al. 2005; Sudo et al. 2000), and clonal hematopoiesis (Jaiswal et al. 2014; Steensma et al. 2015). In addition to these changes, aging is also associated with an increased risk of developing malignancies originating from HSCs including MDS (Busque et al. 2012; Genovese et al. 2014; Jaiswal et al. 2014; Jan et al. 2012; Kwok et al. 2015). Hematologic changes observed in MDS, including anemia, decreased lymphoid output, myeloid bias, increased HSC frequency, and clonal expansion are similar to those observed in the normal elderly population **(Table 2.1)**. We hypothesized that in addition to the somatic mutations and karyotypic alterations, MDS pathogenesis is also dependent upon factors present in normal aging background.

Table 2.1: Hematopoietic Features in Aging and MDS

	Age	MDS
Increased HSC number	✓	✓
Decreased HSC long-term reconstitution potential	✓	✓
Myeloid Differentiation Bias	✓	✓✓✓
Decreased lymphoid production	✓	✓✓✓
Decreased red cell production	✓	✓✓✓
Clonal Expansion	✓	✓✓✓
Risk of MDS/AML	✓	✓✓✓

Given that MDS arises from HSCs and the elimination of MDS HSCs is required for a cure, it is important to identify all the factors that regulate MDS HSC function. In this study we investigated the contribution of aging in the development and severity of MDS phenotypes. We used the Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ and Mx-cre- Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ mouse models of MDS to address two questions: 1) Does the age of the cell-of-origin impact MDS pathogenesis and 2) Does disease chronicity impact MDS stem cell phenotype.

RESULTS

The Cell-of-Origin Impacts MDS Pathogenesis

We proposed to use Mx-cre- Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ conditional null mice to study the effect of varying the age when MDS initiating genetic deletions are induced on MDS disease phenotype and HSC function, predicting that older mice induced to develop MDS would manifest disease with faster kinetics and a more pronounced phenotype.

Initial transplants were set up using 2X10^6 mononuclear cells (MNC) from Mx-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ (CD45.2) mice of varied ages (5, 12, and 22 months) into lethally irradiated CD45.1 recipients. At 16 weeks following stable engraftment, Asxl1 and Tet2 deletions were induced using polyI:polyC **(Figure 2.1a)**. Following establishment of nearly 100% chimeras **(Figure 2.2b)**, mice were tracked long-term for development of disease based on peripheral blood and bone marrow analysis.

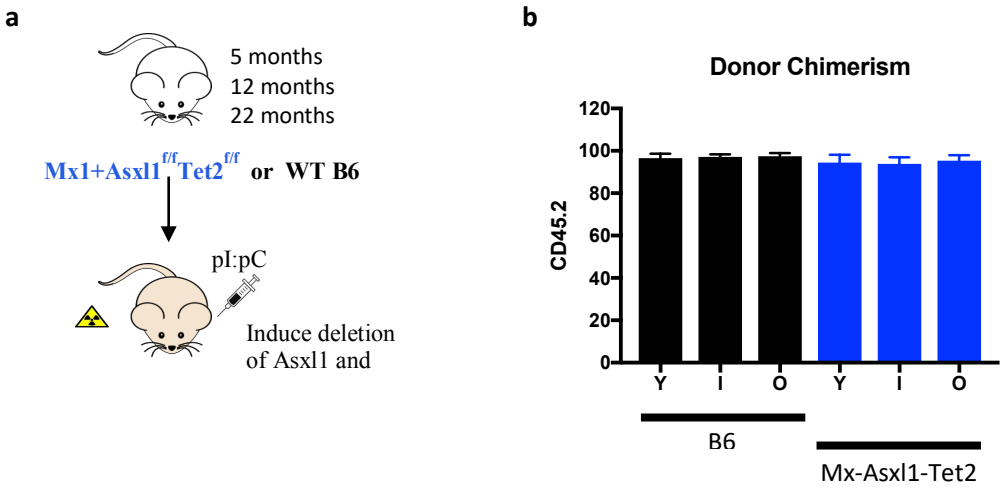


FIGURE 2.1. Generation of Stably Engrafted Recipients

a. Experimental Setup. $2x10^6$ MNC from Mx-cre- $Asxl1^{fl/fl}$-$Tet2^{fl/fl}$ or C57BL6 WT mice were transplanted into lethally irradiated mice. 16 weeks after transplant, deletion of Asxl1 and Tet2 was induced by 5 intraperitoneal injections of polyI:polyC every other day at a dose of 20mg/kg of body weight.

b. 16-week Total Peripheral Blood Donor Chimerism. Donor chimerism represented by frequency of donor cells of total CD45+ cell population.

6 months after the induced deletion of Asxl1 and Tet2 genes, peripheral blood counts show groups receiving old MDS cells exhibit lower white blood cell, specifically in the neutrophil compartment, when compared to groups receiving old WT cells **(Figure 2.2a)**. The neutropenia was confirmed with the reduction of Gr1-+Mac-1+ granulocytes in the peripheral blood by flow cytometry analysis **(Figure 2.2b)**. Lineage analysis in the peripheral blood showed no difference in monocyte, B cell, and T cells at this time point. (data not shown).

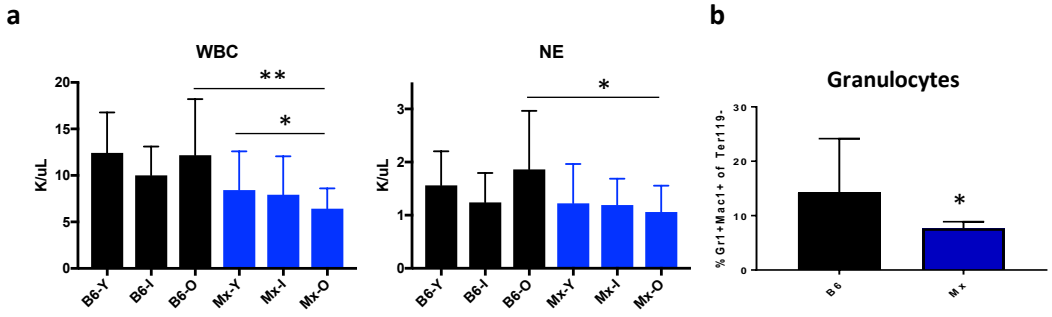


FIGURE 2.2. Recipients of Old Donor HSCs Exhibit Leukopenia

a. **White Blood Cell and Neutrophil Counts.** Complete blood counts measured using HemaVet 950.

b. **Peripheral Blood Flow Cytometry Analysis of Granulocytes.** Granulocyte frequency as measured by Gr-1+Mac-1+ of total Ter119- cells. All data represent mean±s.d. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

As expected, groups receiving young MDS cells had lower hematocrit percentage when compared to groups receiving young WT cells, however, this reduction did not become evident until 12-months post-deletion **(Figure 2.3b)**. At the 6-month time point, hematocrit percentage were elevated in recipients of old MDS cells indicating a possible compensatory mechanism to produce excess RBC in response to an initial decrease in output **(Figure 2.3a and 2.3b)**. Alterations in platelet counts are observed in normal aging and in this transplant, old WT donors exhibited reduced platelet output persistent from 6-months onward **(Figure 2.3a and 2.3b)**. This reduced platelet phenotype was not observed in recipients of old MDS donor cells possibly indicating an intrinsic advantage of MDS cells or the result of an advantage due to a young microenvironment. These findings are consistent with the known myeloid dysplasia and leukopenia in MDS patients and mice.

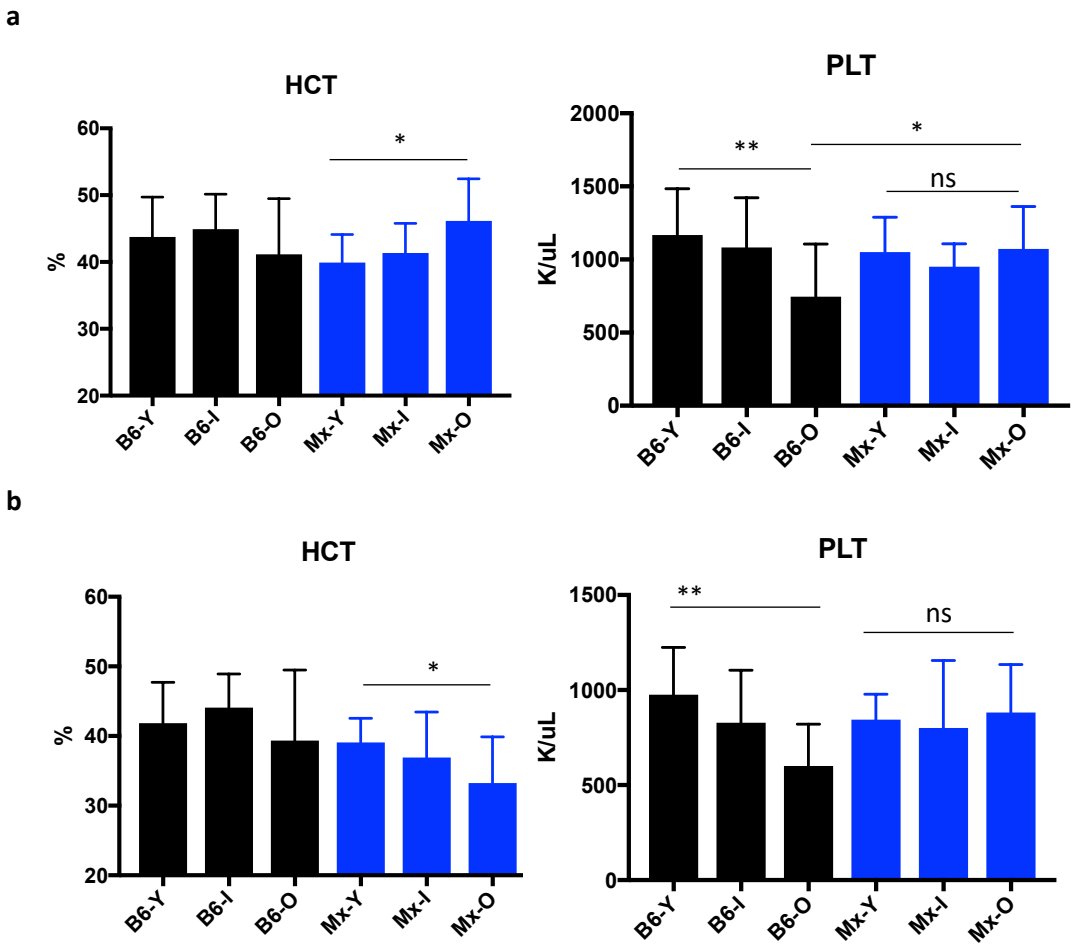


FIGURE 2.3. Recipients of Old Donor HSCs Decrease in RBC Output

a. **Hematocrit % and Plate Count at Six-Months Post-Deletion.**

b. **Hematocrit % and Plate Count at Twelve-Months Post-Deletion.** Complete blood counts measured using HemaVet 950. All data represent mean±s.d. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

As expected, recipients of old MDS cells also exhibited an expansion of the LSK and CMP populations in the bone marrow **(Figure 2.4a)**. This expansion is consistent with the previously described MDS phenotype in humans and mice. Kaplan-Meier survival curves of groups transplanted with young, intermediate, and old WT and mutant cells exhibited reduction in survival among recipients of old donor cells **(Figure 2.4b)**. Together, these data suggest that the age of a stem cell at the time of deletion plays a

role in disease phenotype including neutropenia, defects in red cell compartment, expansion of HSCs, as well as overall survival.

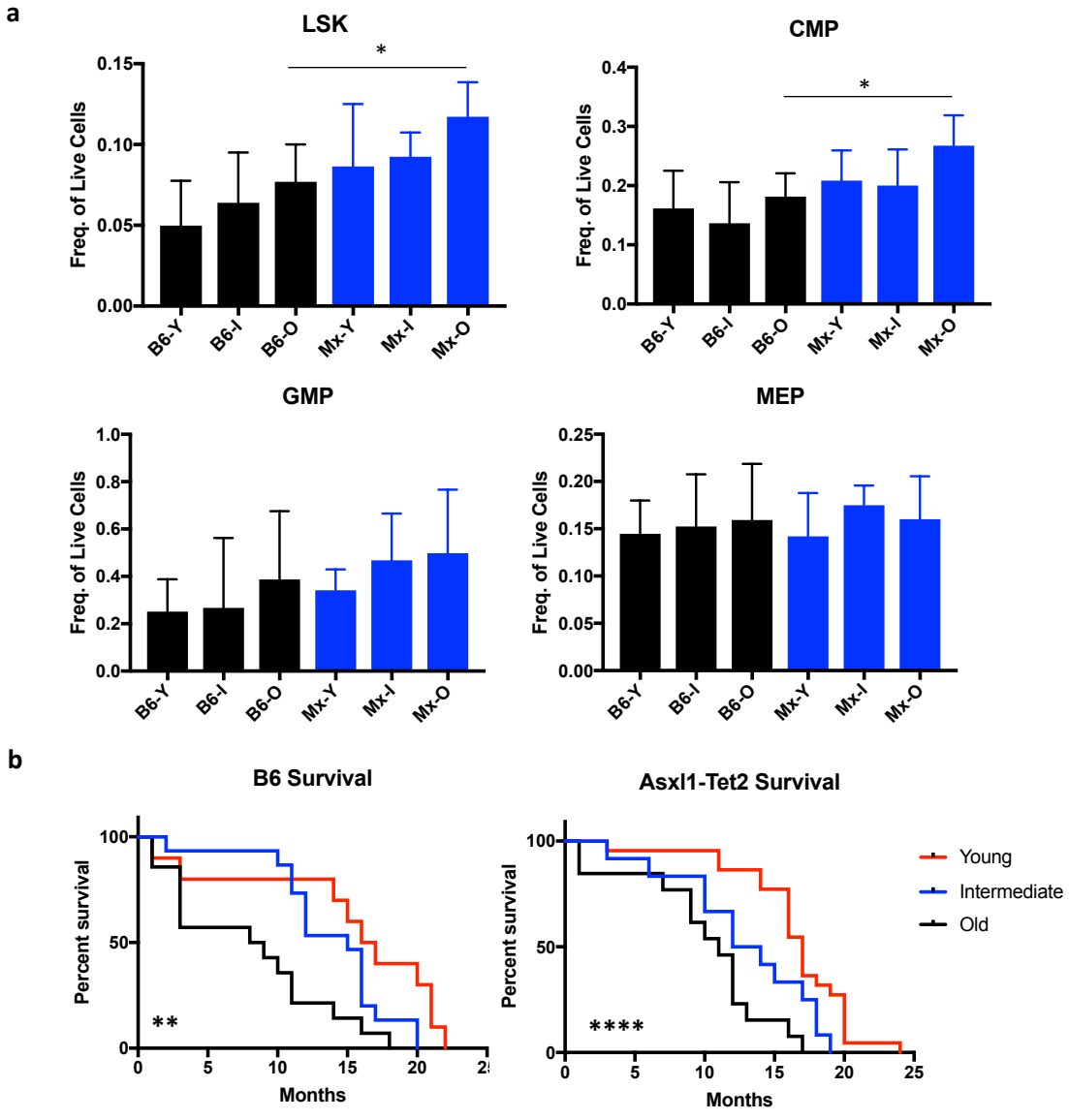


FIGURE 2.4. Deletion of Asxl1-Tet2 Results in the Expansion of HSPCs and Reduced Survival of Recipients of Old Stem Cells

a. Bone Marrow HSPC Flow Cytometry Analysis Twelve-Months Post-Deletion. Bone marrow aspirates were performed and HSPC compartments were analyzed by flow cytometry.

b. Kaplan-Meier Survival Curves. All data represent mean±s.d. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Impact of Disease Chronicity on MDS Stem Cell Phenotype

To assess the impact of disease chronicity on MDS pathogenesis, transplants were set up using mononuclear cells from Vav-creTet2$^{fl/fl}$Asxl1$^{fl/fl}$ (CD45.2) constitutive double knock out mice of varied ages (5, 14, and 23 months) into lethally irradiated CD45.1 recipients. Following establishment of nearly 100% chimeras, mice were tracked long-term for development of disease based on peripheral blood and bone marrow analysis. Long-term cell counts showed no significant differences between the various groups **(Figure 2.5)**. As MDS is characterized by an increase in severity of anemia with disease progression, this similarity in RBC between the young and old mutant recipients was unexpected.

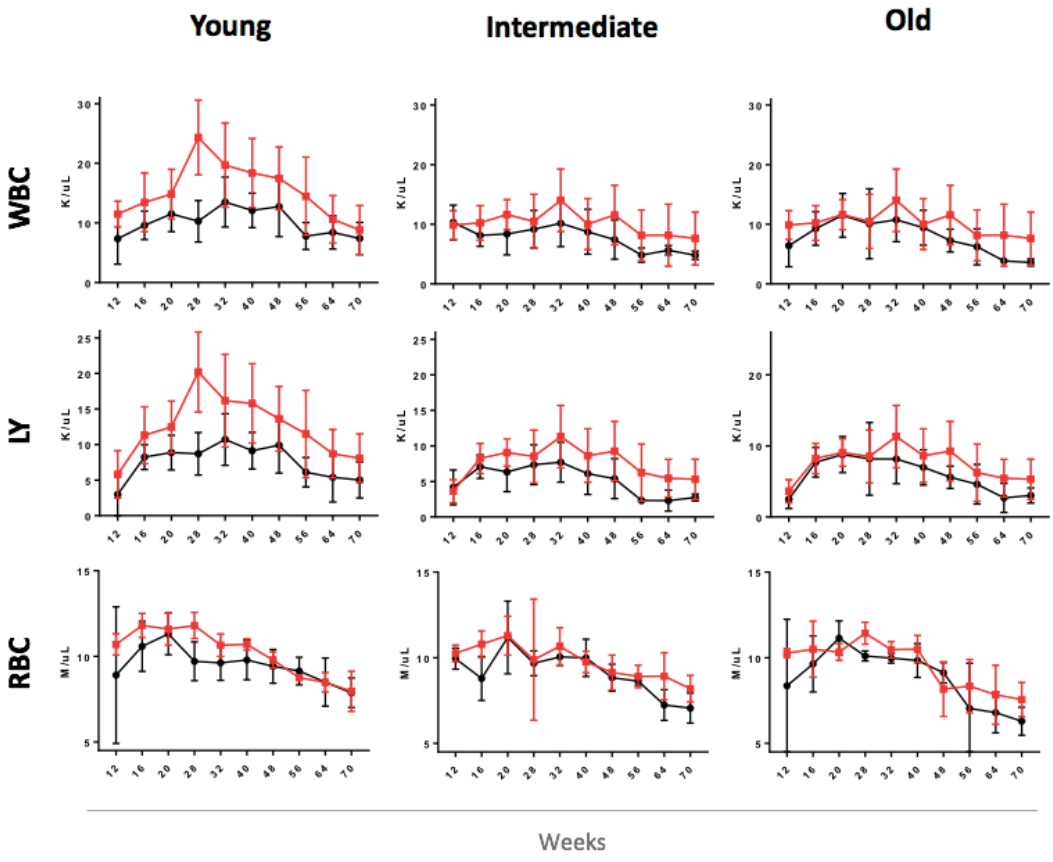


FIGURE 2.5. No Significant Differences in Long-Term Peripheral Blood Counts of Young and Old Mutant Recipients. Complete blood counts measured using HemaVet 950. All data represent mean±s.d. *, p<0.05; **, p<0.01; ***, p<0.001.

Examination of the bone marrow at the 34 and 60-week time point showed maintenance of high over all donor chimerism as well as chimerism within hematopoietic stem & progenitors (data not shown). Further examination showed groups receiving intermediate or old MDS cells exhibit lower GMP frequencies when comparted to groups receiving WT cells **(Figure 2.6).** This is consistent with the previously known findings that the Vav-creTet2$^{fl/fl}$Asxl1$^{fl/fl}$ mice exhibit age-dependent myeloid dysplasia. Surprisingly, we also observed that mice receiving MDS cells exhibited lower LSK frequencies **(Figure 2.6).** Vav-creTet2$^{fl/fl}$Asxl1$^{fl/fl}$ mice have been shown to have rescue the impaired self-renewal seen in the single Asxl1-deficient mice. These findings suggest a non-cell intrinsic mechanism leading to the lower LSK frequencies. Furthermore, when comparing young, intermediate, and old MDS cell recipients, no significant differences in HSCS frequencies was observed

As expected, groups receiving young WT cells showed increased survival when compared to those receiving old WT cells. Interestingly, this difference was not observed when comparing groups receiving young, intermediate, and old MDS cells. Additionally, groups receiving old MDS cells had a significant increase in survival when compared to groups receiving old WT cells **(Figure 2.7)**. As donor chimerism was maintained at equally high levels in these two groups (Data not shown), we have ruled out that this is due to inability of MDS cells to engraft. Together, these data demonstrate that constitute deletion of Asxl1 and Tet2 did not alter disease progression with varying the age of the stem cell. This was evident by similar peripheral blood counts, LSK frequencies, as survival of recipients of mutant cells.

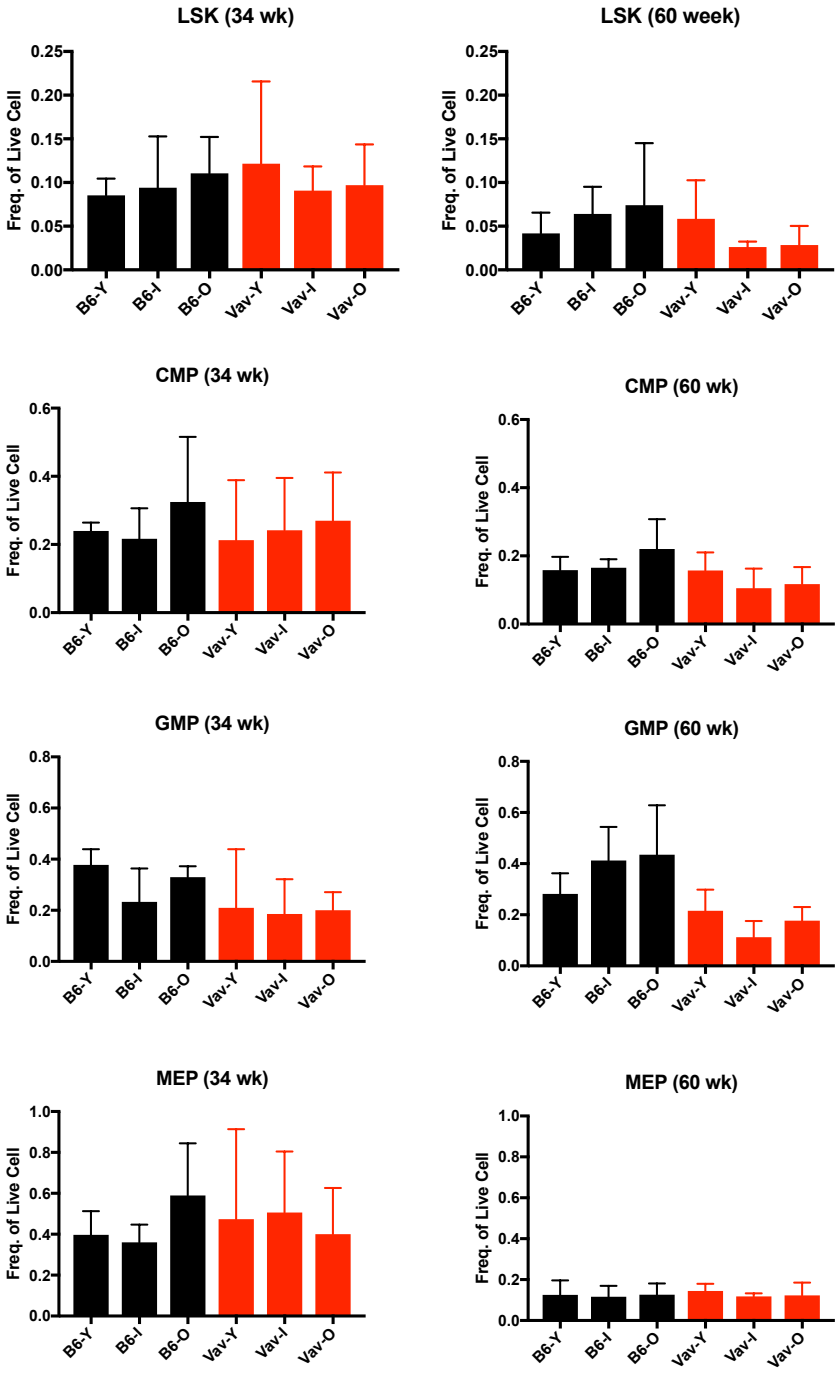


FIGURE 2.6. Bone Marrow HSPC Frequencies. Bone marrow aspirates were performed on recipient mice at 34 and 60-weeks post-transplant. LSK, CMP, GMP, and MEP frequencies were determined by flow cytometry analysis.

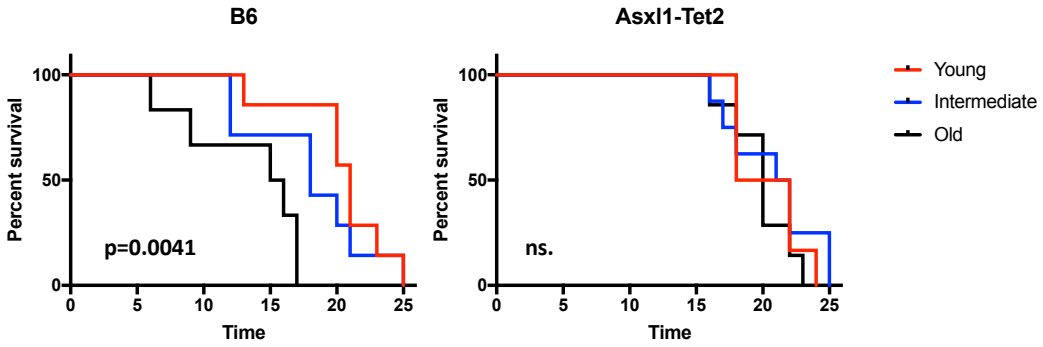


FIGURE 2.7. Kaplan-Meier Survival Curve All data represent mean±s.d. *, p<0.05; **, p<0.01; ***, p<0.001.

DISCUSSION

In this study, we examined the effects of aging stem cells on MDS pathogenesis. Using a genetically accurate model of MDS, we show that hematopoietic specific deletion of Asxl1 and Tet2 in older cells manifests disease with faster kinetics as evident by decreased disease latency, greater disease severity, and decreased survival. Additionally, constitutive deletion of Asxl1 and Tet2 in young, intermediate, and old stem cells resulted in manifestation of disease at a similar rate and recipient mice exhibited no differences in overall survival. While age of the cell of origin impacts disease pathogenesis, disease chronicity itself does not impact MDS phenotype. Cell extrinsic factors may play a role in full manifestation of MDS.

CHAPTER THREE
TRANSLATION IN MDS AND THE DEPLETION OF HSCS WITH CD117-SAPORIN IMMUNOTOXIN

INTRODUCTION

Defects in ribosomal proteins have been reported across different subtypes of MDS (Alkhatabi et al. 2016; Boultwood et al. 2007; Cai et al. 2015; Czibere et al. 2009; Davendra P. Sohal, Andrea Pellagati, Li Zhou, Yongkai Mo, Joanna B. Opalinska, Christina Alencar, Christoph Heuck, Amittha Wickrema, Ellen Friedman, John Greally, Benjamin L. Ebert, Jonathan Warner 2008; Lansdorp et al. 1990; McGowan et al. 2011). As these defects are pronounced in MDS stem cells they may be functioning on limited resources, making the more vulnerable targets of ribosome inhibitors.

Ribosome-inactivating proteins (RIPs) are toxins which specifically and irreversibly inhibit protein synthesis by cleaving a specific adenine residue (A^{4324}) on the 28S ribosomal subunit. Hundreds of RIPs have been isolated from plants and bacteria and have been shown to have a range of toxicity and are classified as type I or type II. Type I RIPs are single A-chain proteins with N-glycosidase enzymatic activity while type II RIPS have two polypeptide chains (A and B chain). The A-chain has enzymatic function and the B-chain is a lectin-like peptide with an affinity for galactose allowing translocation through the plasma membrane making type II RIPS more toxic (Schrot, Weng, and Melzig 2015; Stirpe 2004). In 1891, ricin and abrin became the first RIPs to be identified by Paul Ehrlich who identified the proteins and raised antibodies against them (Olsnes 2004). Several decades later the enzymatic activity was

described and ricin and related proteins were characterized as proteins that inhibit translation by damaging ribosomes, or ribosome-inactivating proteins (Olsens and Pihl 1972).

Saporin, isolated from Saponaria officinalis L (soapwort), is a type I RIP with nine forms. Saporin-6 is the most abundant form and conserves the RNA N-glycosidase activity specific to A^{4324} of the 28S ribosomal subunit. While Saporin-6 lacks the B-chain, some groups have reported internalization of saporin by endocytosis in some cell types (Bagga, Seth, and Batra 2003; Cavallaro et al. 2018). Once internalized, saporin permanently inactivates the 28S subunit, blocking the binding of EF-1 and the formation of EF-2. This prevents the translocation of tRNA from the A site to the P site of the ribosomal complex ultimately leading to cell death (Endo and Tsurugi 1988).

Saporin molecules have been conjugated to antibodies of cell surface markers to generate immunotoxins against several cancer types, including hematologic malignancies (Polito et al. 2013). The first clinical trial used CD30-Saporin conjugates to treat refractory Hodgkin lymphoma patients. While the therapeutic effect was limited to a few months, the immunotoxin was successful in reducing the tumor mass by up to 60% (Falini et al. 1992).

In a recent study, saporin conjugated to a CD45.2 internalizing antibody was used to deplete the BM of C57BL/6 wild-type mice allowing for nearly 100% engraftment of donor cells. Despite CD45.2 being expressed on most hematopoietic cells in the BM, CD45-Saporin conditioning had minimal effects on most of the mature BM cells avoiding toxicity generally caused by irradiation conditioning. This study

demonstrated a novel method of conditioning patients for HSCT (Palchaudhuri R et al 2016).

Here we aim to test whether saporin treatment can deplete MDS stem cells with minimal effects on the rest of the BM architecture. As standard conditioning regimen for HSCT for MDS patients can be too aggressive, affecting cells beyond the target, a more targeted and less aggressive regimen can serve as a replacement, making it possible for older patients with comorbidities to be eligible for HSCT. Additionally, as MDS HSCs have been shown to have global reductions in translation levels when compared to normal counterparts, they may have been more susceptible to further inhibition of translation.

RESULTS

Normal Human and MDS Patient HSCs exhibit decreased translation rates

We first sought to quantify the rate of translation in human HSCs by using an op-puro incorporation assay. While one of the most well-established methods of measuring global changes in protein synthesis is dependent upon the incorporation of radiolabeled amino acids, such as ^{35}S methionine and ^{35}S cystine, in newly synthesized polypeptides, we needed a flow cytometry-based assay to measure protein synthesis of HSCs within a bulk population. A more recent assay using P-propargyl-puromycin (OP-Puro) has been developed to detect protein synthesis by fluorescence and for cells in vitro and in vivo. OP-Puro, an alkyne puromycin analog, when taken up by cells during active protein synthesis forms covalent polypeptide-OP-Puro conjugates and

nascent polypeptides can be quantified using fluorescence-based techniques (Liu et al. 2012).

Umbilical cord blood is a rich source for human HSCs and progenitors. Assessing protein translation in CD34 enriched cord blood cells, we found that when compared to total CD45+ cells, progenitors (CD45+CD34+CD38+) and more strikingly, HSCs (CD45+CD34+CD38) exhibit lower op-puro incorporation **(Figure 3.1a)**. We further assessed op-puro incorporation in cells from two normal human bone marrow samples and observed more than a 50% reduction in translation within the progenitors and HSC compartment when compared to total CD45+ cells **(Figure 3.1b)**. These results demonstrate that human HSC and progenitors from both cord blood and BM, exhibit reduced translation rates.

We next sought to assess translation in stem cells from MDS patient BM samples. We obtained a BM aspirate sample from a patient with refractory anemia with excess blasts (RAEB-1), 6%. Patients with RAEB-1 have multiple cytopenias and have between 5-9% blasts in the BM. Similar to the observations in normal BM HSCS, op-puro incorporation in HSPCs was less than half of the levels as total CD45+ cells **(Figure 3.2a)**.

To further validate the reduced translation in MDS HSCs, we compared monosomes and polysomes from a healthy patient and an MDS patient with RUNX1 and EZH2 mutations. First using CHX to freeze translating ribosomes with mRNA transcripts on FACS sorted CD34 cells, cell lysates were added to sucrose gradients followed by centrifugation and fractionation. Early fractions representing monosomes had higher

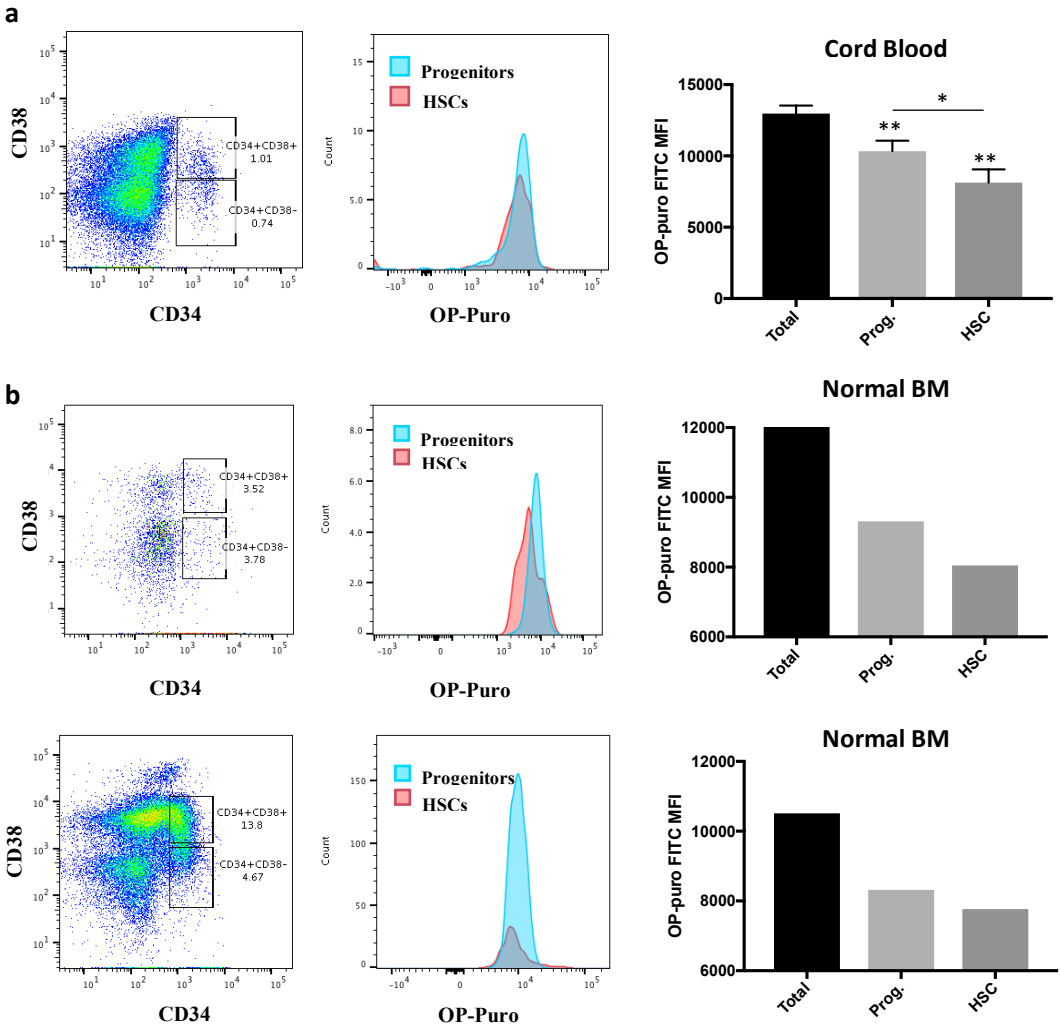


FIGURE 3.1. Cord Blood and BM HSCs Exhibit Reduced Translation
Total fraction represents CD45+ cells. Progenitors cells are phenotypically characterized as CD45+ CD38+ CD34+ fractions and HSCs are characterized by CD45+ CD38- CD34+. Representative histograms show OP-puro MFI comparing progenitor and HSC populations.

a. **OP-Puro MFI in Cord Blood.** Representative gating strategy, OP-Puro MIF histogram, and bar graphs. 300,000 CD34 enriched cord blood cells. N=3. All data represent mean±s.d. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

b. **OP-Puro MFI from Two Normal BM Patient Samples.** Representative gating strategy, OP-Puro MIF histogram, and bar graphs. 300,000 CD34 enriched bone marrow cells.

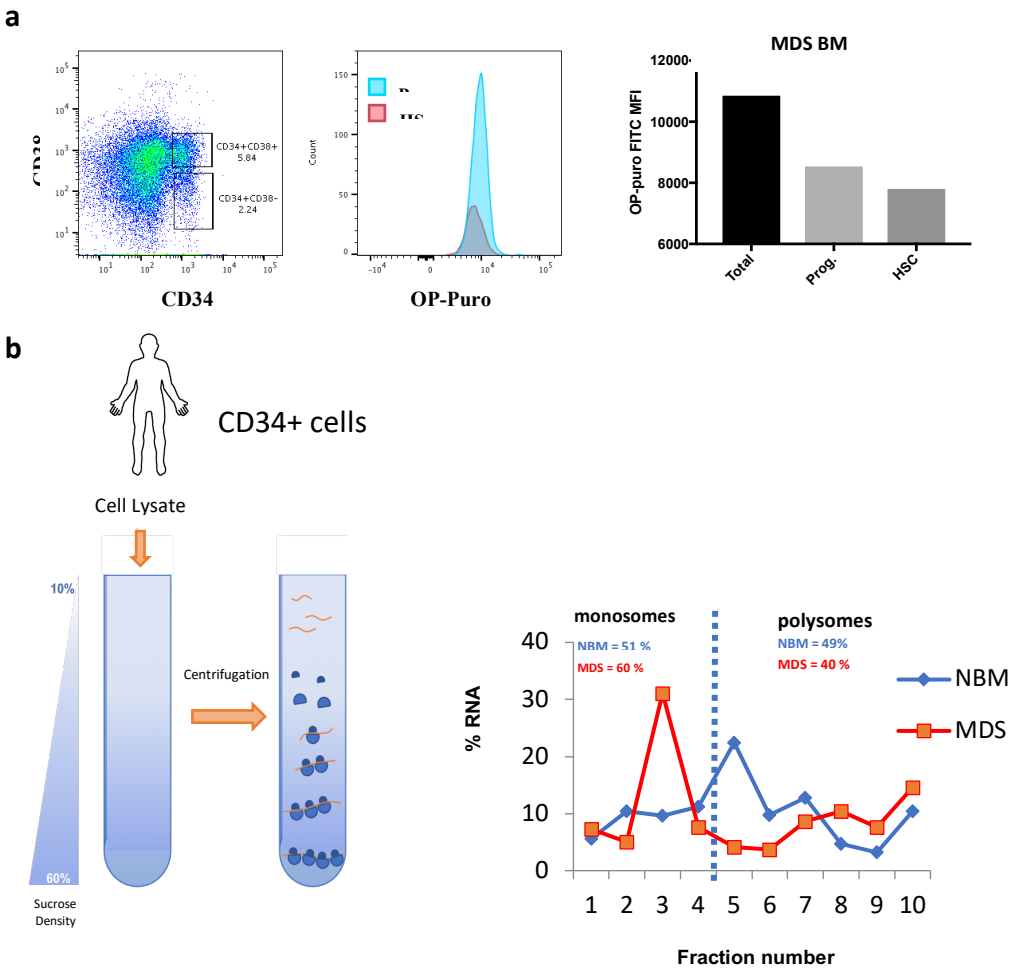


FIGURE 3.2. MDS HSCs Exhibit Reduced Translation

a. **OP-Puro Incorporation in an MDS Patient BM Samples** Total fraction represents CD45+ cells. Progenitors cells are phenotypically characterized as CD45+ CD38+ CD34+ fractions and HSCs are characterized by CD45+ CD38- CD34+. Representative histograms and bar graph show OP-puro MFI comparing total CD45+, progenitor, and HSC populations.

b. **Monosomes and Polysomes of Healthy and MDS patient BM sample.** CD34+ cells from healthy and MDS patient samples were treated with 10mg/ml of CHX followed by centrifugation and fractionation. Ribosomal RNA content is plotted against each fraction.

ribosomal RNA (rRNA) content in CD34+ MDS cells when compared to the healthy control indicating more free ribosomes. Late fractions, representing the polysomes, showed lower rRNA content in CD34+ MDS cells when compared to the healthy control indicating fewer ribosomes bound to mRNA transcripts **(Figure 3.2b)**. These two MDS patient samples both showed reduced translation in HSPCs however, analysis of larger sample number across different MDS subtypes will be necessary to validate these findings.

Mouse Models of MDS Exhibit Reduced Translation

We next wanted to test the efficacy of saporin in depleting MDS stem cells. To investigate this, we first established whether HSCs from mouse models of MDS also exhibit decreased translation. We began with an SRSF2 mouse model which carries a mutation, found in 20-30% of MDS patients (Papaemmanuil et al. 2013; Yoshida et al. 2011), altering its splicing function. cKit enriched cells from an SRSF2 mutant and an SRSF2 wild-type were run through a sucrose gradient rRNA content across ten fractions was measured. cKit enriched cells from the mutant mouse had higher rRNA content within the monosome fractions and lower rRNA content within the polysome fractions when compared to wild-type cells indicating reduced translation in the mutant cells **(Figure 3.3a)**.

Additionally, we measured translation in the Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ mouse model. Wild-type and mutant cKit enriched cells were assessed for op-puro incorporation. Wild-type HSCs (Lineage- Sca-1+ cKit+) trended toward having lower translation than progenitors and mutant HSCs were shown to have significantly lower translation than mutant progenitors. Moreover, when compared to wild-type HSCs, mutant HSCs

had reduced translation **(Figure 3.3b)**. Taken together, the SRSF2 and Asxl1-Tet2 models of MDS both exhibit reduced translation in HSCs.

In Vivo Saporin Treatment Depletes HSCs

To mimic a human MDS patient BM, wild-type mononuclear cells from UBC-GFP mice and mutant cells from Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ mice were transplanted at a 1:1 ratio into lethally irradiated recipients. 12-weeks post-transplant, mutant and GFP donor cells were stably engrafted at nearly the initial transplanted ratio **(Figure 3.4a)**. Monocytes and B cells chimerism levels were equally between donor mutant and donor GFP cells and T cells were mostly derived from mutant cells. Granulocytes were mostly derived from wild-type donor cells instead of mutant donor cells evidence of the neutropenia exhibited by Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ mice **(Figure 3.4b)**.

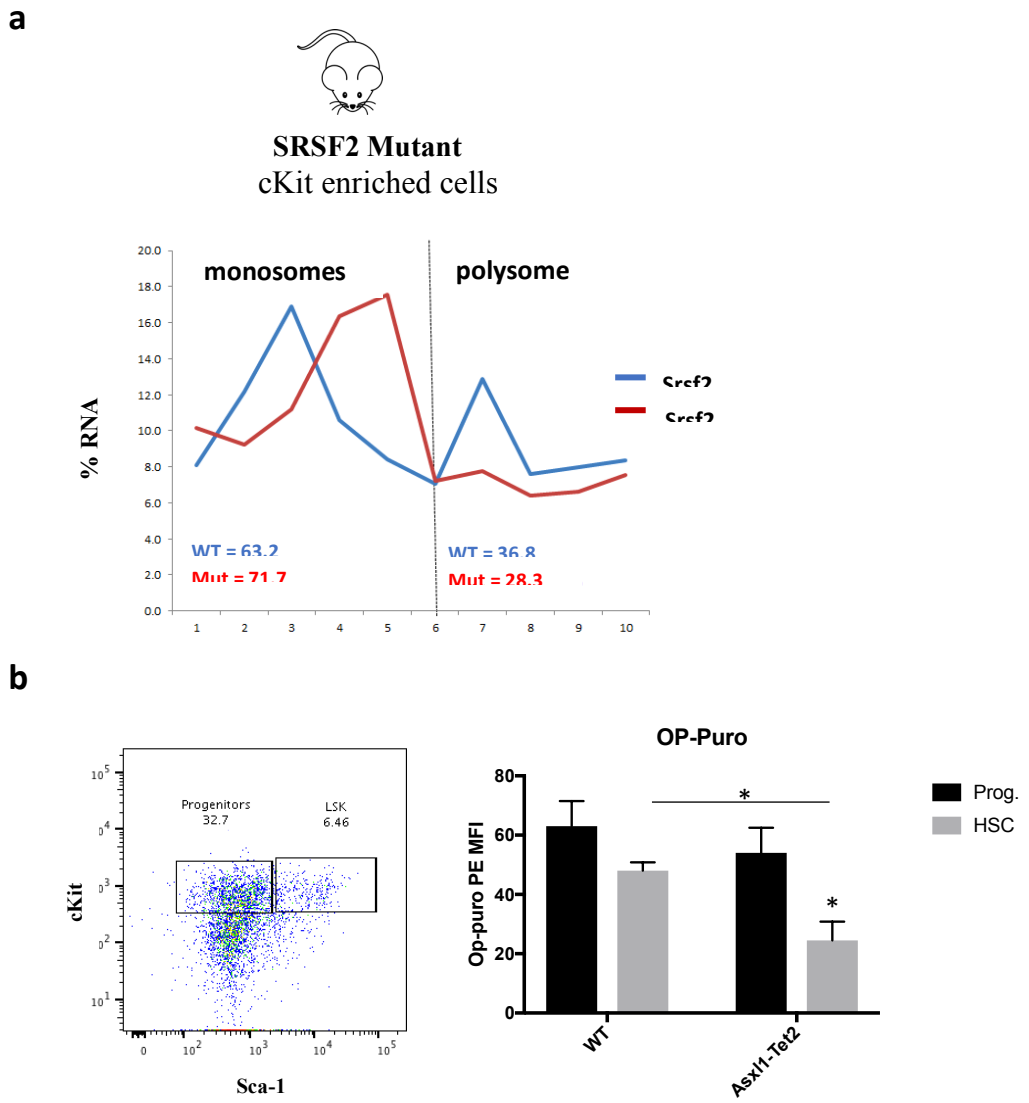


FIGURE 3.3. HSCs in Mouse Models of MDS Have Reduced Translation

a. **Monosome and Polysomes of CKit Enriched BM cells of SRSF2 WT and SRSF2 Mutant.** cKit enriched cells were treated with 10mg/ml of CHX followed by centrifugation and fractionation. Ribosomal RNA content is plotted against each fraction.

b. **OP-Puro Incorporation in Asxl1-Tet2 Mutants.** Progenitors cells are phenotypically characterized as Lineage- Sca-1- cKit+ fractions and HSCs are characterized by Lineage- Sca-1+ cKit+ Representative bar graph shows OP-puro MFI comparing in progenitor, and HSC populations from mutant and WT mice.

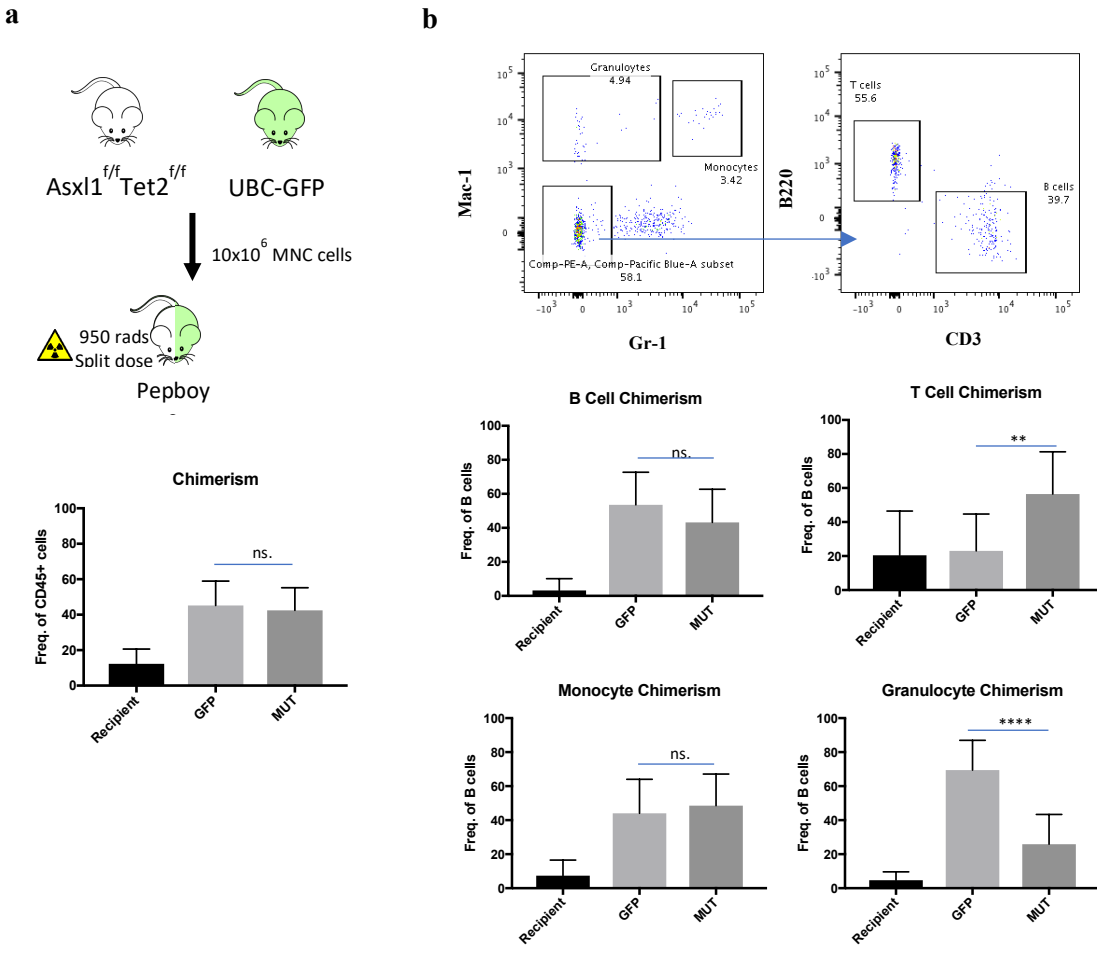


FIGURE 3.4. Generation of WT UBC-GFP and Asxl1-Tet2 Chimeric BM

- **a. Generation of Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ /UBS-GFP Mice.** 10x10^6 MNC from Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ (CD45.2) and 10x10^6 MNC from UBC-GFP (CD45.2) were transplanted via retro orbital injection into lethally irradiated pepboy (CD45.1) recipients.
- **b. Sixteen-week Total Peripheral Blood and Lineage Chimerism.** *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

Following stable engraftment, mice were treated with a biotinylated CD45.2 antibody, CD117, or IgG, conjugated to saporin-streptavidin. The CD45.2-Saporin immunotoxin has been shown to deplete HSPCs specifically from C57BL/6 BM (Palchaudhuri R et al 2016). As CD117 is expressed on mainly immature populations in the BM, we also tested the efficacy of HSC depletion when combined with saporin. BM aspirates were sampled immediate before the immunotoxin treatment and 8 days post-treatment. As previously described, 8 days was sufficient time to observe depletion of HSCs from mouse BM (Palchaudhuri R et al 2016). A reduction in total LSK and LSK CD150+ cells was observed with both CD117-Saporin and CD45-Saporin treatment **(Figure 3.5a)**. Change in chimerism levels of each cell type within the LSK and progenitor compartments was also measured. Post-treatment, recipient (CD45.1) LSK and progenitor chimerism mostly increased, likely a result of the decreasing contributions from t wild-type GFP and mutant cells. Contributions of GFP and mutant cells within the LSK and progenitor compartment trended towards a decrease upon CD117-saporin and CD45-saporin treatment **(Figure 3.5b)** indicating that a single dose of the immunotoxin can deplete HSPCs in vivo.

Treatment with Saporin Allows Engraftment of Donor Cells

Myeloablative conditioning regimen for a HSCT aims to clear the BM of dysplastic cells and is followed by the infusion of healthy stem cells. Engraftment of healthy stem cells indicate sufficient depletion of resident HSCs has opened niche spaces for donor cells. To test whether saporin treatment can serve as a conditioning regimen for HSCT for MDS patients, we treated GFP and mutant chimeric mice with CD117-saporin and CD45-saporin, and followed up with a transplant of F1 donor cells 8 days

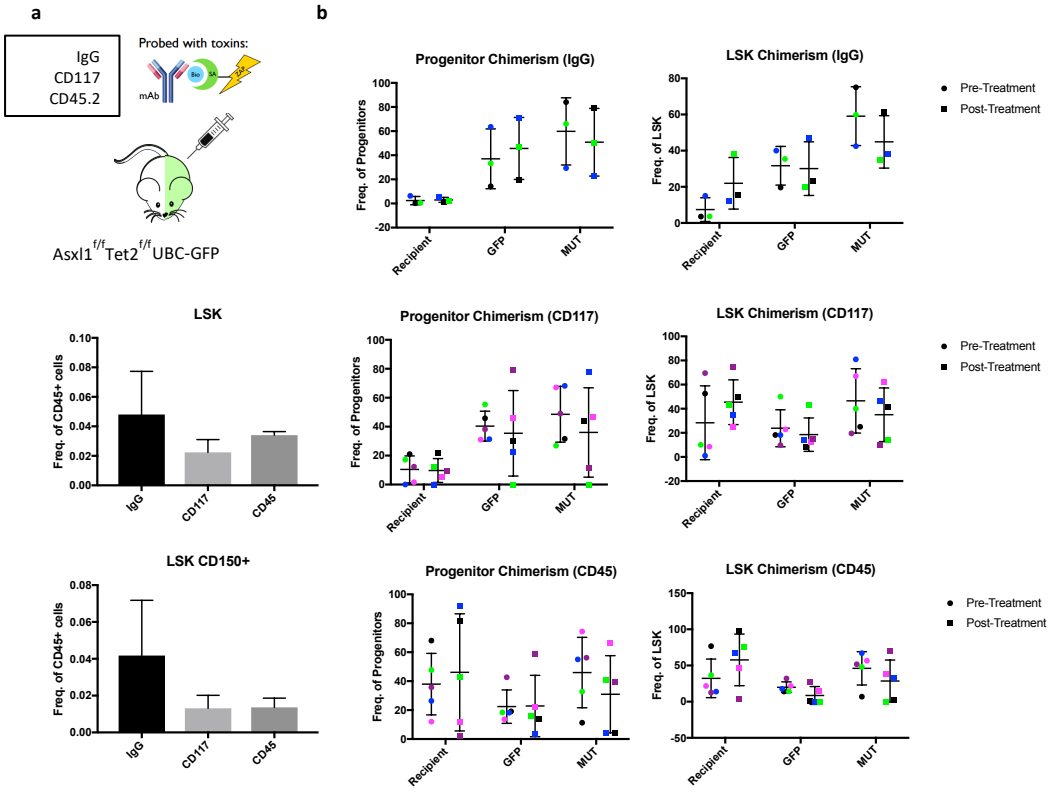


FIGURE 3.5. In Vivo Depletion of WT UBC-GFP and Asxl1-Tet2 HSCs Generation of Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ /UBS-GFP Mice.

a. **Eight-Day Post-Treatment Frequencies**. LSK and LSK CD150+ frequencies within total CD45 cells in IgG, CD117, and CD45.2 treated groups. 12ug of CD117-saporin and 24ug of CD45-saporin were administered retro-orbitally.

b. Change in LSK and Progenitor Chimerism. Pre and post IgG, CD117, and CD45.2 treatment. Each dot represents a single mouse and colors are coordinated to represent the same mouse pre and post-treatment.

later **(Figure 3.6a)**. Following the transplant, peripheral blood analysis showed that CD117 and CD45 – saporin treated groups had approximately 60-70% engraftment of F1 cells 12 weeks after the transplant **(Figure 3.6b)**.

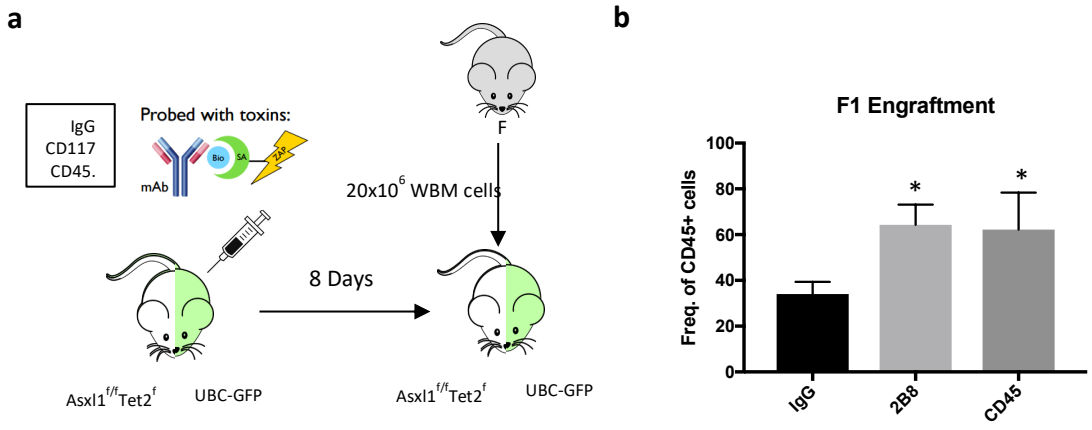


FIGURE 3.6. Treatment with Saporin Allows Engraftment of F1 Donor Cells

a. **Eight-Days after IgG, CD117, and CD45– Saporin Treatment.** Chimeric mice were transplanted with $20x10^6$ WBM cells F1 donor cells.

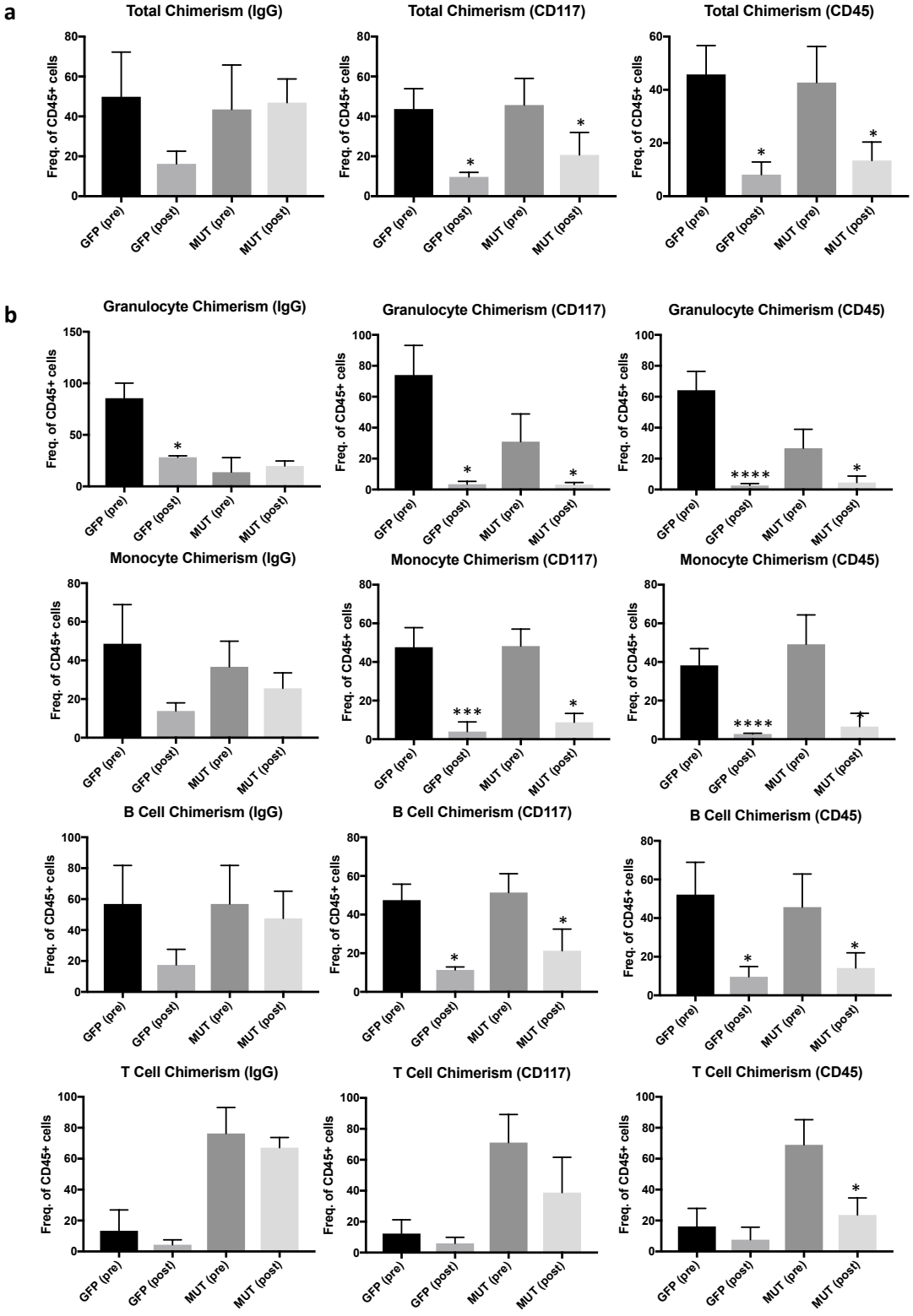
b. Twelve-Week F1 chimerism levels. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

To assess whether engraftment of F1 cells was the result of depletion of more wilt-type GFP cells or mutant cells, we measured changes in GFP and mutant chimerism levels after saporin treatment. Comparing chimerism levels, we observed that both GFP and mutant cells were depleted and mutant cells, despite having lower rates of translation, likely were not more susceptible to saporin treatment **(Figure 3.7a)**. Looking within granulocytes, monocytes, b cells, and t cells, chimerism levels of GFP and mutant cells across all lineages were both reduced **(Figure 3.7b)**. These data demonstrate that while saporin did not preferentially deplete MDS stem cells, it allowed for sufficient depletion of all HSCs to allow for donor cell engraftment.

FIGURE 3.7. GFP and Mutant Peripheral Blood Chimerism Decrease After Saporin Treatment

a. **Change in Total GFP and Mutant Chimerism Twelve Weeks After Saporin Treatment.**

Change in Lineage Chimerism of GFP and Mutant Cells Twelve Weeks After Saporin Treatment. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$


a
Total Chimerism (IgG)
Total Chimerism (CD117)
Total Chimerism (CD45)
b
Granulocyte Chimerism (IgG)
Granulocyte Chimerism (CD117)
Granulocyte Chimerism (CD45)
Monocyte Chimerism (IgG)
Monocyte Chimerism (CD117)
Monocyte Chimerism (CD45)
B Cell Chimerism (IgG)
B Cell Chimerism (CD117)
B Cell Chimerism (CD45)
T Cell Chimerism (IgG)
T Cell Chimerism (CD117)
T Cell Chimerism (CD45)
Freq. of CD45+ cells
GFP (pre)
GFP (post)
MUT (pre)
MUT (post)

DISCUSSION

We have shown using op-puro incorporation and a sucrose gradient assay that in both mouse models of MDS and in human cord blood and BM samples, normal and MDS HSCs exhibit reductions in global translation when compared to bulk and progenitor populations. Reduced translation in HSPCs of the two MDS patient samples evaluated here represent only a small subset of MDS patients. Additional samples will be required to validate the findings and to correlate the reduction in translation to known defects in ribosome biogenesis.

Using mononuclear cells from the genetically accurate Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ MDS model and wild-type UBC-GFP mice we generated chimeric BM representing the normal and dysplastic cells present in MDS patient BM prior to disease progression. We transplanted equal number of cells from GFP and mutant mice and observed that engraftment efficiency of both cells was equal and total chimerism levels remained equal long-term. These mice were treated with the immunotoxin and evidence of GFP and mutant HSPC depletion was observed from BM aspirates 8 days after treatment.

Subsequent donor cell transplant lead to high engraftment indicating elimination of resident HSPCs opened niche space for donor cells. We also observed that the IgG treated groups also exhibited increase F1 engraftment and reduced chimerism levels specifically within the GFP compartment. While the internalization of IgG by some BM cells explains the depletion, the reasons for the specificity of GFP cell depletion is not clear.

We had observed that HSCs from mutant mice have lower translation when compared to wild-type, and hypothesized that perhaps the reduced translation would make them more susceptible to an immunotoxin that targets the translation machinery. However, we did not see a selective depletion of mutant HSCs. Nonetheless, sufficient depletion of mutant HSCs allowed engraftment of donor cells making the treatment a potential non-genotoxic conditioning regimen for transplants.

CHAPTER FOUR
EXPLORING THE MECHANISM OF SAPORIN-INDUCED CELL DEPLETION

INTRODUCTION

While our data shows depletion of wild-type and MDS HSCs in vivo, an understanding of how saporin causes the depletion needs to be determined. In early studies, evidence of saporin cytotoxicity has mostly been obtained by determining inhibition of protein synthesis or determining cell viability of treated cells compared to controls (Bolognesi et al. 2005; Polito et al. 2009; Tazzari et al. 1999, 2018). In an early study, saporin conjugated to anti-CD2 mAb showed inhibition of proliferation and an inhibition of protein synthesis measured by ^{3}H-leucine incorporation by the SKW-3 T-ALL cell line after 48 hours of incubation with the immunotoxin (Tazzari et al. 2018).

We sought to determine whether the immunotoxins CD117-saporin and CD45-saporin also inhibited translation of HSPCs in wild-type and mutant Vav-cre-Asxl1$^{fl/fl}$-Tet2$^{fl/fl}$. We also assessed protein translation rates in cord blood units to determine whether human HSPCs respond to the immunotoxins by the same mechanism. We evaluated protein synthesis by the op-puro incorporation assay and also assessed cell viability as well as functional effects of the immunotoxin treatment.

RESULTS

We began the study by using the multipotent mouse erythroid myeloid lymphoid (EML) cell line to evaluate in vitro effects of the immunotoxins. We verified that EML cells express CD117 receptor and contained both Sca-1+ LSK and Sca-1- progenitor cell populations. Dose titration curve of the CD117-saporin treatment was generated based on cell viability after 72 hours of culture **(Figure 4.1a)**. Using 10nM of both CD117 and saporin, we demonstrated that within 24 hours, saporin molecules were robustly internalized with CD117-saporin treatment. No shift was observed in the saporin only group indicating that only when conjugated with the internalizing antibody can saporin be transported into the cell **(Figure 4.1b)**. To assess whether apoptosis was induced at this time point we did an annexin V and 7AAD stain and it revealed little to no increase in apoptotic cell frequencies **(Figure 4.1c)**. We followed up with a cell cycle stain to determine whether any shifts in cell cycle status occurs with the immunotoxin and observed a decrease in S phase accompanied by an increase in G1 phase indicating a possible G1 arrest **(Figure 4.1c)**. These data demonstrate that at the 24-hour time point CD117-saporin is efficiently internalized and while no induction of apoptosis occurs, cells may enter into a G1 arrest. As EML cells treated with CD117-saporin showed greater changes at 72 hours, apoptosis and cell cycle changes need to me measured at timepoints beyond 24 hours.

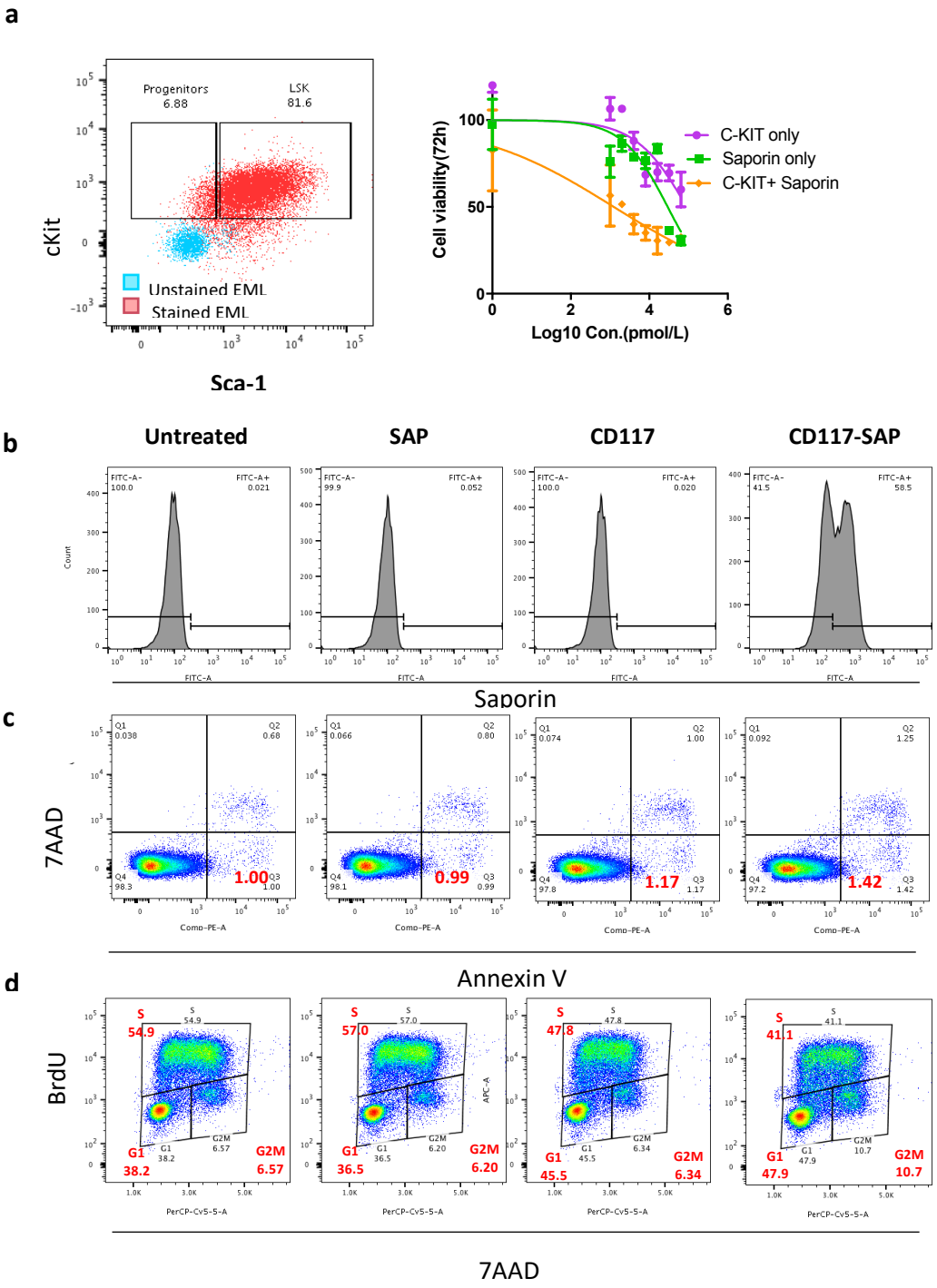


FIGURE 4.1. In Vitro Effects of Saporin on EML Cells

a. EML Cell Flow Cytometry and Dose Titration.

b. **Internalization of Saporin**. EML cells treated for 24 hours with 10nM CD117-saporin followed by an intracellular anti-saporin FITC stain

c. Annexin V and 7AAD Staining After 24 hours.

d. BrdU Staining to Assess Cell Cycle Status 24-hours After Treatment.

Next, we cultured cKit enriched cells from C57BL/6 mice with saporin, CD117-saporin, and CD45-saporin for 72 hours and observed that at the 48-hour time point, we began to see reduced cell counts in the CD117-saporin and CD45-saporin groups which then became significant by the 72 hours timepoint **(Figure 4.2a)**.

Similar to the observation made in EML cells, 24-hour treatment with CD117-saporin let to a decrease in cells in S phase **(Figure 4.2b)**. Moreover, across the progenitor, LSK, and LT-HSC compartments, we observed well over 50% reduction in op-puro incorporation after a 24-hour treatment indicating that saporin treatment inhibits protein synthesis in wild-type HSPCs **(Figure 4.2c)**.

Finally, we wanted to know if human HSC exhibit similar reductions upon treatment with saporin. CD34 enriched cells from cord blood were treated with saporin, CD117, and CD117-saporin for 90 minutes. We have shown that internalization of CD117 occurs within 90 minutes and we wanted to assess how quickly internalized saporin can alter translation. We observed that after 90 minutes, op-puro incorporation was reduced by more than 50% in HSCs and progenitor cells **(Figure 4.3a)**. We wanted to see whether any functional effects take place with short-term treatment and performed CFU assay with treated cells. After a 7-day culture, we observed an increase in total colony output from cells treated with CD117-saporin. A striking increase in erythroid colonies was observed possibly indicating an increase in differentiation **(Figure 4.3b)**.

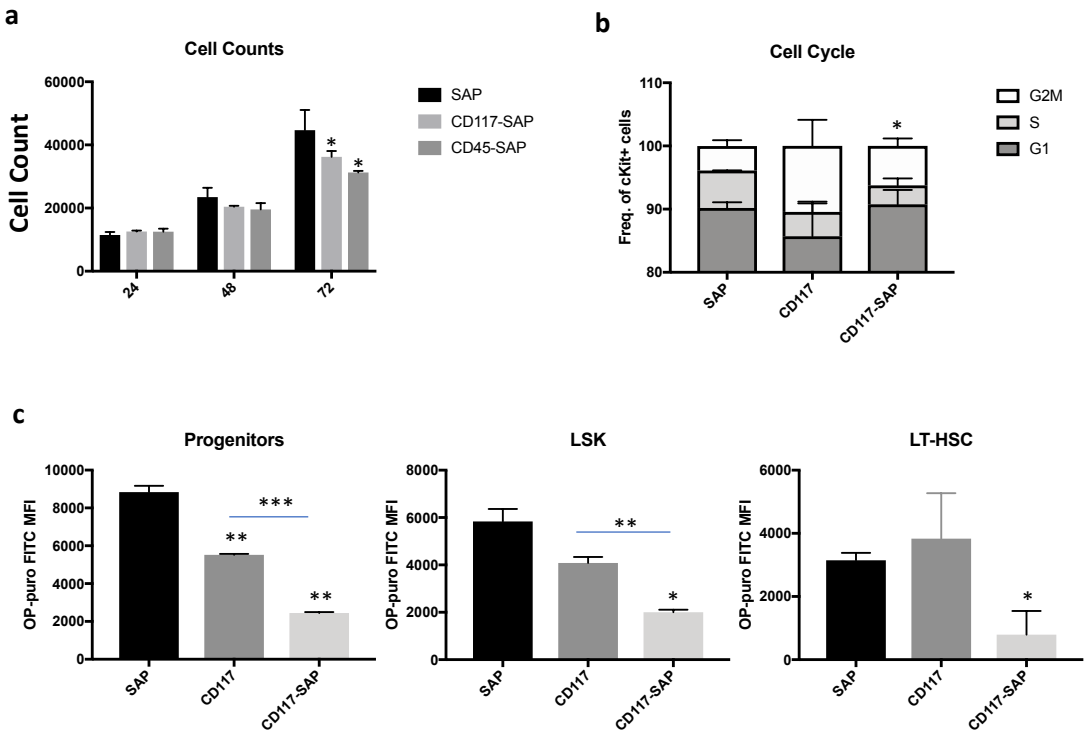


FIGURE 4.2. In Vitro Effects of Saporin on WT BM Cells

a. cKit enriched cells from C57BL/6 mice were plated and treated with saporin, CD117-saporin, and CD45-saporin. Cell counts at 24, 48, and 72 hours.
b. cKit enriched cells from C57BL/6 mice were treated with saporin, CD117, and CD117-saporin and BrdU staining was performed to assess cell cycle status
c. OP-Puro incorporation assay after 24 hour. Progenitors (Lin- Sca-1- cKit+), LSK (Lin- Sca-1+ cKit+), and LT-HSC (Lin- Sca-1+ cKit+ CD34- CD150+)*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

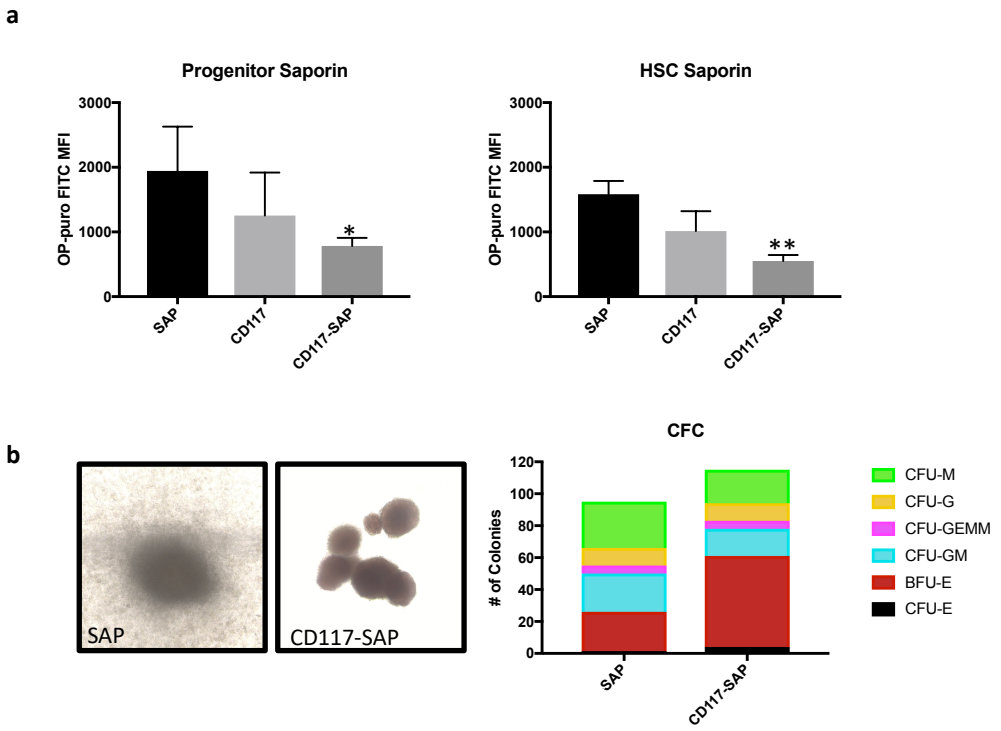


FIGURE 4.3. In Vitro Effects of Saporin on Cord Blood HSPCs

a. OP-Puro incorporation in CD34 enriched cord blood cells following a 90-minute saporin, CD117, and CD117-saporin treatment.
b. 500 CD34 enriched cord blood cells were treated with the immunotoxin for 90-minutes and plated in m3434 methylcellulose for 7 days.

DISCUSSION

Here we show that the HSCs and progenitors from C57BL/6 mice and cord blood are sensitive to saporin treatment. We demonstrated that progenitor, LSK, and LT-HSC populations from mouse bone marrow all have reduced op-puro incorporation after a 24-hour CD117-saporin treatment. While LT-HSCs already have reduced translation rates compared to LSK and progenitor populations, further reduction of translation

with CD117-saporin treatment may exert a functional effect on the cells. We also measured effects of CD117-saporin on EML cells and observed a possible G1 arrest suggesting insufficient components for cell cycle to proceed. A previous study demonstrated that in saporin treatment of two different glioblastoma cell lines lead to a G1 arrest with no increase in apoptosis in one cell line and an increased apoptosis and a block in S phase progression in the other (Cimini et al. 2012). These results indicate that distinct cellular responses can be induced by saporin in different cells. As we proceed forward, mechanism of cell death must be validated across normal and mutant mouse and human cells.

CHAPTER FIVE
MATERIALS AND METHODS

Animal Studies

All animal studies were performed with Memorial Sloan Kettering Cancer Center or NYU Langone Medical Center IACUC approval. Wild-type C57BL/6J (CD45.2) mice, congenic B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) mice, and CByJ. B6-Tg(UBC-GFP)30Scha/J mice were purchased from Jackson Laboratories. Hybrid F1 mice were generated at MSKCC by crossing CD45.2 C57BL/6J mice with CD45.1 Bs.SJL-Ptprca Pepcb/BoyJ mice and were used to distinguish donor, competitor, and host cell contribution. Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ and Mx-cre- Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ mice were generously provided by Dr. Abdel-Wahab, MSKCC. All mice within each experiment were age and sex-matched.

Transplantation

BM cells for transplantation were harvested by crushing freshly isolated femurs, tibia, hips, and spine and were filtered through a 40um strainer. Competitive transplants were performed with 20x10^6 BM mononuclear cells at a 1:1 ratio of CD45.2 Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ and CD45.2 UBC-GFP cells. Cells were injected retro-orbitally into lethally irradiated (9.5Gy split dose) congenic CD45.1 recipient mice.

Mouse Peripheral Blood Analysis

Peripheral blood was drawn from the tail-vein and collected in blood collection tubes with 0.5M EDTA (Fisher Scientific: 22-040-200). Automated peripheral blood counts were obtained using either a HemaVet 950 (Drew Scientific) or ProCyte Dx Hematology Analyzer (IDEXX) according to standard manufacturer's instruction. For

flow cytometry quantification of T, B, and myeloid cells, blood samples were treated with 2% dextran for 30 minutes at 37C flowed by ACK lysis (ThermoFisher Scientific: A10492-01) for 10 minutes at room temperature. Cells were stained with the following antibody cocktail in FACS butter for 30 minutes on ice. Ter119-APC (eBioscience: 17-5921-82), Gr-1-PE (eBioscience: 12-5931-82), Mac-1-e450 (eBioscience: 48-0112-82), B220-PE-Cy5 (BioLegend: 103210), and CD3-APC-Cy7 (eBioscience: 47-0032-82). Donor chimerism analysis were performed using CD45.1-PE-Cy7 (eBioscience: 25-0453-82), CD45.2-A700 (BioLegend: 109822) and for transplants using UBC-GFP cells, chimerism was based on GFP+ events within the CD45+ gate. Propidium iodide (BioLegend – 421301) was used to evaluate cell viability.

Mouse Bone Marrow Analysis

Bone marrow aspirates were performed as previously described (Chung, Kim, and Abdel-Wahab 2014). Briefly, mice were anesthetized with isoflurane using a vaporizer. A 27.5G tuberculin syringe was inserted through the patellar tendon into the shaft of the femur and the plunger was pulled back until approximately 5uL were collected. For flow cytometry analysis of stem and progenitor cells, cells were stained using the following antibody cocktail: Ter119, B220, CD3, CD4, CD8, Gr-1, and Mac-1-PE-Cy5 (BioLegend: 116210), Sca-1-Pacific Blue (BioLegend: 122520), CD117-APC-Cy7 (105826), CD34-FITC (BioLegend 343604), CD150-PE (BioLegend: 115904), and CD16/32-A700 (eBioscience: 56-0161-82). Donor chimerism analysis were performed using CD45.1-PE-Cy7 (eBioscience: 25-0453-82), CD45.2-A700 (BioLegend: 109822) and for transplants using UBC-GFP cells, chimerism was based on GFP+ events within the CD45+ gate. Propidium iodide (BioLegend – 421301) was used to evaluate cell viability.

Immunotoxin preparation

Saporin (Streptavidin-ZAP) was purchased from Advanced Targeting Systems (IT-27). The number of saporin molecules ranged from 1-2.8 molecules/streptavidin depending on the lot. Aliquots of saporin were prepared and stored at -80°C for long-term use. Biotinylated anti-mouse CD45.2 (clone 104 - 109804), biotinylated anti-mouse CD117 (clone 2B8 - 105804), biotinylated anti-mouse IgG1, $_{K}$ Isotype control antibody (clone MOPC-21 400104) and biotinylated anti-human CD117 (clone 104D2 – 313208) were purchased from BioLegend. Immunotoxins were prepared by combining saporin and antibody at a 1:1 molar ratio and after 10 minutes of incubation at room temperature diluted with PBS. In vivo administration of immunotoxin was performed by intravenous injections (300µL volume).

Saporin Internalization Assay

Anti-Saporin FITC labeled (goat polyclonal) antibody was purchased from Advanced Targeting Systems (catalog number: FL-02). Aliquots were prepared and stored at -20°C until use. Cells were first incubated with antibody-saporin conjugate for at least 90 minutes to allow for internalization at in 5% CO_2 at 37°C in culture media. Cells were washed with PBS and were treated with fixation/permeabilization solution (BDbiosciences - 554714) for 10 minutes at 4°C. Cells were then washed with 1x wash buffer provided in the fix/perm kit. Anti-saporin FITC antibody was diluted at 1:000 in 1x wash buffer and incubated with cells for 30 minutes at room temperature in the dark. Internalization of saporin was analyzed by flow cytometry.

Measurement of Antibody Internalization

In vitro antibody internalization was assessed as previously described (Schmidt, Thurber, and Wittrup 2008). Briefly, EML cells and cKit enriched mouse BM cells in respective media were incubated with biotinylated anti-CD117 (clone 2B8) anti-CD45.2 (clone 104), and IgG1K Isotype (clone MOPC-21) antibody and streptavidin-AF488 conjugate (Life Technologies). After 24 hours, cells were washed twice with FACS buffer (PBS, 2% FBS). Cells were incubated with and without 0.25mg.mL polyclonal anti-AF488 quenching antibody (Life Technologies clone A-11094) and AF488 signal was quantified by flow cytometry and allowed for measurement of surface and internal fluorescence.

Mouse BM Cell Culture

BM cells were harvested by crushing freshly isolated femurs, tibia, hips, and spine and were filtered through a 40um strainer. For CD117 enrichment, BM cells were incubated with CD117 MicroBeads, mouse (Miltenyi Biotec: 130-091-224) for 30 minutes at 4°C. Cells were passed through LS columns (Miltenyi Biotec: 130-042-401) on a MACS manual separator. LS column was washed three times with FACS buffer and eluted with 5 mL FACS buffer. CD117 enriched cells were plated in DMEM-F12 with 10% FBS, 10 ng/μL SCF (Peprotech: 250-03), 10 ng/μL Flt3 ligand (Peprotech: 250-31L), 10 ng/μL TPO (Peprotech: 315-14), 10ng/μL IL-3 (Peprotech: 213-13), and 10 ng/μL IL-6 (Peprotech: 216-16). All cytokines were obtained from Peprotech.

Cord Blood Preparation and Culture

Cord blood units were obtained through the National Cord Blood Program at the New York Blood Center. Individual cord blood units were diluted with equal volume PBS, 2% EDTA buffer and mononuclear cells are subsequently isolated by density gradient

centrifugation. 30mL of diluted cord blood were layered onto 15mL Ficoll-Paque solution (Fisher 45-001-750 and centrifuged at 1500 rpm for 40 minutes at room temperature. Mononuclear cells are carefully collected from the interphase layer between the plasma layer and ficoll layer and pooled together. Cells are washed with PBS, 2% EDTA buffer and spun for 30 minutes at 1500 rpm at room temperature. For CD34 enrichment after density gradient centrifugation, MNCs were incubated with CD34 MicroBead Kit, human (Miltenyi Biotec: 130-046-702) for 30 minutes at 4°C. Cells were passed through LS columns (Miltenyi Biotec: 130-042-401) on a MACS manual separator. LS column was washed three times with FACS buffer and eluted with 5 mL FACS buffer. CD34 enriched cord blood cells were plated in StemSpan (Stem Cell Technologies: 09600) supplemented with 10% FBS, 20ng/mL TPO (Peprotech: 300-18), 20ng/mL IL-3 (Peprotech: 200-03), 20ng/mL G-CSF (Peprotech: 300-23) and 1% Pen Strep.

Patient Bone Marrow Cell Preparation and Culture

Patient BM samples were obtained from the Department of Pathology NYU Medical Center. BM cells were diluted in FACS buffer and layered onto Ficoll-Paque and spun at 1500 rpm for 40 mins at room temperature. Mononuclear cells are carefully collected from the interphase layer between the plasma layer and ficoll layer and pooled together. Cells are washed with PBS, 2% EDTA buffer and spun for 30 minutes at 1500 rpm at room temperature. For CD34 enrichment after density gradient centrifugation, MNCs were incubated with CD34 MicroBead Kit, human (Miltenyi Biotec: 130-046-702) for 30 minutes at 4°C. Cells were passed through LS columns (Miltenyi Biotec: 130-042-401) on a MACS manual separator. LS column was washed three times with FACS buffer and eluted with 5 mL FACS buffer. CD34 enriched cord blood cells were plated in StemSpan (Stem Cell Technologies: 09600) supplemented

with 10% FBS, 20ng/mL TPO (Peprotech: 300-18), 20ng/mL IL-3 (Peprotech: 200-03), 20ng/mL G-CSF (Peprotech: 300-23) and 1% Pen Strep.

EML Cell Culture

EML cell line, clone 1 was purchased from ATCC (ATCC CRL-11691). Cells were cultured in Iscove's modified Dulbecco's medium with 4mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate (ATCC: ATCC 30-2005) containing 200 ng/ml mouse stem cell factor, and 20% FBS. Culture was maintained by replacement of media every 2 days to maintain a cell concentration of $1x10^6$ viable cells/mL.

Cell-Cycle and Apoptosis Analysis

Cell cycle analysis was performed using BD Pharmingen BrdU Flow Kits APC (BD Biosciences: 552598). Cells were incubated with 10uL of BrdU solution (1nM BrdU in 1x PBS) and incubated for 1 hour followed by staining with cell surface marker antibodies for 30 minutes and in the case of staining with mouse CD34 antibody for LT-HSCs, cells were stained for 2 hours. Cells were washed and incubated with 100uL of BD cytofix/cytoperm buffer at 4°C for 10 minutes. Cells were subsequently washed with 1x perm/wash buffer and analyzed by flow cytometry. For evaluation of apoptosis, the Annexin V–PE apoptosis detection kit (BD Biosciences: 556421) was used. Cells were incubated with 5uL of PE Annexin V and 5uL of 7-AAD and incubated for 15 minutes at room temperature in the dark. Cells were resuspended in 1x binding buffer and analyzed by flow cytometry.

CFU-Assay

Colony assays on CD34 enriched cord blood cells were performed using MethoCult H4434 Classic (StemCell: 04434). After cells are isolated, 500 CD34 enriched cells

were added to 2mL of H4434 and vigorously vortexed. Using a syringe with a 16-gauge blunt-end needle, methocult mixture with cells are dispensed into 6-well plates. The plate is gently tilted to allow to distribute evenly. Sterile water is added to the plate and plate is incubated with 5% CO_2 at 37°C for 7-10 days. Colony phenotyping was performed using manufacturer's instructions. Methocult and cells were collected and washed twice with 1xPBS and 20,000 cells were plated for secondary plating. The remaining cells were stained for flow cytometry analysis.

Cell Viability Assay

CellTiter-Glo Luminescent Cell Viability Kit (Promega: G7571) was used to determine the number of viable cells after immunotoxin treatment. Cells were first treated with antibody-saporin conjugates for 72 hours in culture media at 37°C. CellTiter-Glo Reagent was added to the cells in equal volume and mixed for two minutes on a shaker to induce cell lysis. The plate was incubated for 10 minutes at room temperature to stabilize the luminescent signal. Luminescence was recorded on a spectrophotometer.

OP-Puro Incorporation Assay (in vitro & in vivo)

In vitro OP-Puro incorporation assay was performed using either the Click-iT Plus OPP Alexa Fluor™ 488 Protein Synthesis Assay Kit (ThermoFisher Scientific: C10456) or the Click-iT Plus OPP Alexa Fluor™ 594 Protein Synthesis Assay Kit (ThermoFisher Scientific: C10457). Cells were cultured with Dilute Click-iT OPP (Component A) at 1:1000 for 30 minutes at 37°C. Cells were fixed and permeabilized with 100uL of the BD Fix/perm solution. Cells were washed with 1x wash buffer and

incubated with OPP detection solution for 30 minutes at room temperature. Cells were washed with Click-iT Reaction Rinse Buffer and subsequently analyzed by flow cytometry.

Sucrose Gradients

10%, 20%, 30%, 40%, 50%, and 60% sucrose solution was prepared in lysis buffer. Gradients were prepared by layering each concentration of sucrose followed by incubation at -80C for 45 minutes to allow for solidification before adding the next layer. Cells of interest were treated with 10mg/ml CHX for 10 minutes at 37°C to freeze translating ribosomes in place on the mRNA transcripts. Cell were lysed and on top of the sucrose gradient solution and centrifuged at 4°C for 3 hours at 50,000 rpm. After centrifugation, 300uL fractions were removed from the top of the gradient and placed into pre-chilled microcentrifuge tubes. RNA was isolated using RNeasy Mini Kit (Qiagen: 74104) and quantified.

CHAPTER SIX
DISCUSSION & FUTURE DIRECTION

Deregulation of translation has been implicated in many hematologic malignancies including MDS. Here we demonstrate in mouse models of MDS and in two MDS patient samples, wildtype- and MDS HSCs exhibit decreased translation rates as measured by op-puro incorporation. We also show that using an immunotoxin that targets the 28S ribosomal subunit, we are able to deplete MDS HSPCs in vivo allowing access to open niches for donor HSCs. While it is promising to propose using saporin as a conditioning regimen of HSCT for MDS patients or as an adjunct therapy to standard care, many questions still remain unanswered.

How does saporin affect the cell and its cell cycle status? In EML cells, treatment with CD117-saporin lead to an increase in the G1 phase and a decrease in S phase suggesting a G1 arrest. As a cell progresses through the cell cycle, protein production, including production of ribosomal proteins, is highest in the G1 phase making the phase most sensitive to translation inhibition. As saporin inhibits translation initiation by preventing the 60S subunit from binding to the elongation factor 2 (EEF2), cells decide to arrest in G1 instead of committing to the next cell cycle. This arrest in G1 would also be accompanied by increased doubling time as cells would spend more time in the G1 phase and fewer cells would be cycling. With increasing concentration of our immunotoxin, we observed a decreasing EML cell viability (Figure 3.1a). Whether this is due to cell death or reduced cell growth is unclear. We measured apoptosis after a 24-hour treatment and did not observe any significant increase however, this does not exclude the possibility of cell death. We need to assess apoptotic as well as necrosis pathways at different time points ranging from 24-72

hours to allow sufficient time for saporin to exert its effect. Additionally, by measuring saporin internalization using a range of saporin concentrations, a correlation between amount of toxin can be made to its direct effect.

Our in vivo study we demonstrated that wild-type GFP and Vav-cre-Asxl1$^{fl/fl}$-Tet2 $^{fl/fl}$ HSCs were depleted upon treatment with both CD117-saporin and CD45-saporin and subsequently, donor cells were able to be engrafted. Our data and previously published data (Palchaudhuri R et al 2016) show that in vivo depletion takes 6-9 days. What happens to HSCs in this time is still unclear. If cells undergo cell death or exhaustion during this time, assessing BM in at intermediate timepoints for apoptosis or necrosis and functional assays such as CFU and transplantation of phenotypic HSCs may provide insight into the process of cell depletion.

While many studies suggest that defects in ribosome biogenesis exist across MDS subtypes, how that affects global translation rates and cell function of HSCs has not been validated. Assessment of op-puro incorporation in large cohorts of MDS patient samples would allow access to this information and may provide insight into the biology of MDS stem cells. However, large cohorts of patient samples are not readily available, so an alternative is to study mouse models with ribosomal defects. Previously studied Rpl24$^{bst/+}$ mice have defects in 60S subunit biogenesis. These mice exhibit reduced translation in the HSCs and exhibit reduced HSC function (Signer et al. 2014). In addition to these mice, the rps19 knockdown, rps6, and rps14 all demonstrate that defects in ribosomal proteins are associated with HSC exhaustion or reduced HSC function (Choesmel V, Fribourg S, Aguissa-Toure AH, Pinaud N, Legrand P and Gleizes PE 2008; McGowan et al. 2011; Schneider et al. 2016). Using such mouse models, we would be able to test the efficacy of saporin treatment on cells with defects in ribosome biogenesis. Knowing whether the defects in translation make

these cells more susceptible to depletion would be important finding to demonstrate the specificity by with the Ab-saporin conjugate exerts its effects. As observed in a recent study, the effect of CD45-saporin were very specific to the HSC compartment. Mice treated with the immunotoxin did not have any significant changes to bulk CD45+ cells (Palchaudhuri R et al 2016). A possible reason for this specific depletion may be the low translation levels of HSCs compared to other CD45 cells. If true, this would allow MDS stem cells to be targeted more so than healthy HSCs as they have lower translation rates.

Translational control is a highly regulated process and aberrations in the process are linked with cancers. Ribosomopathies, including MDS, exhibit defects in ribosomal proteins and patients have an increased risk of developing a hematologic malignancy. On the other hand, increase protein synthesis has also been shown to also promote hematologic malignancies, including T cell leukemias (Barna et al. 2008; Hsieh et al. 2010). Deregulation of translation. While changes in translation leading to a malignancy is disease specific, targeting the machinery may serve as a powerful tool for malignancies described with either an increase or a decrease in translation.

A critical question here is whether a defect in ribosome only leads to reduced translation or do the defects altered ribosome function leading to the increased risk of developing a malignancy? The well understood, housekeeping function of a ribosomal protein has been to act as components of the ribosomal structure enabling protein synthesis. However, studies have suggested that ribosomal proteins have additional physiological functions and defects in these proteins might contribute to disease pathogenesis (Ruggero 2013).

Saporin has been used as an immunotoxin for many years now and several studies have shown its efficacy in targeting malignant stem cells in pre-clinical studies (Ferreras et al. 2011; Polito 2004). Several clinical trials have also been conducted with saporin containing immunotoxins and have been well tolerated. The first trial used a CD30-saporin conjugate in patients with advanced refractory Hodgkin's disease. Side effects including fever, myalgias, thrombocytopenia, and transient liver damage were observed in 70% of patients (Falini et al. 1992; Pasqualucci et al. 1995). A second trial using bispecific F(ab') antibodies with saporin in lymphoma patients and only mild fever, weakness, and myalgia for 1-2 days after the treatment were described (French et al. 1995, 1996).

The use of an immunotoxin such as CD117-saporin has some advantages compared to other therapies. Unlike treatment with lenalidomide or myeloablative conditioning for HSCT, the immunotoxin activity is not limited dividing cell. Both dividing and quiescent cells have been demonstrated to be affected by the saporin treatment by showing reduction in translation. Moreover, an immunotoxin can only exert its effects on the targeted cells based on the antibody making it a non-ablative method of eliminating disease-initiating cells.

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