A SURROGATE IN VITRO SYSTEM TO DEFINE INITIATING EVENTS OF MDS AND TRANSITION TO AML DISEASE

A SURROGATE IN VITRO SYSTEM TO DEFINE INITIATING EVENTS OF MDS AND TRANSITION TO AML DISEASE

By GENA MARKOUS, B.Sc. (Honours)

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

McMaster University

Descriptive Note

McMaster University MASTER OF SCIENCE (2021) Hamilton, Ontario (Biochemistry and Biomedical Science)

TITLE:	A surrogate *in vitro* system to define initiating events of MDS and transition to AML disease
AUTHOR:	Gena Markous, B.Sc. (Honours)
SUPERVISOR:	Dr. Mick Bhatia
NUMBER OF PAGES:	xi, 65

Abstract

Acute myeloid leukemia (AML) is an aggressive cancer that begins in the rare blood-forming stem cell subpopulation of the bone marrow, in which the ability to produce functionally specialized, mature blood cells becomes impaired. The prognosis for patients with AML is vastly grim due to a low survival rate, rapid onset, and limited, non-curative treatment options. The understanding of how healthy blood cells progress to lethal leukemic cells is lacking, making it difficult to identify biological predictors and develop novel therapeutics. However, an intermediate state termed myelodysplastic syndrome (MDS) from healthy hematopoiesis provides an opportunity to unravel the mechanisms involved in the initiation and progression of this disease to AML. Much like other cancers, the accumulation of gene mutations in blood-forming stem cells is the driving force behind initial malignancy of this tissue. Recent studies have shown that many of those recurrent mutations are directly related to abnormal DNA methylation, a type of epigenetic modification, that alters gene expression resulting in aberrant cell development. To investigate this, we have taken advantage of our previously described *in vivo* mouse model of MDS-to-AML transition, governed by induced genetic mutation of GSK3, and have developed an *in vitro* system that uniquely allows for the selection, growth, and expansion of the rare blood cells responsible for initiating disease. Here, our *in vitro* system has brought to light specific phenotypes that could be the culprits of early transformation and a platform to explore causal genetic and epigenetic factors that govern disease progression. I propose that the system presented in this thesis serves as a surrogate that mimics MDS-to-AML transition and can be used to perform causal experimental studies. The overarching goal is to move the field forward by identifying tangible targets that have therapeutic intervention or predictive biomarker potential.

Acknowledgments

Firstly, I want to extend my gratitude and appreciation to my supervisor, Dr. Mick Bhatia. Thank you for taking a chance on me, someone who had never worked in a cell culture lab. You have an innate ability to inspire and motivate, which is what makes you an exceptional leader and I feel lucky to have had the opportunity to experience this. Thank you for your trust in my ability and your guidance throughout these past couple of years. I've learned so much.

I would also like to thank my supervisory committee, Drs. Clinton Campbell, Brian Leber, Mark Larche, and Tobias Berg, for their dedication and helpful advice especially during the tougher times. Thank you for providing a clinical perspective, which reminded me of the overall purpose of this work and helped to keep me motivated. You each have my admiration and respect.

Thank you, from the bottom of my heart, to all the members of the Bhatia lab that I have had the wonderful pleasure of working with. I feel so privileged to have spent this time among truly brilliant and inspirational people. Juan, I can't thank you enough for everything you have taught me. Thank you for being my constant cheerleader and staying optimistic, even when things weren't going as planned. You placed your trust in me and gave me the confidence to push forward. Meaghan and Charisa, I truly did not expect to form the insanely strong bond that we did - I have so much love for you both. Same goes for my colleague and friend, Yeonjoon. To say I couldn't have done this without you would be an understatement. I miss you and genuinely hope that we have the opportunity to work together again in the future.

To my family and friends outside of the lab, your undying support means the world to me. Thank you for your genuine interest in my work and for always rooting for my success. A special thanks goes out to my sister, Rita – I can't imagine my life without you in it.

I must also acknowledge my dog who recently passed, Skippy, and my rabbit, Bubs. Your voiceless but essential love and support has made all the difference for my mental health.

Lastly, my deepest appreciation goes out to my life partner, Emmett Borg. Emmett, your unwavering belief in me has kept me going. You are my best friend and my role model. Thank you for being with me every step of the way.

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List of Abbreviations

A	AAV	Adeno-associated virus
	AML	Acute myeloid leukemia
	ARCH	Age-related clonal hematopoiesis
B	BM	Bone marrow
C	CCUS	Clonal cytopenia of undetermined significance
	CH	Clonal hematopoiesis
	CHIP	Clonal hematopoiesis of indeterminate potential
D	DNMT	DNA methyltransferase
E	$eGFP^{lenti}$	Lentivirus expressing eGFP
	eGFP	Enhanced green fluorescent protein
F	FDA	Food and drug administration
G	GOI	Gene of interest
	GSK3	Glycogen synthase kinase 3
	$GSK3\beta^{lenti}$	Lentivirus expressing GSK3β
H	HEK	Human embryonic kidney
	HIV-1	Human immunodeficiency virus type 1
	HMA	Hypomethylating agent
	HSC	Hematopoietic stem cell
	HSP90	Heat shock protein 90
	HSPC	Hematopoietic stem and progenitor cell
	HSPC	Hematopoietic stem and progenitor cell
I	IP	Intraperitoneal
	IPSS-R	International prognostic scoring system-revised
	IRES	Internal ribosome entry site
L	Lin-	Lineage-negative
M	MDS	Myelodysplastic syndrome
	MDS-ICs	Myelodysplastic syndrome-initiating cell
	MMHCC	Mouse Models of Human Cancers Consortium
	MNC	Mononuclear cell
	MOI	Multiplicity of infection
N	NOD-SCID	Non-obese diabetic-severe combined immunodeficiency
P	PB	Peripheral blood
	PDX	Patient-derived xenotransplantation
S	sAML	Secondary acute myeloid leukemia
T	*TET2*	Ten-eleven translocation 2
V	VAF	Variant allele frequency
W	WES	Whole exome sequencing

Declaration of Academic Achievement

This thesis was completed mainly by the work of Gena Markous with the following contributions from the members of Dr. Mick Bhatia's laboratory:

- Dr. Juan Luis Garcia-Rodriguez: optimized the conditions for translating the *in vivo* GSK3 system to an *in vitro* system (data shown in Figure 3.2). Performed the *in vivo* transplantation experiments (data shown in Figure 3.3B and Figure 3.7B). Analyzed the whole exome sequencing data (Table 3.1).
- Dr. Borko Tanasijevic: designed primers for PCR analysis, provided technical training.

1.0 Introduction

1.1 Normal hematopoiesis

1.1.1 Hierarchical organization of the blood system

The adult human loses over 1 billion blood cells daily (Fuchs 2009). To compensate for this loss, hematopoietic stem cells (HSCs), a rare subpopulation of cells in the bone marrow (<0.01%) (Curtis et al. 2004), are tasked with the lifelong responsibility of replenishing all lineages of the blood system. This is accomplished through division of HSCs, whereby the daughter cells produced will either self-renew to produce more HSCs or differentiate to a hematopoietic progenitor cell (HPC) that is committed to maturation (Morrison and Kimble 2006; Y. M. Yamashita 2009). The hierarchical process by which HSCs differentiate to produce all functionally specialized, mature blood cells through intermediate progenitors is called hematopoiesis (Orkin 2000; Pandolfi, Barreyro, and Steidl 2013). Early HPCs and HSCs are oftentimes referred to as hematopoietic stem and progenitor cells (HSPCs) (Granick, Simon, and Borjesson 2012; Orkin 2000). As cells move down the hierarchy, their self-renewal capacity is progressively reduced and they become more committed to a single lineage (Seita and Weissman 2010). Broadly, the two lineages of hematopoiesis are myeloid and lymphoid, each of which consist of several distinct cell types (Iwasaki and Akashi 2007).

In the clinic, HSPCs can be obtained from three sources: bone marrow (BM), peripheral blood (PB), and umbilical cord blood. BM aspiration, the process by which HSPCs are extracted via a biopsy needle, is a relatively uncomfortable procedure. A less invasive method involves the use of granulocyte colony-stimulating factor or plerixafor to “mobilize” HSPCs from the BM into the PB for easier access. The third source of HSPCs comes from

umbilical cord blood which is collected post-delivery and poses no harm to the donor (Panch et al. 2017).

1.1.2 Functional testing of hematopoietic stem and progenitor cells

HSCs and HPCs can be functionally tested through *in vivo* and *in vitro* assays that measure the capacity for self-renewal/hematopoietic repopulation and proliferation/differentiation, respectively.

1.1.2.1 In vivo assays

HSCs were discovered during the atomic era, at which time it was realized that radiation-induced mortality was due to bone marrow failure (Jacobson et al. 1951). Several animal transplantation experiments performed at this time demonstrated the ability of healthy allogeneic donor bone marrow cells to recapitulate the blood system that was destroyed in irradiated mice, guinea pigs, and dogs (Ferrebee, Lochte, Jaretzki, Sahler, & Thomas, 1958; Ford, Hamerton, Barnes, & Loutit, 1956; Lorenz, Uphoff, Reid, & Shelton, 1951; Nowell, Cole, Habermeyer, & Roan, 1956; Smith, Makinodan, & Congdon, 1957). It was not until the 1960s that multipotent, self-renewing HSCs were functionally proven to exist. Till and McCulloch demonstrated that the injection of single bone marrow cells into irradiated recipient mice results in highly proliferative spleen colonies containing cells capable of multi-lineage differentiation (Till and Mcculloch 1961; Wu et al. 1967, 1968). In subsequent studies, chromosomal analysis of spleen colonies confirmed that each colony is composed of clones (Becker, Mcculloch, and Till 1963) and, through secondary transplantation experiments, it was shown that these spleen colonies contain cells with the property of self-renewal (Siminovitch, McCulloch, and Till 1963). As such, the *in vivo* approach used to measure long-term hematopoietic repopulation potential involves transplanting the putative HSCs

into irradiated, immunosuppressed mice, such as non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice. NOD-SCID mice harbor mutations that result in impaired NK cell, T-cell, and B-cell function (Bosma, Custer, and Bosma 1983; Kataoka et al. 1983). Host and donor hematopoietic cells are distinguished by the expression of allelic variants of a hematopoietic cell marker, CD45, which is expressed on all blood cells(Altin and Sloan 1997). The ability to distinguish host hematopoietic cells (CD45.1) from donor hematopoietic cells (CD45.2) allows for the determination of engraftment efficiency and repopulation capacity of the donor cells(Mercier, Sykes, and Scadden 2016).

1.1.2.2 In vitro assays

The *in vitro* colony forming unit (CFU) assay, also known as the methylcellulose assay, is commonly used to assess the differentiation and proliferative ability of lineage-restricted progenitors to form colonies in semi-solid media. Depending on the growth factors supplemented in the media, colonies of a particular lineage form from individual progenitor cells and are identified based on morphology. The different types include burst-forming unit-erythroid (BFU-E), CFU-erythroid (CFU-E), CFUs consisting of granulocytes and macrophages (CFU-GM), and CFUs consisting of granulocytes, erythrocytes, macrophages, and megakaryocytes (CFU-GEMM) (Pamphilon et al. 2013).

1.1.3 Clonal succession in human hematopoiesis

HSPCs acquire random somatic mutations which accumulate over time as they age. While most of these somatic mutations are neutral, some are positively selected for and provide the affected cell with a fitness advantage thereby resulting in increased expansion of that clonal lineage i.e., the progeny of the single initiating cell (Bowman, Busque, and Levine 2018; Calvillo-

Argüelles et al. 2019; Welch et al. 2012). When this event occurs in the context of hematopoiesis, it is referred to as clonal hematopoiesis (CH). CH is a common condition that does not always manifest clinically and can only be confirmed via DNA sequencing. The results of an extensive, whole-exome sequencing study performed on peripheral blood revealed that CH was present in approximately 10% of individuals over the age of 65 (Genovese et al. 2014); work by others also show that the incidence of CH continues to rise with age (Jaiswal et al. 2014; Zink et al. 2017).

Individuals with CH can be categorized into specific groups based on the type of somatic mutation(s) present and whether abnormal hematopoiesis is present/detected (R. Bejar 2017). For this, it is important to make the distinction between background or "passenger" mutations and driver mutations. Blood cells harbor hundreds of randomly acquired background mutations that, by definition, do not contribute to the pathogenesis of disease, are not selected for, and are simply passed on through cell division (passengers). Conversely, driver mutations are ones known to directly result in disease development, confirmed through studies in model systems (more in *Section 1.5*) (Bowman, Busque, and Levine 2018; Stratton, Campbell, and Futreal 2009; Welch et al. 2012). The risk of an individual with CH developing a hematologic malignancy depends on the mutation burden i.e., the percentage of affected cells. If a leukemia-associated driver gene is mutated and present at a variant allele frequency (VAF) of at least 2%, the annual rate of progression to disease is estimated to be 0.5-1% (Genovese et al. 2014; Jaiswal et al. 2014; Steensma 2018a). The presence of CH without specifying type of somatic mutation or burden in individuals with otherwise normal hematopoiesis is simply referred to as age-related CH (ARCH). If the VAF cut-off value of 2% is reached, the term CH of indeterminate potential (CHIP) is used. Following this, the development of one or more cytopenias i.e., reduced cell count of mature blood cell lineage will result in a clonal cytopenia of undetermined significance (CCUS) diagnosis.

Furthermore, if dysplasia i.e., abnormal cell morphology is detected and sustained, myelodysplastic syndrome (MDS), a pre-leukemic disease, is diagnosed (R. Bejar 2017; Bowman, Busque, and Levine 2018; Sperling, Gibson, and Ebert 2017; Steensma 2018a; Steensma et al. 2015; Valent and Valent 2019).

Progression to overt leukemia, namely acute myeloid leukemia (AML), requires additional mutations that cooperate with already existing "initiating" mutations, i.e., ones that are not capable of causing disease on their own (Xie et al. 2014). This may explain why many healthy individuals over the age of 70 harbour mutations in DNMT3A and TET2 (Buscarlet et al. 2017), epigenetic regulators that are recurrently mutated in MDS and AML, but never go on to develop disease (Xie et al. 2014). Without the acquisition of subsequent cooperating mutations, it is thought that DNMT3A and TET2 variants merely provide the affected cell with a clonal growth advantage, inducing a state of CH (Bowman, Busque, and Levine 2018; Busque et al. 2018; Challen et al. 2012a; Quivoron et al. 2011). The process of disease initiation and progression is not fully understood as the complete set of driver mutations is yet to be defined; however, this list will continue to grow as more sensitive sequencing technologies become available.

1.2 Development of myelodysplastic syndrome

1.2.1 Myelodysplastic syndrome (MDS)

The World Health Organization (WHO) defines myelodysplastic syndrome (MDS) as a bone marrow failure disease characterized by clonal hematopoiesis (abnormal karyotype, somatic mutations), ineffective development of one or more myeloid lineages leading to peripheral blood cytopenia(s), and myelodysplasia (Arber et al. 2016). Another defining feature is the risk of developing acute myeloid leukemia (AML), which occurs in approximately one third of MDS cases. There are several subtypes of MDS associated with varying degrees of risk for

transformation to AML or death (Steensma and Stone 2020; Valent et al. 2007); however, in general, patients can be broadly classified as low- or high-risk MDS (Platzbecker 2019).

1.2.2 Incidence and Clinical features of MDS

The largest risk factor for MDS is aging and the median age of diagnosis is 70 years old (Cazzola 2020). The recently updated WHO classification of MDS provides detailed guidelines for defining the subtypes of MDS and is commonly used by clinicians to make important decisions regarding diagnosis (Arber et al. 2016). Additionally, the revised International Prognostic Scoring System (IPSS-R) is used to assess risk of disease progression and mortality in MDS patients at time of diagnosis (Greenberg et al. 2012). There are several other classification and prognostic tools available that provide varying definitions of MDS, which may partly explain why it has been difficult to determine the true incidence of MDS (Sekeres 2010). MDS is also likely underreported to cancer registries (Cazzola 2020). Taking these inconsistencies into consideration, Cogle and colleagues designed and implemented an algorithm to reassess data from cancer registries which computed an estimated incidence of 75 MDS cases per 100,000 people aged 65 or older, per year (Cogle et al. 2011). If accurate, this makes MDS one of the most common hematologic malignancies (Steensma and Stone 2020).

A patient is considered to be suspected of MDS if they present with, otherwise unexplained, cytopenia(s). While MDS is characterized by at least one cytopenia (anemia, neutropenia, and/or thrombocytopenia), it is important to first eliminate other potential causes of cytopenias such as viral infections or other similar hematologic disorders (Cazzola 2020; Steensma and Stone 2020; Valent et al. 2007). The presence and duration of several clinical features are assessed through a number of procedures including peripheral blood smear and counts, bone marrow aspiration, bone

marrow biopsy, and cytogenetic analysis (Cazzola 2020; Greenberg et al. 2017). To make a diagnosis of MDS the following "prerequisite" criteria are, at minimum, required: (1) marked cytopenia persisting for at least 6 months, reduced to 2 months if a chromosomal abnormality or bilineage dysplasia is confirmed and (2) all other potential causes for cytopenia and/or dysplasia are dismissed. Additionally, at least one of the following "decisive" criteria is required: (1) blast cell count of 5-19% in BM; (2) dysplasia in at least 10% of cells in BM; and/or (3) karyotype commonly associated with MDS. Furthermore, some clinics use the following "co-criteria" to aid in diagnosis: (1) abnormal immunophenotype of BM cells measured by flow cytometry and (2) mutation analysis revealing myeloid clonality (Greenberg et al. 2017; Steensma and Stone 2020; Valent et al. 2007). Despite peripheral blood cytopenia(s) due to increased apoptosis in myeloid progenitors, most patients present with a normocellular or hypercellular BM (Adès, Itzykson, and Fenaux 2014; Steensma and Stone 2020). Diagnostic tests are redone periodically to monitor the patient's progress or when only some diagnostic criteria are met in a patient suspected of MDS (Valent et al. 2007).

1.2.3 Pathogenesis of MDS

The clonal nature of MDS makes it a heterogenous disease in the clinical and biological sense. Clinically, it is sometimes challenging to make a diagnosis. Biologically, disease heterogeneity has made it difficult to (1) identify the cell of origin and (2) pinpoint the exact events involved in the pathogenesis of disease (Sperling, Gibson, and Ebert 2017; Steensma and Stone 2020). However, owing to the growing development and use of next-generation sequencing (NGS) technologies in the last decade, the field has made considerable progress by uncovering recurrently mutated genes implicated in MDS (Haferlach et al., 2014; E. Papaemmanuil et al., 2011; E. Papaemmanuil et al., 2013; Walter et al., 2012; Yoshida et al., 2011). The identification of over

40 MDS-associated gene mutations has enabled researchers to better understand the complex events involved in the initiation of progression of disease (Haferlach et al., 2014; Papaemmanuil et al., 2013; Xie et al., 2014). Furthermore, it is generally agreed that it would be beneficial to add profiling of somatic mutations to the IPSS-R to assist with conventional diagnosis, risk stratification, monitoring progression, and measuring residual disease (Rafael Bejar et al. 2015; Cazzola 2020; Malcovati et al. 2014; Steensma and Stone 2020; Thol and Platzbecker 2019). As previously mentioned, cytogenetic analysis is already a consideration of the IPSS-R. The following two subsections will discuss/detail the most common cytogenetic abnormalities and somatic mutations involved in the pathogenesis of MDS and how this information can be used to inform clinical decisions.

1.2.3.1 Recurrent cytogenetic abnormalities

As previously mentioned, cytogenetic (karyotypic or chromosomal) abnormalities are considered in the IPSS-R. Cytogenetic abnormalities are present in approximately half of MDS patients (Steensma and Stone 2020). Recurrent cytogenetic abnormalities associated with MDS are listed in Table 1 (not exhaustive).

Table 1: Commonly acquired MDS-associated chromosomal abnormalities

IPSS-R risk category	Chromosomal abnormality	Frequency in MDS (%)
Very good	-Y	2.2
	del(11q)	0.7
Good	Normal karyotype	55.1
	del(5q) ±1 other abnormality	8.0
	del(20q)	1.7
Intermediate	Trisomy 8	4.7
Poor	-7/del(7q)	2.8
Very poor	Complex (≥3 abnormalities)	7.0

Data in table adapted and modified from Steensma, DP and Stone, RM. Myelodysplastic Syndromes. *Abeloff's Clinical Oncology*. 2020;1798-1820.

1.2.3.2 Recurrent somatic mutations

While the complete set of somatic mutations involved in MDS initiation and progression have not been fully defined, current knowledge may still provide insight into the pathogenesis of disease and prove useful for classification and prognostication of MDS patients (Haferlach et al. 2014). With the exception of SF3B1 used to diagnose a subtype of MDS with ring sideroblasts, molecular profiling of target genes is not yet incorporated into the IPSS-R nor is a part of routine clinically testing (Greenberg et al. 2017; Steensma and Stone 2020).

Large-scale sequencing studies have reported that approximately 78-90% of patients harbour at least one recurrent MDS-associated mutation (Haferlach et al. 2014; Elli Papaemmanuil et al. 2013). Another study reported that most mutations contained C-to-T base pair transitions at CpG dinucleotides, a phenomenon indicative of age-related methylcytosine deamination (Makishima et al. 2017). Mutations in pre-mRNA splicing factors (e.g., *SF3B1*, *U2AF1*, *SRSF2*, and *ZRSR2*) are the most common, found in 60-70% of MDS patients (Higgins and Shah 2020; Inoue, Bradley, and Abdel-Wahab 2016). The second most common group of mutations are in epigenetic regulators (e.g., *TET2*, *DNMT3A*, *IDH1/2*, *EZH2*, *ASXL1*) involved in DNA methylation and chromatin remodelling (Higgins and Shah 2020; Sperling, Gibson, and Ebert 2017; Steensma and Stone 2020). There are many other, albeit less common, MDS-associated mutations affecting certain signaling pathways, cohesin complex, and some transcription factors. The following commonly mutated genes in MDS patients have been recently proposed as potential clinically relevant biomarkers either due to their high occurrence (>10%) or association with poor prognosis: *SF3B1*, *TET2*, *SRSF2*, *ASXL1, TP53*, *NRAS*, *DNMT3A,* and *RUNX1* (Cazzola 2020; Steensma and Stone 2020). However, it is important to emphasize that the presence of these mutations are intended to help confirm a suspected MDS diagnosis and are not diagnostic on their

own (Steensma and Stone 2020). Additionally, the results of a large-scale sequencing study proposed a set of mutations involved in early initiation (i.e., *DNMT3A, TET2, JAK2, ASXL1, GNAS, PPM1D, SF3B1,* and *SH3B3*) and cooperating mutations (i.e., *FLT3, NPM1, WT1, IDH1, RUNX1, NRAS, CEBPA, U2AF1, PHF6,* and *STAG2*) which, in theory, could be used to screen for disease progression (Xie et al. 2014). Makishima et al., made similar conclusions in their study as well (Makishima et al. 2017).

1.2.3.3 Epigenetics of MDS

Epigenetics is defined as the study of heritable gene expression changes caused by DNA methylation, histone modification, and/or nucleosome composition/placement, but not by modifications of the underlying DNA sequence (Pon and Marra 2015; Yamazaki and Issa 2013). While some epigenetic alterations are transient, only the heritable ones that provide a growth advantage can be candidate drivers of disease (Pon and Marra 2015). MDS, like many other cancers, is characterized by epigenetic dysregulation which is thought to result in a gene signature that may provide insight into the mechanisms that cause disease (Nagata and Maciejewski 2019; Yamazaki and Issa 2013).

As previously mentioned, many MDS patients harbor mutations in DNA methylation regulators (*DNMT3A*, *TET2*, and *IDH1/2*) and histone modifiers (*EZH2* and *ASXL1*) (Yamazaki and Issa 2013). DNA methylation is accomplished via DNMTs (DNA methyltransferases) that catalyze the methylation of cytosines in CpG islands which commonly occur in gene promoter regions, thereby resulting in gene silencing. *De novo* DNA methylation is mediated and subsequently maintained by *DNMT3A/3B* and *DNMT1*, respectively (Itzykson and Fenaux 2014; Nagata and Maciejewski 2019; Yang, Rau, and Goodell 2015). Loss-of-function mutations in *DNMT3A* occur in 10-15% of MDS patients and result in hypomethylation of cytosines at enhancer

sequences (Sperling, Gibson, and Ebert 2017). *DNMT3A* has been shown to play an important role in HSC differentiation (Challen et al. 2012b; Trowbridge and Orkin 2012). In contrast, DNA demethylation is carried out by *TET2* (ten-eleven translocation 2) that catalyzes the conversion of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) which is unrecognizable by DNMT1 (Itzykson and Fenaux 2014). Loss-of-function *TET2* mutations occur in 35% of MDS patients resulting in hypermethylation and repression of genes involved in myeloid differentiation (Itzykson and Fenaux 2014; Sperling, Gibson, and Ebert 2017). Mutations in DNMT3A and TET2 arise in early stages of disease development and, despite their opposing mechanisms in epigenetic regulation, they commonly co-occur in MDS patients (Buscarlet et al. 2017; Haferlach et al. 2014; Sperling, Gibson, and Ebert 2017). Furthermore, mutated IDH (isocitrate dehydrogenase) 1 and 2 (in 5% of MDS patients) catalyzes the production of a metabolite that inhibits the function of *TET2,* contributing further to aberrant DNA methylation patterns (Itzykson and Fenaux 2014). Mutations in the histone modifiers *EZH2* (enhancer of zest homolog 2) and *ASXL1* (additional sex combs-like 1) occur in 5% and 20% of MDS patients, respectively, (Itzykson and Fenaux 2014) and are both associated with decreased overall survival (Rafael Bejar et al. 2011).

1.2.4 Current standard of care

Currently, there is no curative treatment for MDS other than allogenic HSC transplant which has a success rate of 20-50% and only few patients (<10%) qualify for (Deeg and De Lima 2013). Alternative therapies include the Food and Drug Administration (FDA)-approved DNA hypomethylating agents (HMAs), azacitidine and decitabine (DNMT inhibitors), and an immunomodulatory agent called lenalidomide (Steensma and Stone 2020).

Standard of care differs when treating MDS patients classified as low-risk vs high-risk and depending on the type of cytopenia present. Also, in some cases, chromosomal abnormalities and

somatic gene mutations can predict a MDS patient's response to certain treatments (Sperling, Gibson, and Ebert 2017; Weinberg and Hasserjian 2019). For example, the success of a transplantation can be predicted through genetic testing; namely, patients with mutations in *TP53*, a recurrently mutated tumor suppressor gene, are more likely to relapse (Rafael Bejar et al. 2015). For low-risk MDS patients, the main goal is to prevent and manage cytopenia-related complications, such as bleeding and infections, and improve quality of life (Adès, Itzykson, and Fenaux 2014; Kantarjian et al. 2007; Platzbecker 2019; Steensma 2015). Some low-risk MDS patients are monitored regularly without the need for therapeutics and others qualify for low-intensity treatment with hematopoietic growth factors (Platzbecker 2019; Steensma and Stone 2020). The results of a clinical trial show that treatment of lenalidomide in low-risk MDS patients with chromosome 5q deletion significantly reduced the need for blood transfusions (List et al. 2006). However, lenalidomide treatment is less effective in patients with more complex karyotypes (Steensma 2018b). In contrast, MDS patients with a high risk of progression to AML and poor prognosis require transplantation or, in most cases, treatment with HMAs; the priority is to delay progression to overt leukemia and improve survival (Platzbecker 2019; Steensma and Stone 2020). Only a proportion of high-risk MDS patients respond to treatment with azacitidine and decitabine, in which the effect is transient, only offering a few months of prolonged survival as the founder clone(s) are not successfully eradicated (Fenaux et al. 2009; Merlevede et al. 2016; Unnikrishnan et al. 2017; Zeidan et al. 2017). The unmet clinical need of maintaining quality of life in low-risk MDS patients and preventing disease progression to AML in high-risk MDS patients is greatly hampered by the lack of effective treatment options.

1.3 Progression to secondary acute myeloid leukemia

1.3.1 Diagnosis and distinction from MDS and de novo AML

Approximately one third of MDS patients develop AML (secondary AML; sAML) (Menssen and Walter 2020). Clinically, sAML is diagnosed in patients with BM blast cell counts of ≥20%, as defined by the WHO (Arber et al. 2016). At the cellular level, there is a shift from apoptosis to proliferation in the progression from MDS to sAML, suggesting increased cell survival possibly explaining the increased accumulation of blasts in the bone marrow (Adès, Itzykson, and Fenaux 2014; Corey et al. 2007). In MDS, the HSCs differentiate to a point then they undergo apoptosis and die, or they become dysplastic, whereas in sAML, there is a block in differentiation in which HSCs differentiate to an early point then stop differentiating and accumulate as immature cells (Corey et al. 2007). Also, karyotypes become more complex in sAML, as chromosome translocation are more frequent (and also associated with poorer response to treatment) (Adès, Itzykson, and Fenaux 2014; Corey et al. 2007). While these observations highlight fundamental differences between MDS and sAML, there remains a great deal unknown regarding the mechanism(s) driving disease progression. Interestingly, mutations originating from MDS remain highly recurrent in sAML patients, including *RUNX1* (25-30%), *SRSF2* (20%), *ASXL1* (35%), *EZH2* (9%), and *IDH1/2* (11%) (Dicker et al. 2010; Kakosaiou et al. 2018; Lindsley et al. 2015; Makishima et al. 2017; Patnaik et al. 2012; Tefferi et al. 2017; Thol et al. 2011). Namely, the persistence of mutations involved in epigenetic regulation underscores its potential mechanistic role in development of disease. Also, *FLT3* becomes frequently mutated in sAML (12-20%), whereas in MDS it occurs less than 1% of the time (Dicker et al. 2010; Makishima et al. 2017).

Clinically, sAML and primary (*de novo*) are considered different diseases for a number of reasons (Arber et al. 2016; Sperling, Gibson, and Ebert 2017). The prognosis for sAML patients is grim compared to patients diagnosed with primary (*de novo*) AML (Lowenberg, Downing, and Burnett 1999), as the former respond more poorly to therapy (Saultz and Garzon 2016). Biologically, patients with *de novo* AML can be distinguished from those with sAML through distinct methylation patterns (Figueroa et al. 2009) and by the presence of certain mutations (Lindsley et al. 2015).

The use of NGS technologies in recent years has granted the field novel insights to explore with regard to the mechanistic evolution of disease from healthy to MDS to sAML. Genetic engineering can help fill the gaps in the current understanding of disease based on putative driver mutations that emerge from NGS studies, with the ultimate goal of developing highly effective therapeutics.

1.4 Genetic engineering of mouse HSCs

1.4.1 Perturbing gene expression

As previously mentioned, putative driver genes can only be functionally validated in model systems in which the gene expression is disrupted, and the resulting phenotype can be assessed (Beachy and Aplan 2010; Boettcher and McManus 2015; El-Brolosy and Stainier 2017). In the context of BM diseases, there are two general methods used to test the role of a putative driver gene: (1) introduction of transgene into germline of mice and (2) perturbing expression of HSPCs *in vitro* followed by transplantation into immunodeficient mice (Beachy and Aplan 2010; Bedell, Jenkins, and Copeland 1997). In the first method, transgenic mice are generated by pronuclear injection of the gene of interest (GOI) into the germline of a fertilized egg, or by homologous recombination in embryonic stem cells subsequently injected into blastocysts. The second method

involves harvesting murine BM mononucleated cells (MNCs), using a delivery system to either delete (knockout) or introduce (knock-in or overexpress) the GOI, then transplanting the altered cells into immunodeficient mice (Beachy and Aplan 2010). Several methods are available to knockout or knockdown (loss of function) or overexpress (gain of function) a GOI. In general, loss of function studies involves technologies that either alter the GOI's transcriptional process or result in excision from the genome. Whereas for gain of function studies, the general approach is to integrate the GOI into the host genome or infect the target cell with a plasmid containing the open reading frame under the control of a strong promoter (Boettcher and McManus 2015; Kallunki et al. 2019).

1.4.2 Inducible expression systems

Gene expression systems that can be induced at a specific time are generally preferred over germline mutations and are sometimes the only option considering some germline mutations are lethal. This is especially useful for creating models of diseases in which mutations are somatically acquired (Rajewsky et al. 1996). This can be accomplished using the Cre-*loxP* recombination system. Cre recombinase facilitates site-specific deletion of DNA sequences through the recognition of flanking *loxP* sites (Kim et al. 2018). To generate inducible mice using this technology, two strains are needed. The first mouse strain harboring a modified Cre recombinase gene is bred with a second mouse strain containing the functional GOI flanked (floxed) by *loxP* sites (Kim et al. 2018; Rajewsky et al. 1996). This system is made inducible by the modified Cre fusion protein, called CreER, consisting of Cre and an estrogen receptor with a mutated ligand binding domain. The CreER complex is bound by heat shock protein 90 (HSP90) in the cytoplasm where it remains until the administration of tam (via intraperitoneal injection) which displaces HSP90, thereby allowing the CreER-tam complex to translocate to the nucleus where it can carry-

out the deletion of the floxed GOI (Kim et al. 2018).

1.4.3 Delivery and control methods of genetic engineering

There are three general methods used to deliver a gene into a cell: (1) physical, (2) chemical, and (3) via viral vectors (Giacca 2010; Mali 2013). The use of viruses to deliver a modified vector is preferred over physical and chemical methods because it provides relatively high gene transfer efficiency *in vivo* and *in vitro*. The main viral vectors used include adenoviral, adeno-associated virus (AAV), and lentiviral vectors. Adenoviruses are DNA viruses that are able to transduce dividing and non-dividing cells. Unlike lentiviruses and some AAVs, adenoviruses do not integrate their DNA into the genome of the target cell which results in transient expression. AAVs are also DNA viruses that can be designed to be integrating or non-integrating, are not immunogenic, can infect non-dividing cells *in vivo*. Several capsid serotypes are available for gene delivery to specific organs of interest. AVVs however have a limited cloning capacity due to its small genome size (Giacca 2010; Mali 2013). Lentiviruses, namely human immunodeficiency virus type 1 (HIV-1), have been extensively researched over the last decade and several generations have been designed to ensure safety regarding pathogenicity (Giacca 2010). They are able to transduce dividing and non-dividing cells both *in vivo* and *in vitro*, providing long-lasting, stable gene expression via integration of vector DNA into the target genome (Giacca 2010; Mali 2013).

1.4.4 Limitations of mouse models

Discoveries made in the mouse system must ultimately be validated in the human system, given the differences in the cellular biology between the two species. A mutation in the mouse may have significantly different outcomes than it does in the human (Pon and Marra 2015). As

such, a limitation of mouse models in general is the potential lack of translation and relevance to humans. For example, this lack of translation was exemplified by the differing effect of ectopic expression of *HoxB4* in mice vs humans. Specifically, Kyba et al., showed that ectopic expression of *HoxB4* in mouse embryonic stem cells promoted the development of functional HSCs (Kyba, Perlingeiro, and Daley 2002). However, Wang et al., demonstrated that this is not the case when performed in human embryonic stem cells (Wang et al. 2005). Despite this limitation, the mouse remains a valuable tool as oftentimes it is the only alternative system; however, it is imperative that caution be exercised when extrapolating findings from the mouse system to the human.

1.5 Modelling myeloid malignancy evolution in mice

Several mouse models of AML have been established but there are not as many available for MDS. In order to study the initiation of the disease at early stages, emphasis should be placed on developing MDS models that allow for the real-time monitoring of the events driving the transformation to overt leukemia. This section is focused on the advancements and shortcomings of MDS murine models published in recent years.

1.5.1 Methods used to model MDS in mice

Common methods used in attempt to create mouse models of MDS include patient-derived xenotransplantation (PDX) and genetic engineering of MDS-implicated genes in mouse HSCs, as described earlier (Beachy and Aplan 2010; Côme et al. 2020). Xenotransplantation of primary patient samples or MDS cell lines into immunodeficient mice is an intriguing avenue but is met with considerable challenges. Numerous attempts by several groups have failed to engraft human MDS cells into immunodeficient mice (Benito et al. 2003; Martin et al. 2010; L. Nilsson et al. 2000; Lars Nilsson et al. 2002). In some cases where

sufficient engraftment was achieved, it was found to be done so by karyotypically normal cells instead of the MDS clone(s) (Benito et al. 2003; Thanopoulou et al. 2004). Culturing MDS-derived clones *in vitro* is challenging as well (Corey et al. 2007). The use of MDS cell lines is not a reliable option either, as many were found to be "false", i.e., they were cross-contaminated with an already established leukemia cell line or were non-malignant (Drexler, Dirks, and MacLeod 2009).

1.5.2 Bethesda criteria for diagnosing MDS in mice

As previously discussed, mouse models of MDS should be clinically relevant i.e., resemble human pathology. Accordingly, the hematopathology subcommittee of the Mouse Models of Human Cancers Consortium (MMHCC) defined a set of criteria (Bethesda) for diagnosing murine myeloid dysplasia in accordance with the WHO criteria for human MDS (Kogan et al. 2002). According to the Bethesda criteria, to diagnose MDS in mice, one or more of the following must be satisfied: mice display cytopenia in (1A) neutrophils (neutropenia), (1B) platelets (thrombocytopenia), or (1C) erythrocytes (anemia). Another criterion is (2) ineffective maturation of nonlymphoid cells which can present itself as dysgranulopoiesis (impaired granulocyte maturation), dyserythropoiesis (impaired red blood cell maturation), and/or dysplastic megakaryocytes which may or may not be accompanied by increased blast counts. Additionally, (2) ineffective maturation of nonlymphoid cells which manifests as dysgranulopoiesis (impaired granulocyte maturation), dyserythropoiesis (impaired red blood cell maturation), and/or dysplastic megakaryocytes and may or may not be accompanied by increased blast cell counts. Lastly, (3) the mice must not satisfy the criteria for nonlymphoid leukemia otherwise it cannot be considered an MDS model (Kogan et al.

2002). The Bethesda criteria can also be used to identify whether transformation to overt leukemia has occurred. Akin to human AML classification, a mouse is diagnosed with myeloid leukemia when (1) the non-lymphoid blast count makes up at least 20% of the total cell count in the blood, spleen, or bone marrow. Furthermore, (2) leukemic onset should be rapid, resulting in death by 4 weeks or less, and (3) disease is transplantable (Kogan et al. 2002).

1.5.3 Current MDS to AML mouse models and limitations

An ideal MDS model would be one that is capable of capturing the transformation to AML, allowing for mechanistic events to be studied. This would be especially useful if the model could be designed in such a way that allows for the MDS-to-AML transformation to be experimentally controlled. Additionally, the model should resemble human pathology and be able to recapitulate features of disease. While many have attempted to generate such models, most only meet some criteria; disease progression either never occurs or takes months or disease phenotype is not consistent with human pathology.

In 2016, our group developed an *in vivo* mouse model where MDS and AML features reminiscent of clinical disease are produced through the sequential deletion of the glycogen synthase kinase-3 (GSK-3) homologs, *Gsk3b* then *Gsk3a*, in HSCs (Guezguez et al. 2016). The GSK-3 homologs are players in a number of pathways such as Hedgehog, Wnt, and Notch that, when impaired, are involved in the onset of leukemia (McCubrey et al. 2014). Although there have been reports that GSKα and GSK3β are functionally redundant in other contexts (Doble et al. 2007; Gillespie et al. 2011; Itoh et al. 2012), Guezguez et al. show that the deletion of *Gsk3b* in mice HSCs impaired hematopoiesis and resulted in the formation of MDS-initiating cells (MDS-ICs), while the deletion of both homologs led to the development

of AML through the generation of AML-ICs. Interestingly, they observed that mice with only *Gsk3a* knocked-out in HSCs did not have an effect on hematopoiesis and resembled WT mice in regard to phenotype and function. Additionally, this group designed the mouse model such that expression of GSK-3 is conditionally controlled under a Cre-*loxP* system, which is a highly advantageous option as it allows researchers to study the stepwise initiation and progression. Moreover, the abolishment of Gsk3b expression produced a dysregulated epigenetic signature, as the targets of DNA methyltransferase 3A (DNMT3A) were upregulated, suggesting that DNMT3A is a player in the progression of disease in this model. This observation further supports that the GSK3 mouse model is reminiscent of human disease, as DNMT3A mutations have been previously reported in MDS and AML patients (Ley et al. 2010; Y. Yamashita et al. 2010; Yan et al. 2011). Therefore, the GSK3 model can be used as a unique tool to study the MDS to AML evolution based on the dysregulation of multiple pathways involved in leukemia.

1.5.4 Need and rationale to develop in vitro GSK mouse model vs. in vivo systems

As discussed, the lack of understanding of how MDS progresses to AML hampers the development of much-needed, highly effective therapeutics. Our group's model clearly defines MDS and AML states that would permit mechanistic understanding of the initiator and driver events (Guezguez et al. 2016). Translation to *in vitro* system would allow for the growth, expansion, and direct investigation of MDS-ICs.

1.6 Study Rationale

As discussed, the development of effective therapeutics is hampered by the incomplete mechanistic understanding of MDS initiation and progression, which ultimately requires relevant

MDS-to-AML models of disease to move the field forward. Capitalizing on of our group's previously described *in vivo* GSK3 mouse model (Guezguez et al. 2016), we have developed a novel *in vitro* system that uniquely allows for the selection, growth, and expansion of the rare blood cells responsible for initiating disease. This system can serve as a surrogate to understand the initiating events involved in the stepwise transition from healthy to MDS to AML phenotype. Preliminary whole exome sequencing work conducted by our group revealed a lack of consistent mutations in the *in vivo* system, suggesting that the nature of disease in this model is epigenetic and not simply driven in a clonal evolution model by driver mutations. Translation to a high-content *in vitro* model will allow us to investigate this further by studying the outcome of what reconstitution of GSK3β in MDS-ICs (GSK3β-KO cells) may do. The work presented in this thesis is part of a larger, collective effort by our group with the long-term goal of identifying targets for the development much-needed, effective therapeutics.

1.7 Hypothesis

I hypothesize that GSK3β knockout in the *in vitro* model can act as a surrogate of MDS disease and induces an epigenetic state that requires further manipulation beyond restoring GSK3β to reverse disease onset.

1.8 Experimental objectives

This thesis aims to:

1. Generate GSK3β-knockout cells in culture with MDS-IC properties.
2. Achieve temporal re-expression of GSK3β in the KO cells by lentivirus.
3. Assess properties of MDS vs. healthy restoration by measuring hematopoietic progenitors.

2.0 Materials and Methods

2.1 GSK-3 mouse genotyping

The generation of conditional Gsk3β-knockout mice carrying Cre recombinase has been previously described (Guezguez et al. 2016). Genomic DNA was extracted from ear notches and amplified using the Extract-NAmp™ Tissue PCR Kit (Sigma Aldrich). The genotype of each mouse was determined via PCR analysis using primers designed to amplify GSK-3α, GSK-3β and Cre (Table 2).

Table 2: Primer sequences used for genotyping

Gene Name	Primer Sequences
Gsk3a	F: 5'-CCC CCA CCA AGT GAT TTC ACT GCT A-3'
	R: 5'-AAC ATG AAA TTC CGG GCT CCA ACT CTA-3'
Gsk3b	F: 5'-AAC CAC AGT AGT GGC AAC TC-3'
	R: 5'-CCA GTC ACA AAT CGT ACT GC-3'
	Neo F: 5'-CGT GCT ACT TCC ATT TGT CAC G-3'
Cre	Rosa26 F: 5'-AAA GTC GCT CTG AGT TGT TAT-3'
	Rosa26 R: 5'-GGA GCG GGA ATG GAT ATG-3'
	CreER: 5'-CCT GAT CCT GGC AAT TTC G-3'

2.2 Mouse harvest and BM processing

Mice were sacrificed by cervical dislocation and bone marrow was harvested from the iliac crests, femurs, tibiae, fibulae, and spine using sterile technique. Cells were mechanically dissociated/homogenized using a mortar and pestle in IMDM (Gibco) supplemented with 3% FBS (Performance, Wisent, Canada) and 1 mM EDTA (Invitrogen). Lysis of red blood cells was accomplished by resuspending and incubating the centrifuged bone marrow cells in ammonium chloride (STEMCELL Technologies) for 10 minutes at 4°C, leaving the BM mononuclear cells (MNCs) intact. An aliquot of the isolated BM MNCs were kept aside for flow cytometry analysis of linage-negative (Lin-) and LSK cells before lineage-positive cell depletion, i.e. cells expressing mature markers. The Direct Lineage Cell Depletion Kit (Miltenyi Biotec) was used to deplete

lineage-positive cells via incubation with a mature cell lineage antibody cocktail conjugated to magnetic MicroBeads, for 10 minutes at 4°C. Unlabelled Lin- cells were then purified via column separation using the QuadroMACS™ Separator (Miltenyi Biotec). Flow cytometry analysis was performed to assess the efficiency of the column lineage-positive cell depletion.

2.3 Culture conditions of floxed and GSK3β^{KO} cells

Freshly isolated Lin- cells were resuspended in StemSpan™ serum-free hematopoietic cell expansion media (STEMCELL Technologies) supplemented with mouse IL-6 (10 ng/mL), mouse IL-3 (10 ng/mL), and mouse stem cell factor (100 ng/mL, Miltenyi Biotec). Lin- cells were then plated in 6-well flat bottom ultra-low attachment cell culture plate (1.5-2x10^6 cells in 3 mL media per well; Corning). Cells were incubated at 37°C in a 5% CO_2 incubator. Cells were gently pipetted daily to discourage attachment to the bottom of the plate and clumping, to avoid differentiation. After 3-4 days in culture, 0.5 mL of fresh media was added. At day 7, cells were treated with 4-hydroxytamoxifen (5μM, Sigma-Aldrich) at a density of 2x10^6 cells/well for 48 hours, then kept in culture for another 4-7 days (specified in figure legends) and pipetted daily.

2.4 RNA Extraction, cDNA synthesis and RT-qPCR

RNA was extracted from approximately 3x10^5 cells using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific) was used to quantify the RNA. 1000 ng of RNA was used for cDNA synthesis, performed using the iScript™ cDNA Synthesis Kit (Bio-Rad), following the manufacturer's protocol. Quantitative PCR was accomplished using the PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific). SimpliAmp™ Thermal Cycler (ThermoFisher

Scientific) was for all reactions. Primer sequences used are listed in Table 3. Relative gene expression levels were normalized to housekeeping gene HPRT calculated using the delta-delta Ct method.

Table 3: Primer sequences used for qPCR analysis

Gene Name	Primer Sequences
Hprt	F: 5′-AGG GTG TTT ATT CCT CAT GGA CTA A-3′
	R: 5′-TCC TTC ATC ACA TCT CGA GCA A-3′
Gsk3b	F: 5′-GCA GCA GCC TTC AGC TTT TG-3′
	R: 5′-TGG TTA CCT TGC TGC CAT CTT-3′

2.5 Western blots

Protein was isolated from 0.5-1x10^6 cells by lysis with RIPA buffer (10 mM Tris/HCl, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS) with fresh Halt Protease and Phosphatase Inhibitor Cocktail (1:100, ThermoFisher Scientific) and sonication. Protein was quantified using the DC™ Protein Assay (Bio-Rad). Protein samples (15 µg) were boiled at 95°C for 5 minutes to denature and then run in a freshly prepared 10% acrylamide SDS-PAGE gel. Protein from the gel was then transferred onto nitroceullose membrane which was blocked with EveryBlot Blocking Buffer (Bio-Rad). The membrane was then incubated with primary antibodies overnight at 4°C and secondary antibodies for 1 hour at room temperature. Imaging and analysis were performed using the ChemiDoc Imaging System and Image Lab™ Software (Bio-Rad). Primary antibodies used: rabbit anti-GSK3α/β (1:1000, #5676, Cell Signaling Technology); mouse anti-β-actin (1:1000, MAB1501, Sigma-Aldrich). Secondary antibodies used: anti-rabbit and anti-mouse HRP conjugates (1:5000, Bio-Rad).

2.6 Flow cytometry (surface staining and intracellular flow) and analysis

Cells were pelleted and their surfaces were stained with antibodies (Table 4) diluted in PEF (PBS, 3% FBS (Performance, Wisent, Canada), 1 mM EDTA (Invitrogen)) for 1 hour at 4°C. Cells were then washed with PEF and stained with 7-Amino-Actinomycin D (7-AAD, Beckman Coulter) diluted at 1:50 in PEF, to distinguish viable and from non-viable cells. For intracellular staining, cells were first stained with LIVE/DEAD™ Fixable Violet Dead Cell Stain (ThermoFisher Scientific) diluted at 1:7000 in PBS for 30 minutes at room temperature in the dark, followed by extracellular staining 1as previously described. The cells were fixed, permeabilized and washed using the fixation/permeabilization solution and the Perm/Wash™ buffer from the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences). Cells were stained with GSK3β antibody conjugated to PE (NBP1-47470PE, Novus Biologicals) diluted at 1:100 in Perm/Wash™ buffer and incubated for 30 minutes at room temperature in the dark. UltraComp eBeads (Affymetrix eBioscience) were used to prepare samples for compensation. LSRII (BD Biosciences) or Cytoflex LX (Beckman Coulter) cytometers were used to run the samples and subsequent analysis was performed using FlowJo™ v10.6.1 (BD Biosciences).

Table 4: Antibodies used for extracellular flow cytometry

Antigen specificity	Fluorochrome	Dilution	Catalogue	Supplier
Lin+ (CD3/Gr-1/CD11b/CD45R(B220)/Ter119)	Fitc Ms	1:500	78022	BioLegend
Lin+ (CD3/Gr-1/CD11b/CD45R(B220)/Ter119)	PB/eFluor 450	1:500	88-7772-72	Invitrogen
Lin+ (CD3/Ly-6G(Ly-6C)/CD11b/CD45R(B220)/Ter119)	AF700	1:500	79923	BioLegend
Sca1 (Rat Anti-Mouse Ly-6A/E)	PE	1:5000	553336	BD Biosciences
Sca1 (Rat Anti-Mouse Ly6A/E)	PE-Cy7	1:5000	558162	BD Biosciences
cKit (Rat Anti-Mouse CD117)	APC	1:1500	561074	BD Biosciences
Ter119 (Rat Anti-Mouse TER-119)	Fitc	1:500	557915	BD Biosciences
CD11b (Rat Anti-CD11b)	PE-Cy7		552850	BD Biosciences
Gr-1 (Rat Anti-Mouse Ly-6G and Ly-6C)	APC		553129	BD Biosciences
CD45 Rat Anti-Mouse CD45	PE-Cy7	1:1000	553082	BD Biosciences
Mouse Anti-Mouse CD45.1	eFluor 450/PB	1:1000	48-0453-82	eBioscience
Mouse Anti-Mouse CD45.2	APC	1:500	558702	BD Biosciences

2.7 Wright-Giemsa-stained Cytospins– morphological analysis

Cells were centrifuged onto glass microscope slides using the Shandon Cytospin 3 (Block Scientific, Inc.). Wright's Giemsa differential stain was accomplished using the Shandon Kwik-Diff™ Stain Kit (Thermo Scientific). Images were scanned with an Aperio CS2 and viewed using Aperio Image Scope software and ObjectiveView™ software.

2.8 CFU plating and counting

At endpoint, cells were resuspended in Methocult™ M3434 (STEMCELL Technologies) and plated at $5.0x10^4$ cells/0.5 mL per well of a 12-well non-treated cell culture plate (Corning). Cells were incubated at 37°C in a 5% CO_2 incubator for 14 days and manually scored.

2.9 Transplantation of MDS-ICs/GSK3β^{KO} cells

Immunodeficient NOD.Cg-Prkdc^{scid} (NOD/SCID CD45.1) mice from Jackson Laboratory were housed and transplanted in an animal barrier facility; all procedures were approved by the Animal Research Ethics Board of McMaster University. Lin- bone marrow cells were obtained from floxed CD45.2 donor mice (*Gsk3a*$^{+/+}$*b*$^{flx/flx}$) as previously described in section *2.2 Mouse harvest and BM processing* and cultured for a total of 8 days. GSK3β deletion was induced at day 7 to generate GSK3β^{KO} cells which were transplanted into lethally irradiated (11 Gy; 24 hours pre-injection) NOD/SCID CD45.1 recipient mice via intravenous tail vein injection at day 8 (N=4, 1.5-$2.5x10^6$ cells). Mice were irradiated 24 hours pre-transplantation.

Bl6/SJL (CD45.1) mice from Jackson Laboratory were housed and transplanted in an animal barrier facility; all procedures were approved by the Animal Research Ethics Board of McMaster University. Lin- bone marrow cells were obtained from floxed CD45.2 donor mice (*Gsk3a*$^{+/+}$*b*$^{flx/flx}$) as previously described in section *2.2 Mouse harvest and BM processing* and transplanted into congenic CD45.1 recipient mice (N=4, $1x10^6$ CD45.2+ LSK cells) irradiated at 11 Gy via intravenous tail vein injection (Guezguez et al. 2016). Mice were irradiated 24 hours pre-transplantation. GSK3β deletion was induced 14 days post-transplantation by three daily consecutive intraperitoneal (IP) injections of 6.64 mg/kg 4OH-tamoxifen in 10% Capstiol. At

endpoint, BM cells were harvested, sorted for CD45.2+ LSK cells, and processed for downstream analysis.

2.10 Whole exome sequencing and transcriptome analysis

Sorted LSK cells from mice transplanted with *in vivo* generated MDS-ICs, previously described in Section *2.9,* were used for whole exome sequencing (WES) analysis (Agilent technologies). The GATK Best Practices workflow was used for the analysis of the raw data and an open-source tool called SnpEff was used for variant annotation and prediction of high impact variants. The list of putative high impact variants was generated by using the floxed mice as the reference genome.

2.11 Plasmid design

Murine *Gsk3b* cDNA was subcloned into the pHIV-eGFP plasmid (Welm et al. 2008) (gifted from Bryan Welm and Zena Werb, #21373, Addgene) using the InFusion HD Cloning Kit (Takara Bio Inc.), following the manufacturer's protocol. The internal ribosome entry site (IRES) allows for bicistronic expression of *Gsk3b* and enhanced green fluorescent protein eGFP (pHIV-*Gsk3b*-IRES-eGFP). Empty plasmids (pHIV-IRES-eGFP) were used as experimental controls. Plasmid constructs were verified by sequencing.

2.12 Lentivirus production and titration

Plasmids (23 µg) constructed in Section *2.11* were transfected into human embryonic kidney (HEK) 293FT cells in the presence of packing plasmid psPAX2 (13 µg; #12260, Addgene) and VSV-G envelope expressing plasmid pMD2.G (9 µg; #12259, Addgene) using Opti-MEM™

media (ThermoFisher Scientific) and Lipofectamine^{TM} LTX reagent (ThermoFisher Scientific). 72 hours post-transfection, lentiviral particles were purified and concentrated using Amicon Ultra-15 Centrifugal Filter Units (100 kDa; Millipore Sigma), then stored at -80°C. Lentivirus titer was determined by a dilution series on HEK293FT cells with polybrene (8 µg/mL; Sigma Aldrich). Lentiviral transduction efficiency was determined by percentage of eGFP expression in living cells, measured via flow cytometry.

2.13 Lentiviral transduction

Two methods of lentiviral transduction were tested in floxed and GSK3β^{KO} cells. In the first method, the cells were transduced in growth media (as described in section *2.3 Culture conditions of floxed and GSK3β^{KO} cells*) supplemented with polybrene (8 µg/mL; Sigma Aldrich) at a multiplicity of infection (MOI) of 5 for 24 hours at 37°C in a 5% CO_2 incubator. In the second method, 6x10^5 cells were resuspended in growth media with lentivirus at an MOI of 9 and plated in freshly prepared 6-well RetroNectin-coated plates (5 µg/cm^2; Takara Bio Inc.) with spinoculation (600 g for 1 hour) then incubated at 37°C in a 5% CO_2 incubator for 48 hours, as previously described (Millington et al. 2009). In both methods, lentiviral transduction efficiency was determined by percentage of eGFP expression in living cells, measured via flow cytometry.

2.14 Statistical analysis

All results are represented as mean±SEM, unless stated otherwise. Prism 7 (GraphPad Software, Inc.) was used to manage data and perform statistical analyses (unpaired Student t-test or one-way ANOVA with Tukey's multiple comparisons test), with the criterion for significance set at $P<0.05$.

3.0 Results

3.1 Translation of the *in vivo* GSK3 model to an *in vitro* system

3.1.1 In vitro LSK cell expansion can be achieved in 14 days

The *in vitro* GSK3 system was modified from the *in vivo* mouse model of MDS-to-AML transition to allow for the selection, growth, and expansion of the cells responsible for disease initiation (Guezguez et al. 2016). In the *in vivo Gsk3β* deletion model, it was found that transplantation of mouse HSCs specifically (commonly identified as lineage-negative (Lin-), Sca-1+, c-Kit+ (LSK) (Ikuta, Koichi. Weissman 1992; Spangrude, Heimfeld, and Weissman 1988)) resulted in disease initiation whereas mice transplanted with only progenitors (non-LSKs) did not display MDS features (Gusezguez et al. 2016). As such, the *in vitro* GSK3 system was designed to support the growth of mouse HSCs (LSK cells). Figure 3.1A shows a simplified schematic of BM cells harvested from $Gsk3a^{+/+}b^{flx/flx}$ mice, herein referred to as floxed, prior to deletion of GSK3β. LSK cells were expanded *in vitro* with mouse interleukin (IL)-3 and IL-6, and stem cell factor (SCF). A member of Dr. Mick Bhatia's lab, Dr. Garcia-Rodriguez, cultured these cells for a total of 21 days (Figure 3.1B). There is an increase in the Lin- and LSK subsets over the first 14 days; however, the Lin- fraction decreased after day 14 while the total cell count continued to increase, suggesting differentiation (Lin+ cells). Consequently, experiments were limited to 14 days to avoid the loss of Lin- cells. Furthermore, this preliminary data was analyzed without the consideration and exclusion of mast cells. The growth factors used in this system, IL-3 (Ihle et al. 1983; Lantz et al. 1998; Razin et al. 1983), IL-6 (Gyotoku et al. 2001), SCF (Galli et al. 1995) are well-established in their ability to promote the growth of mast cells. Not only would this result in the presence of contaminating mast cells in the *in vitro* system, but it would also make it difficult to distinguish LSK cells from unwanted mast cells as they are phenotypically similar i.e., they both

express c-Kit and Sca-1 on their surfaces (Drew et al. 2002; Haddon et al. 2009). Therefore, in order to make this distinction, the constitutively expressed mast cell receptor, FcεR1α, was measured and excluded in subsequent experiments (Shin and Greer 2015; Wodnar-Filipowicz, Heusser, and Moroni 1989). A large discrepancy can be seen in the number of LSK cells when mast cells were not excluded (Figure 3.1C) vs when they were (Figure 3.1D). In the former, the growth of unwanted mast cells contributes to the LSK population and therefore must be considered when phenotyping for LSK cells.

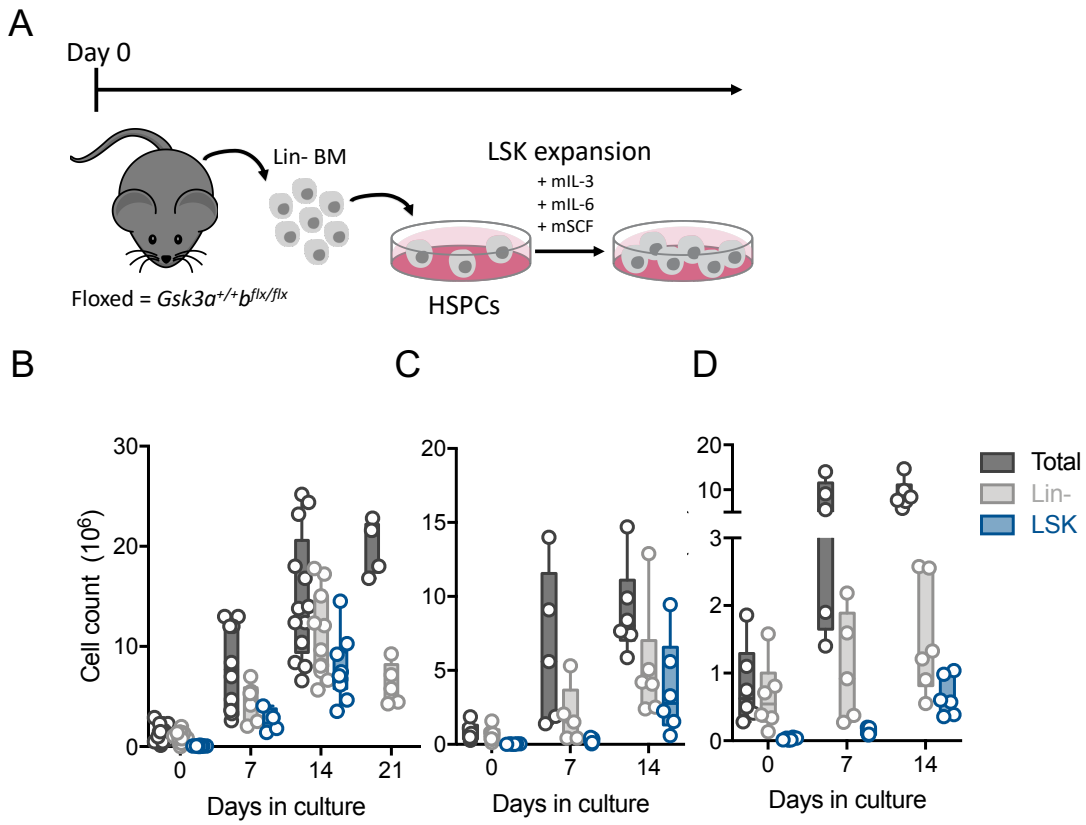

Figure 3.1: *In vitro* expansion of LSKs. (A) Schematic of bone marrow (BM) cells harvested from *Gsk3a*$^{+/+}$*b*$^{flx/flx}$ Bl6/SJL mice, lineage depleted, and cultured in StemSpan media supplemented with growth factors promoting LSK expansion over 14 days. Total, lineage negative (Lin-), and LSK cell counts in (B) preliminary data conducted by Dr. Garcia-Rodriguez with mast cells not accounted for, (C) data produced containing mast cells, and (D) with mast cells gated out. Each dot represents one biological replicate.

3.1.2 Optimization of GSK3β-knockout

Using the inducible Cre-*loxP* system as previously described in *Section 1.4.2,* the optimal tamoxifen concentration was found to be 5 μM as this resulted in sufficient knockout of *Gsk3b* mRNA expression (Figure 3.2A). *Gsk3b* knockout was sustained when tamoxifen was used continuously for a minimum of 48 hours (Figure 3.2B) but resulted in a reduced cell count (Figure 3.2C). At the protein level, it was also shown that GSK3β levels were decreased, with very little residual expression indicative of a homozygous knockout, i.e., deletion of both alleles (Figure 3.2D). A heterozygous knockout, i.e., deletion of one allele, would produce about half residual protein expression, which is not seen here.

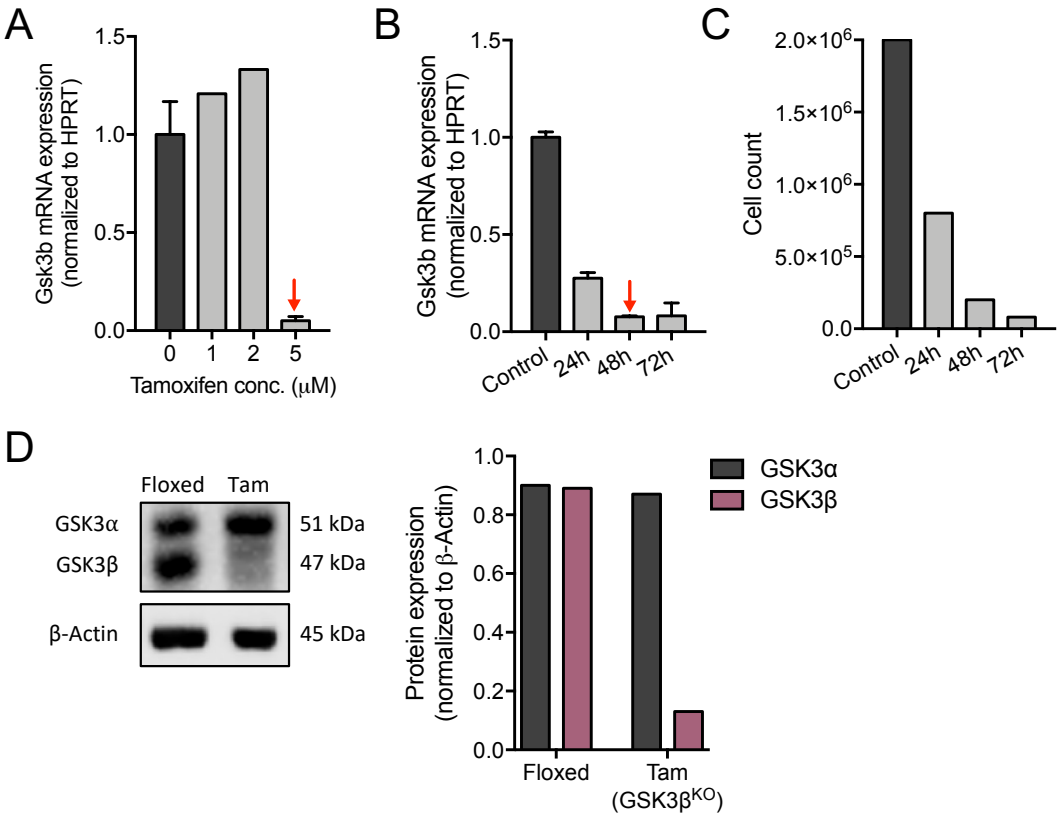

Figure 3.2: Optimization of tamoxifen dosage and duration for GSK3β knockout in floxed BM mononuclear cells. (A) Tamoxifen concentration of 5 μM results in sufficient knockout of *Gsk3b* expression, as measured by qPCR. (B) Duration of tamoxifen concentration at 5 μM. A minimum of 48h of continual tamoxifen treatment is required to maintain stable *Gsk3b* knockout. (C) Cell counts measured 7 days post-tamoxifen treatment. (D) Western blot of GSK3α and GSK3β protein levels (*left*) and densitometry of expression, normalized to β-actin (*right*).

3.1.3 In vitro GSK3β-knockout cells reproduce disease features in mice

The workflow used to generate *in vitro* GSK3β knockout ($GSK3\beta^{KO}$) cells is shown in Figure 3.3A. Cells were treated with tamoxifen at day 7, at which time there it was previously shown that the Lin- subset is enriched. At day 8, $GSK3\beta^{KO}$ cells were transplanted into NOD/SCID mice. The cells were treated with tamoxifen for 24 hours, instead of 48 hours, to avoid further reduction of cell count at the time of the transplantation to promote higher level of engraftment from donor cells. The results from the transplantation (Figure 3.3B) reveal that *in vitro* generated $GSK3\beta^{KO}$ cells were capable of reproducing disease features *in vivo* (higher blast cell percentage), consistent with our group's previous reports in the *in vivo* system (Guezguez et al. 2016). These *in vitro* generated $GSK3\beta^{KO}$ cells are referred to as MDS initiating cells (MDS-ICs) in the *in vivo* system and herein because they have been proven through transplantation to possess disease initiating capacity.

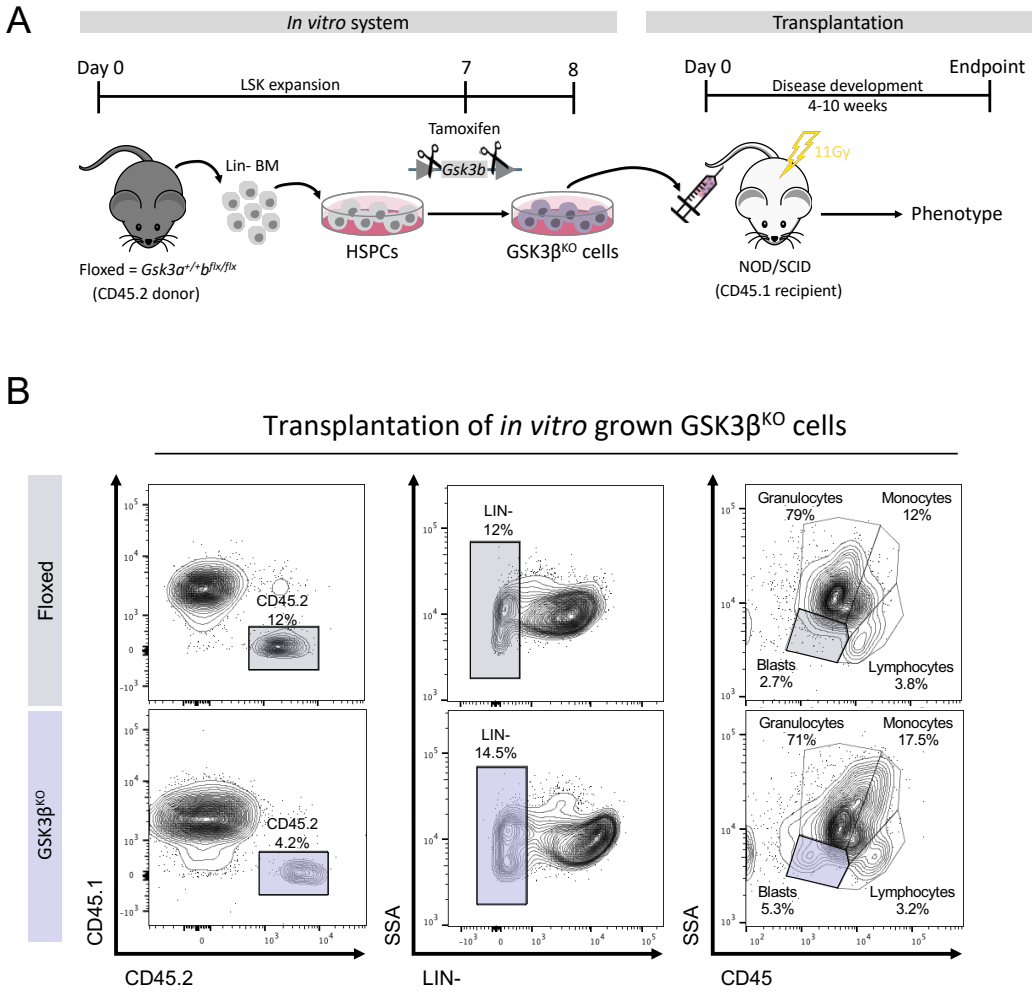

Figure 3.3: GSK3β^{KO} cells generated *in vitro* reproduce disease features *in vivo*. (A) Experimental timeline of GSK3β^{KO} cell production using the *in vitro* system *(left)* and transplantation into NOD/SCID recipient mice (*right*). *In vitro* grown Lin- BM cells were treated with tamoxifen (5 μM) at day 7 for 24 hours. Day 7 was chosen for administration of tamoxifen because by this time there is an enrichment of LSK cells relative to day 0. At day 8, GSK3β^{KO} cells were administered into NOD/SCID mice via tail vein injection. (B) Flow cytometric analysis of engraftment, Lin-, and blast fractions.

3.2 Phenotypic and functional observations of how the disease propagates *in vitro*

3.2.1 LSK gating strategy

An increase of the mast cell fraction was seen in both untreated (floxed) and tamoxifen treated (GSK3β^{KO}) cell conditions (Figure 3.4A). Representative flow cytometry gating strategy of floxed and GSK3β^{KO} cells is shown in Figure 3.4B and C, respectively.

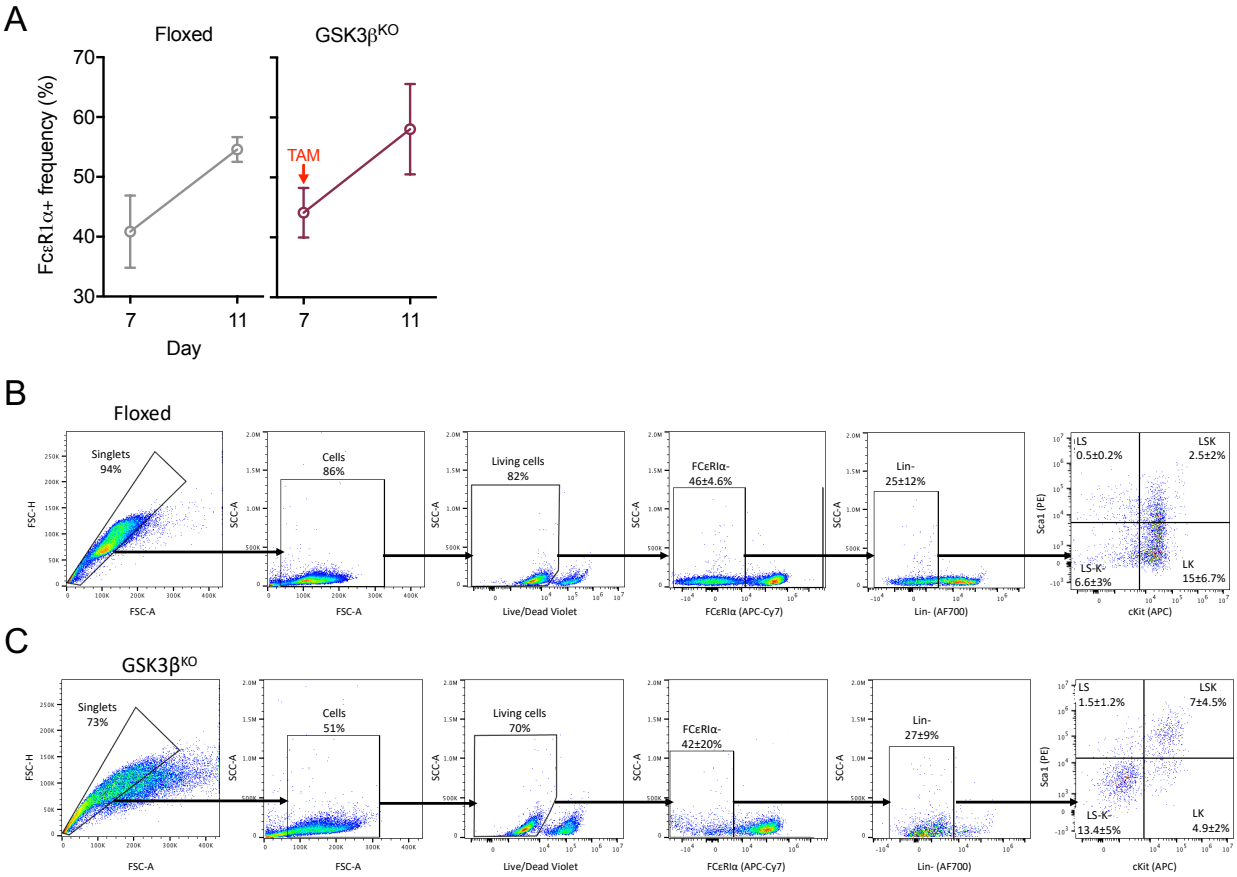

Figure 3.4: Mast cell marker, FcεR1α, must be considered in LSK gating strategy. (A) Frequency of FcεR1α+ (marker for mast cells) in floxed and GSK3β^{KO} overtime. LSK gating strategy used for (B) floxed and (C) GSK3β^{KO} cells. Mean±S.D. N=4, n=1-2 for floxed cells and N=6, n=1-3 for GSK3β^{KO} cells.

3.2.1 Phenotypic difference in floxed vs GSK3β^{KO} cells

A marked reduction in cell count can be observed following tamoxifen treatment in GSK3β^{KO} cells (Figure 3.5A); however, while the Lin- subset was reduced, there was a slight expansion of the LSK subset as shown in Figure 3.5B. Interestingly, GSK3β^{KO} cells also exhibit a slight increase of granulocytes, defined as CD11b+/Gr1+, and a more substantial increase of monocytes, defined as CD11b+/Gr1-, (An et al. 2018) (Figure 3.5C and D) compared to the floxed cells.

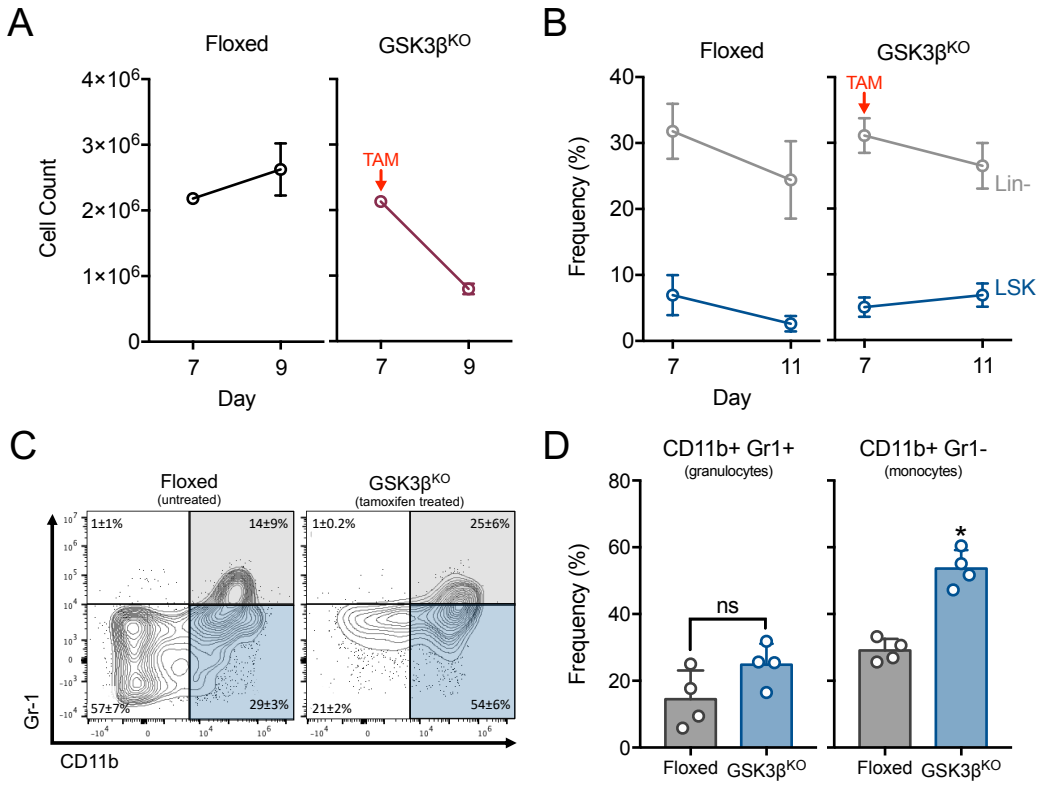

Figure 3.5: Tamoxifen treatment results in total cell count reduction and enrichment of LSK cells and monocytes. (A) Cell counts after tamoxifen treatment. (B) Frequency of Lin- and LSK cells measured at day 7 and day 11. Data is represented as mean ± SEM. N=4, n=1-2 for floxed cells and N=6, n=1-3 for GSK3β^{KO} cells. (C) Flow cytometric analysis of CD11b and Gr1 expression, markers of myeloid differentiation, measured in floxed and GSK3B^{KO} cells at endpoint. Granulocytes (CD11b+Gr1+) and monocytes (CD11b+Gr1-). N=2, n=2 Mean±S.D. (D) Bar graphs representing flow cytometric analysis from (C). Two-tailed t-test, *p=0.0003.

3.2.2 GSK3β^{KO} cells are functionally and morphologically distinct from floxed cells

Interestingly, the GSK3β^{KO} cells did not form CFUs when plated in methylcellulose (Figure 3.6A), whereas floxed cells retained this ability in their functioning progenitors (Figure 3.6B). Also, differences in morphology between floxed and GSK3β^{KO} were noted as shown by representative Wright-Giemsa images in Figure 3.6C. Floxed cells contained healthy looking primitive and some mature cells while the GSK3β^{KO} cells appeared abnormal, granular, and relatively larger.

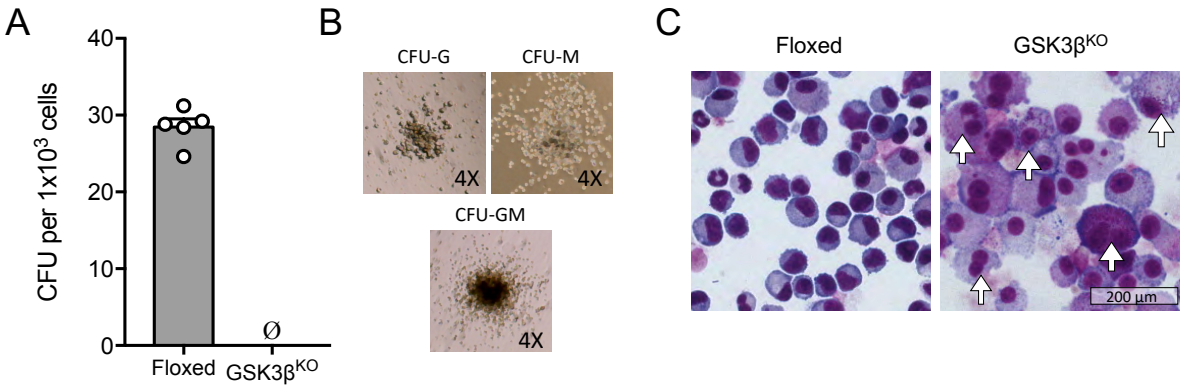

Figure 3.6: GSK3β^{KO} cells are functionally and morphologically distinct from floxed cells. (A) GSK3β^{KO} cells do not produce hematopoietic colonies (CFUs). Data is represented as mean±SEM. N=4, n=1-2 for floxed cells and N=6, n=1-3 for GSK3β^{KO} cells, Ø is zero. (B) Representative images of CFU-types produced by healthy floxed progenitor cells taken 14 days post-plating. (C) Representative images of fixed cells stained with Wright-Giemsa. GSK3β^{KO} cells appear larger in size and many are binucleated and granular, as indicated by the white arrows.

3.3 There are no consistent mutations in the *in vivo* GSK3 system

In order to better understand the pathogenesis of disease in the GSK3 deletion model, whole exome sequencing (WES) analysis from mice transplanted with floxed cells was compared to those transplanted with GSK3β^{KO} cells to uncover potential driver mutations. Specifically, only the LSK cells were considered for genomic analysis. The workflow used to transplant BM cells from floxed mice into congenic recipients for subsequent disease initiation and genomic analysis is shown in Figure 3.7A. Results of the transplantation (Figure 3.7B) revealed greater Lin- and blast cell populations in mice that received MDS-ICs (GSK3β^{KO} cells), consistent with our group's previously published work (Guezguez et al. 2016). Furthermore, whole exome sequencing analysis revealed that although variants were found, there was a lack of common acquired variants among the diseased mice (Table 3.1).

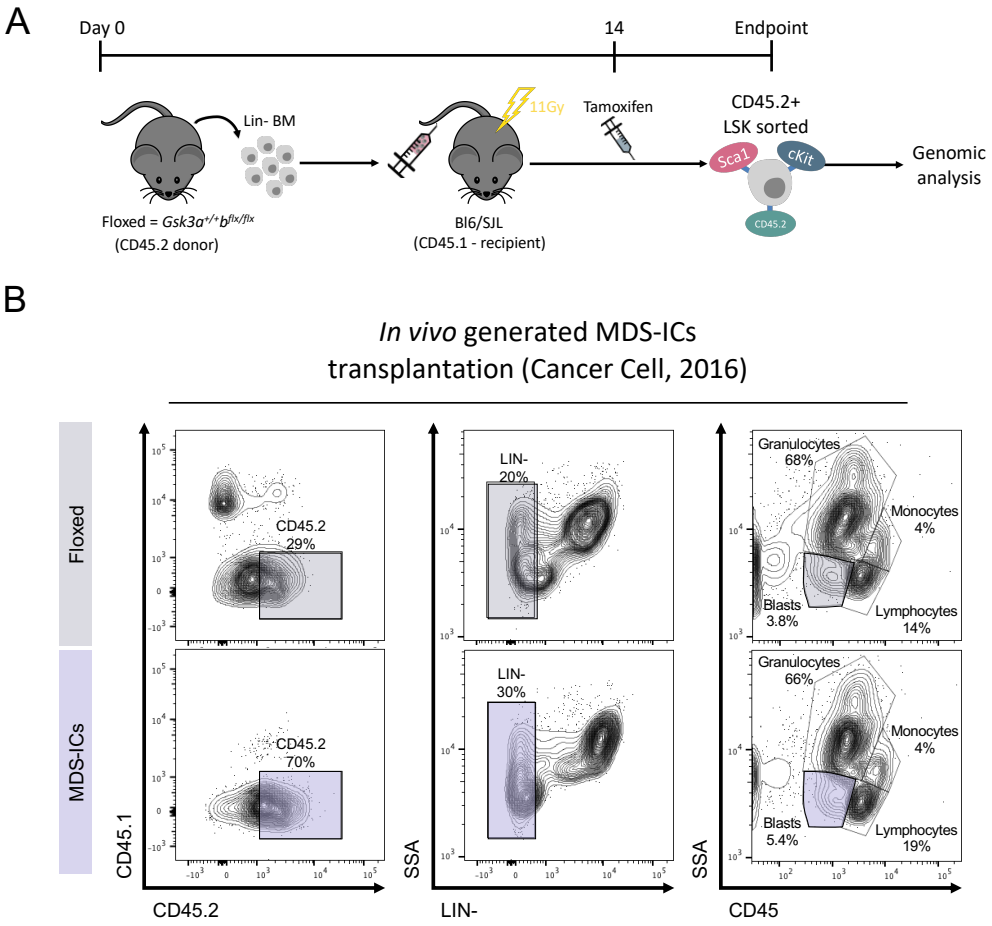

Figure: 3.7 *In vivo* generated MDS-ICs (GSK3β^{KO}) result in MDS-phenotype. (A) Experimental design of *in vivo* generation of MDS-ICs (GSK3β^{KO} cells) following the Cancer Cell (2016) method. MDS-ICs were administered into congenic recipients via tail vein injection and tamoxifen was administered via IP injection. At endpoint, mice were sacrificed, and BM cells were harvested and sorted for LSK cells which were used for whole exome sequencing (WES) analysis. (B) Flow cytometric analysis of engraftment, Lin-, and blast fractions.

Table 3.1: Lack of common single nucleotide variants found in donor cells from mice transplanted with MDS-ICs (GSK3β^{KO} cells). Whole exome sequencing (WES) analysis of donor (CD45.2+) sorted LSK cells from *in vivo* generated MDS-ICs transplanted in Bl6/SJL mice was performed (N=4). Bl6/SJL mice were used to stay consistent with the Cancer Cell method. SnpEff functional tool for variant annotation was used to predict putative high impact variants (thought to cause deleterious gene effects). The data in this table was generated using floxed mice as the reference.

Gene	Gene name	Type of variant	Detected in
Hspa14	Heat shock protein 14	splice_donor_variant&intron_variant,upstream_gene_variant, non_coding_transcript_exon_variant	GSK3β^{KO} mouse 1
Kmt2d	Histone methyltransferase 2D	stop_gained	GSK3β^{KO} mouse 2
Ptpdc1	Protein tyrosine phosphatase domain containing 1	frameshift_variant	GSK3β^{KO} mouse 3
Pacs1	Phosphofurin acidic cluster sorting protein 1	splice_donor_variant&intron_variant	GSK3β^{KO} mouse 3
Tspyl3	TSPY-like 3	frameshift_variant, downstream_gene_variant	GSK3β^{KO} mouse 4
Hsf3	Heat shock transcription factor 3;	splice_donor_variant&intron_variant	GSK3β^{KO} mouse 4
Pan2	PAN2 polyA specific ribonuclease subunit homolog	stop_gained	GSK3β^{KO} mouse 4

3.4 Re-gain of function by re-expressing GSK3β in GSK3β^{KO} cells

Since there were no candidate driver mutations identified from the WES analysis, it was of interest to determine whether the re-introduction of GSK3β in GSK3β^{KO} cells could reverse or "rescue" the disease phenotype.

3.4.1 Transduction efficiency strategy

During the optimization phase, polybrene and RetroNectin were both tested for their ability to facilitate efficient transduction. RetroNectin is often the preferred agent for improving transduction efficiency as it is less toxic to the cells compared to polybrene in some cases (Pay et al. 2018). Figure 3.8A shows the workflow used for transduction with RetroNectin-coated plates, as previously described (Millington et al. 2009). Cells were transduced with lentivirus expressing eGFP (empty-IRES-eGFP; eGFP^{lenti}) or GSK3β (*Gsk3b*-IRES-eGFP; GSK3β^{lenti}). The use of RetroNectin provided relatively higher and more consistent transduction efficiency (Figure 3.8B).

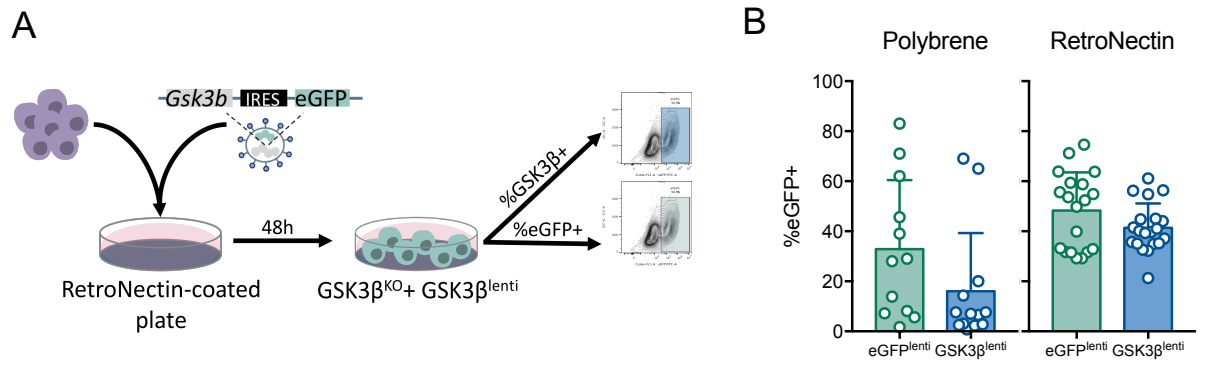

Figure 3.8: RetroNectin reagent allows higher and more consistent lentiviral transduction efficiency. (A) Workflow of *in vitro* lentiviral transduction using RetroNectin-coated plates. GSK3β^{KO} cells were treated with lentivirus (MOI=9) expressing GSK3β (GSK3β^{lenti}) for 48h on retronectin-coated plates (5 μg/cm^2) via spinoculation (600 g for 1 h). (B) Comparison of lentiviral transduction efficiency using polybrene (N=8, n=1-2) vs RetroNectin (N=8, n=1-3).

3.4.2 GSK3β-knockout cells massively overexpress GSK3β

Re-introduction of GSK3β into GSK3β^{KO} cells and GSK3β deletion was measured by GSK3β and eGFP expression (Figure 3.9). Intracellular flow cytometry results showed that the GSK3β expression levels in transduced GSK3β^{KO} cells were about 3-fold greater than that in the floxed controls (Figure 3.9A, *right*). Nearly 50% of the total LSK population in GSK3β^{KO} cells were successful in re-expressing GSK3β (Figure 3.9D). However, a large proportion of total transduction occurred in the mast cells; on average 66% of total mast cells re-expressed GSK3β (Figure 3.9D).

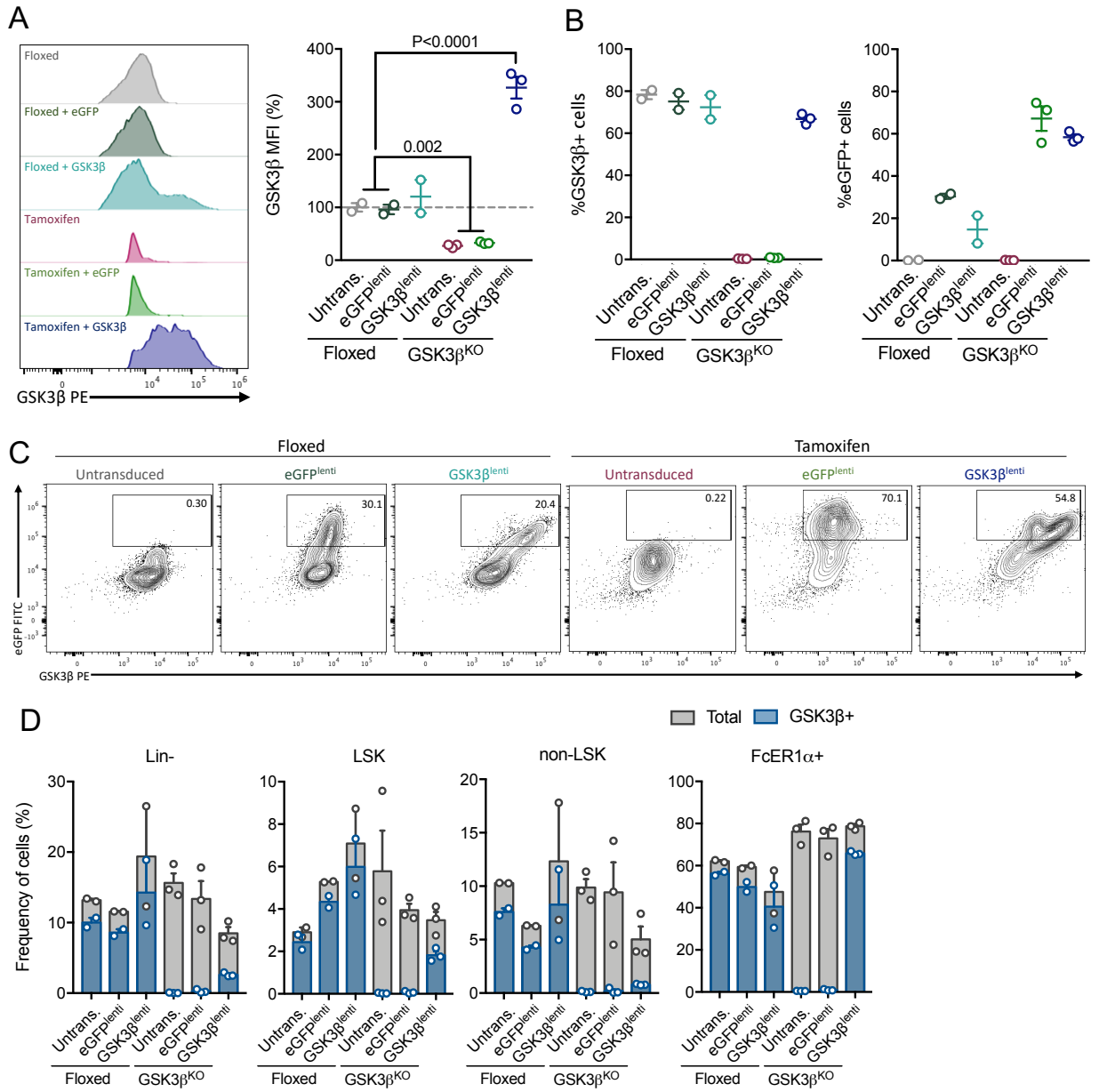

Figure 3.9: Quantification of GSK3β by intracellular flow cytometry confirms re-expression in LSK cells. (A) Flow cytometry plots (*left*) and mean fluorescence intensity (MFI) of GSK3β expression (*right*) in total live BM MNCs. MFI is relative to mean of untransduced floxed cells (100%). Each dot is one biological replicate. One-way ANOVA with Tukey's multiple comparisons test. (B) %GSK3β+ (*left*) and %eGFP+ (*right*) of total living cells assessed by flow cytometry. (C) Flow cytometry plots of eGFP and GSK3β co-expression. (D) GSK3β expression measured via intracellular flow cytometry.

3.4.3 Timeline for overexpressing GSK3β in the in vitro system

Figure 3.10 outlines the timeline used for the re-introduction of GSK3β in the *in vitro* system. Day 9 was chosen for lentiviral transduction because by this time the tamoxifen treatment would have successfully resulted in sufficient knockdown of GSK3β. Day 11 was chosen as an endpoint directly following the 48h incubation with lentivirus.

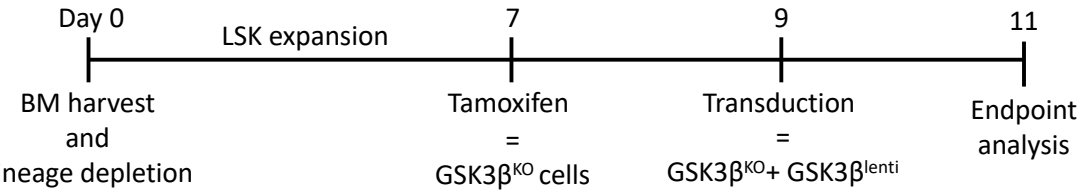

Figure 3.10: Simplified experimental timeline of GSK3β re-expression in GSK3β^{KO} cells. *In vitro* generated GSK3β^{KO} cells were transduced with lentivirus expressing GSK3β on day 9. Day 9 was chosen for lentiviral transduction because by this time the tamoxifen treatment would have successfully resulted in sufficient knockdown of GSK3β. Day 11 was chosen as an endpoint directly following the 48h incubation with lentivirus.

3.4.4 GSK3β^{KO} cells overexpressing GSK3β show no signs of disease rescue

While lentiviral transduction alone did not result in cell death (Figure 3.11A), the morphological assessment revealed a toxic response in the transduced floxed cells, as judged by their reactive appearance, which is further exacerbated when combined with tamoxifen treatment in the transduced GSK3β^{KO} cells (Figure 3.11B). The re-expression of GSK3β in GSK3β^{KO} cells did not result in healthy looking cells, as compared to the untransduced floxed cells.

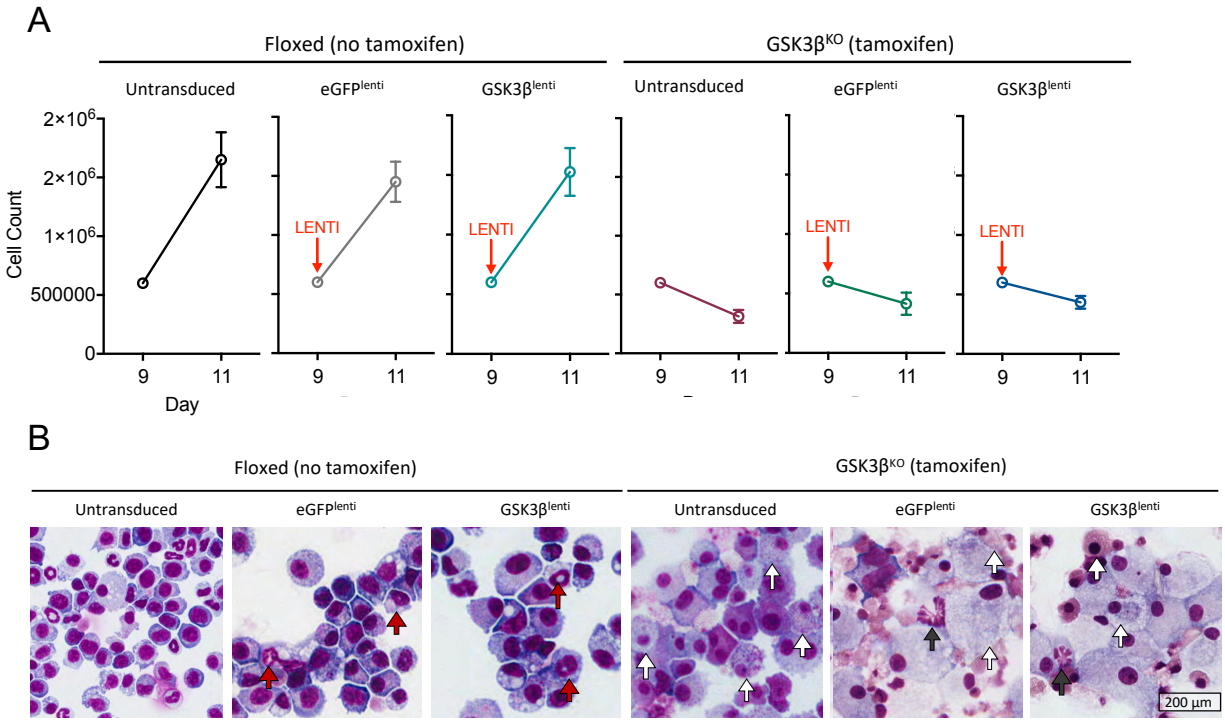

Figure 3.11: Combination of tamoxifen treatment and lentiviral transduction leads to cellular toxicity. (A) Cell counts of floxed cells (no tamoxifen) and GSK3β^{KO} cells (tamoxifen-treated) following lentiviral transduction. Data is represented as mean ± SEM. N=4, n=1-2 for floxed cells and N=6, n=1-3 for GSK3β^{KO} cells. (B) Representative images of fixed cells stained with Wright-Giemsa. Cells treated with tamoxifen appear granular and reactive due to the stress of the transduction, indicated by the white arrows. Hypergranular and hypersegmented neutrophils are indicated by the red and grey arrows, respectively.

Consistent with data previously reported in Figure 3.9, Figure 3.12 shows that about 50% of the total LSK cells in culture are efficiently transduced and a large proportion of total transduction occurs in the mast cells. GSK3β^{KO} cells treated with lentivirus, either eGFP^{lenti} or GSK3β^{lenti}, have a relatively higher granulocyte population. There are no differences between GSK3β^{KO} cells treated with eGFP^{lenti} or GSK3β^{lenti}, suggesting that the re-expression of GSK3β is insufficient for rescue of disease phenotype. Interestingly, a greater granulocyte fraction is observed in transduced GSK3β^{KO} cells compared to all other conditions. Table 3.2 displays representative total cell populations to supplement Figure 3.12.

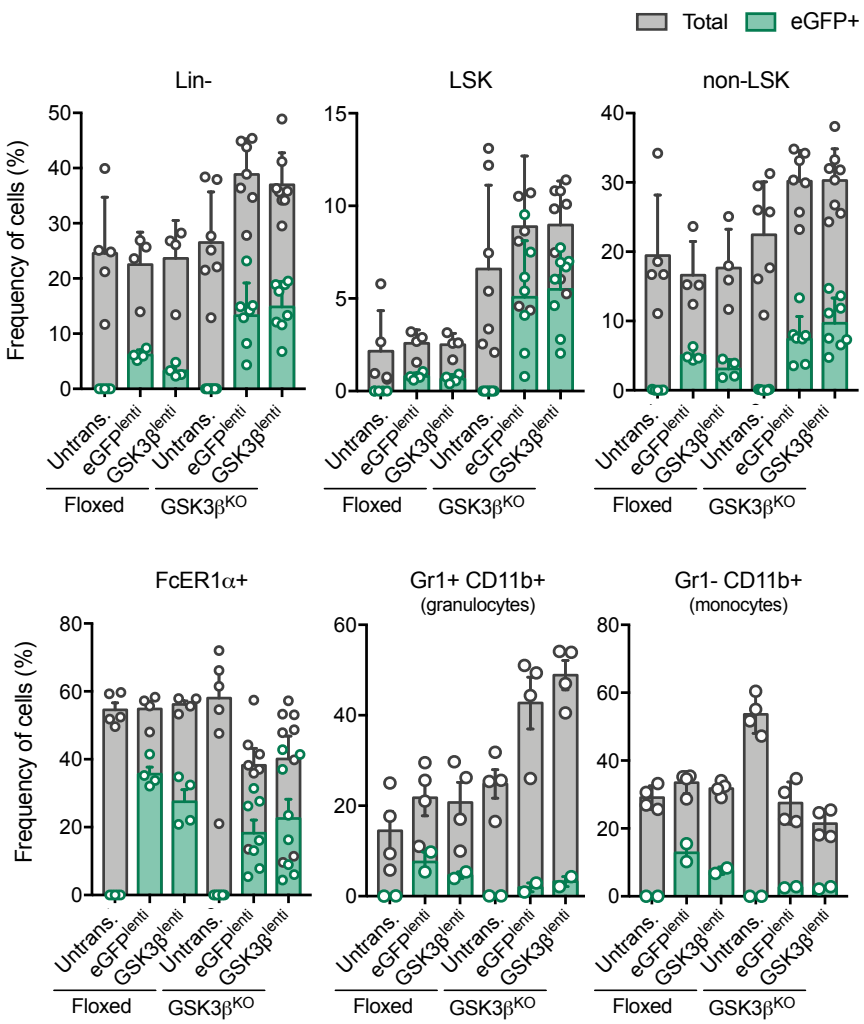

Figure 3.12: Re-expression of GSK3β in GSK3β^{KO} cells does not rescue disease phenotype. Flow cytometry measurement of total and eGFP+ cells of several phenotypes measured at endpoint, day 11. Data is represented as mean ± SEM. Floxed cells (N=4, n=1-2) and GSK3β^{KO} cells (N=6, n=1-3).

Table 3.2: Representative total cell populations

Condition	FcεR1α+	FcεR1α-	Lin-	LSK	LS	LK	LS-K-	Gr1+CD11b+	Gr1-CD11b+
Floxed (untransduced)	52	48	21	3	0	10	8	18	31
Floxed + eGFP^{lenti}	48	52	14	2	0	6	6	26	35
Floxed + GSK3β^{lenti}	53	47	13	2	0	5	6	26	34
GSK3β^{KO} (untransduced)	55	45	25	7	1	3	13	26	52
GSK3β^{KO} + eGFP^{lenti}	37	63	36	11	1	14	11	51	22
GSK3β^{KO} + GSK3β^{lenti}	40	60	36	10	1	16	9	54	18

Data is represented as the total percentage of cell populations, as assessed via flow cytometry.

Furthermore, GSK3β^{KO} cells re-expressing GSK3β failed to re-constitute progenitor function, as determined by the CFU assay (Figure 3.13).

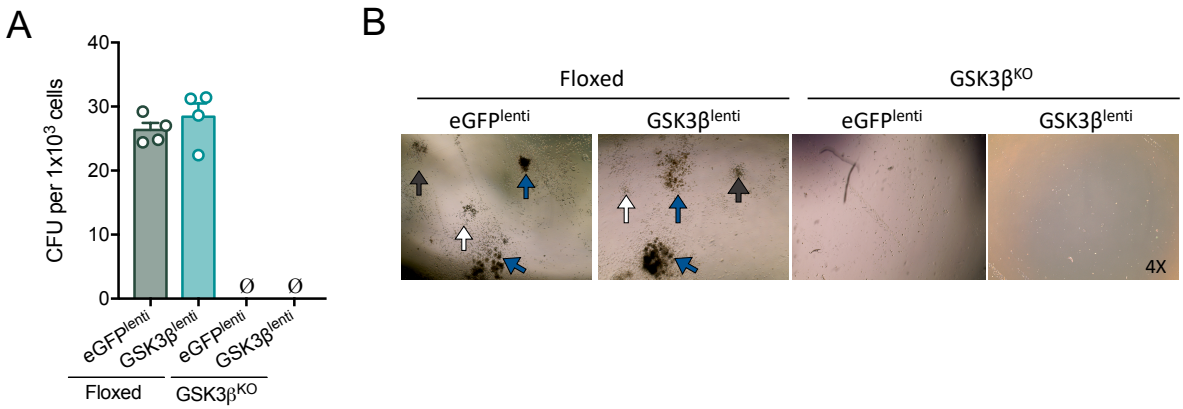

Figure 3.13: Re-expression of GSK3β in GSK3β^{KO} cells does not rescue function. (A) CFUs per 1x10^3 cells. Each dot represents one biological replicate, Ø is zero. (B) Representative images of CFU-types produced by floxed progenitor cells treated with eGFP^{lenti} and GSK3β^{lenti} taken 14 days post-plating. CFU-G, CFU-M, and CFU-GM are indicated by the white, grey, and blue arrows, respectively.

3.5 Rescue of disease phenotype is difficult to detect *in vitro* because the hematopoietic repopulating stem cell frequency is rare

The repopulating stem cell frequency is rare, estimated to be 1 in 230,697 and 1 in 257,697 for floxed and MDS-ICs, respectively (Figure 3.14). These cells are the ones capable of engraftment and as such would be the ones that initiate disease in mice, i.e., MDS-ICs. *In vitro*, while there are many LSK cells, it is difficult to specifically study the cells capable of engraftment directly because of their rare frequency; therefore, *in vivo* repopulation assay would provide insight into whether disease phenotype can be rescued or at least be ameliorated by the re-introduction of GSK3β.

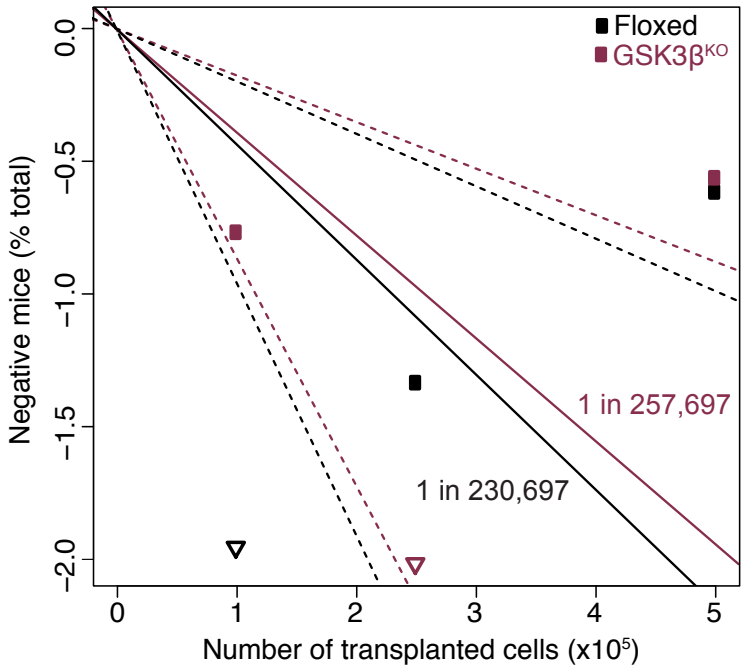

Figure 3.14: Hematopoietic repopulating stem cell frequency is rare. Log-fraction plot of a limiting-dilution assay from transplantation of 3 different bone marrow doses of *in vitro* grown floxed and MDS-ICs cells into NOD/SCID mice (N=4 per group, P=0.83,T-test). The frequency of hematopoietic repopulating stem cells (1 in 230,697 and 1 in 257,697 for floxed and MDS-ICs, respectively) was estimated by plotting the number of transplanted cells by the percentage of mice that were unsuccessfully engrafted. The slope of the line represents the cell frequency, and the 95% confidence interval is displayed by the dotted lines.

4.0 Discussion

The overarching goal of this study was to show the potential use of an *in vitro* surrogate system that mimics MDS-to-AML transition in human patients to perform causal experiments. This model offers a unique opportunity to investigate the pathogenesis by direct manipulation of the cells proven to cause disease. The long-term goal is to use this model to move the field forward by identifying tangible targets that have therapeutic intervention or predictive biomarker potential.

The translation of the *in vivo* GSK3 mouse model to an *in vitro* system allows us the opportunity to expand the rare LSK population responsible for initiating an MDS-like phenotype upon induced genetic mutation of GSK3β. This *in vitro* GSK3 model is unique because it offers a way to perform in-depth analysis on the disease initiating cells that otherwise cannot be practically done in an *in vivo* system. The first part of my hypothesis was that this *in vitro* system can act as a surrogate of MDS disease state initiation. Thus, my first aim was to generate GSK3β-knockout cells in culture with MDS-IC properties. The GSK3β^{KO} cells generated using the *in vitro* system (Figure 3.3A) did indeed reproduce disease features when transplanted *in vivo* (Figure 3.3B). This is significant as it provides confirmation that the pathogenesis of disease is consistent in both the *in vivo* and *in vitro* models. In just 14 days, an expansion of LSK cells is achieved (Figure 3.1B) and is more enriched in GSK3β^{KO} cells compared to floxed cells (Figure 3.5B), which is as expected due to the inability of these cells to differentiate, and was also observed in the *in vivo* system (Guezguez et al. 2016).

The *in vitro* system presented here has brought to light specific phenotypes that could be the culprits supporting early transformation. We show that there is an accumulation of monocytes (CD11b+/Gr1-) and a slight increase of granulocytes (CD11b+/Gr1+) in GSK3β^{KO} cells (Figure 3.5C and D), consistent with what other MDS mouse models have reported, and the latter have

been previously referred to as myeloid-derived suppressor cells (MDSCs) (An et al. 2018; Chen et al. 2013). MDSCs are thought to cause an inflammatory response, contributing to the MDS phenotype.

Interestingly, GSK3β^{KO} cells lost their ability to form CFUs (Figure 3.6A), which was not the case in the *in vivo* system where a significant increase in CFUs was observed (Guezguez et al. 2016). Previously, it was shown that inhibition of GSK3 via RNA interference activated Wnt signaling and resulted in increased proliferation and self-renewal of hematopoietic progenitor cells by 4-fold (Huang et al. 2009). Morphologically, the GSK3β^{KO} cells are distinct from the healthy, floxed cells (Figure 3.6C).

The finding that no consistent mutation was detected in the WES analysis of GSK3β^{KO} mice (Figure 3.7 and Table 3.1) suggests that there may be an epigenetic basis of disease at play in this system. Thus, this formed the basis for the second part of my hypothesis, which was that further manipulation of the genome beyond GSK3 drives disease onset from normal HSCs. Complete rescue by restoring the genetic flaw would suggest that this model of disease is genetically driven; however, if the deletion of GSK3β creates an epigenetic signature responsible for driving disease, then hypothetically, it cannot be restored simply by overexpressing GSK3β. Therefore, my second aim of this thesis was to achieve temporal re-expression of GSK3β in the GSK3β^{KO} cells by lentiviral transduction. Overall, approximately 60% of total cells were successfully transduced, of which were shown to expression GSK3β about 3-fold higher than the basal level in the untransduced floxed cells (Figure 3.9). While this expression is ectopic and does not represent normal conditions, it serves to answer the question of whether a reversion of disease phenotype is achievable. However, the combined treatment of tamoxifen and lentivirus resulted in cellular toxicity (Figure 3.11B) and an increase of granulocytes (Figure 3.12) suggesting an

inflammatory response. This was observed in GSK3β^{KO} cells treated with either eGFP^{lenti} or GSK3β^{lenti}. The cellular toxicity brought on by tamoxifen could be due to the relatively high dosage used (5μM). Previously, tamoxifen dose of 1μM was not shown to elicit adverse outcomes *in vitro* (Abukhdeir et al. 2008); however, a higher dosage of 10μM in mouse BM Lin- cells *in vitro* caused a pro-apoptotic effect on HSPCs (Sánchez-Aguilera et al. 2014). Therefore, further optimization of the tamoxifen dosage and duration may be required to mitigate the adverse effects.

My next aim was to assess the properties of MDS vs. healthy restoration by measuring hematopoietic progenitors. Despite the slight increase in progenitors (non-LSKs; Figure 3.12), overexpression of GSK3β failed to re-constitute functionality (Figure 3.13). Overall, the results of the work presented in thesis show that there are no identifiable differences between GSK3β^{KO} cells treated with eGFP^{lenti} or GSK3β^{lenti}, suggesting that either the re-expression of GSK3β is insufficient for rescue of disease phenotype or simply that any changes cannot be appreciated due to low frequency of initiating cells in culture. Additionally, the toxicity induced by tamoxifen and lentivirus treatment is likely hampering our ability to assess these cells. Therefore, to focus only on the initiating cells, *in vivo* repopulation assays should be performed. Indeed, the hematopoietic repopulating stem cell frequency from *in vitro* grown MDS-ICs was proven to be rare (Figure 3.5); therefore, if the overexpression of GSK3β truly had an effect, it is nearly impossible to capture this using the *in vitro* system alone. That is not to say that the *in vitro* system should not be used; on the contrary, the *in vitro* system is invaluable for its ability to expand and manipulate cells of interest but requires further validation through *in vivo* transplantation assays.

It was also of interest to observe the outcome of overexpressing GSK3β prior to the knockout, as this may indicate whether disease onset could be prevented. This experiment was performed with two biological replicates; however, healthy progenitor function, as determined by

the CFU assay, was not retained after inducing GSK3β knockout (data not shown). Further investigation is required to determine whether the tamoxifen treatment is suppressing the ability of the GSK3β knockout cells to form CFUs. Additionally, in parallel to this work, our group has conducted methylome and transcriptome analysis (data not shown) which has revealed a few candidate genes (e.g., CLEC11A, AHNAK, TMEM109) that correlate with MDS patient survival. However, the relevance in disease initiation and progression of these genes is unknown and difficult to study given that MDS cells from patients do not engraft in immunodeficient mice and there are limited, relevant MDS transition models. Therefore, the system presented in this thesis serves as a surrogate that mimics MDS-to-AML transition in human patients and can be used to perform these causal experiments *in vitro*, and perhaps be assessed for effects on MDS-ICs and HSCs as well if transplanted into recipients after genetic intervention of these candidate genes. Specifically, unlike most other models, this *in vitro* model is unique in that the MDS and AML disease states can be studied as separate entities, allowing for the opportunity to define biomarkers associated with initiation vs progression of disease. Taking it one step further, with the biomarkers identified, the *in vitro* system can then be used to measure response to drugs in a high-throughput manner. This will shed light on the molecular basis of non-responders and resistant clones and could eventually help improve patients' response to treatment with newly tested drug targets.

5.0 Conclusion

In conclusion, the use of GSK3 knockout HSCs in the *in vitro* model can act as a surrogate of MDS disease state initiation and AML progression. This unique model serves a platform to explore causal genetic and epigenetic factors that govern disease progression. Here, for the first aim, we demonstrated that *in vitro* generated GSK3β^{KO} cells were capable of reproducing MDS-ICs properties with a clear distinction from healthy, floxed cells. For the second aim, we were able to achieve temporal massive over-expression of GSK3β in the GSK3β^{KO} cells by lentivirus. This demonstrates the potential use of this system, which can be easily manipulated to perform causal experiments for candidate genes identified, such as those revealed in recent methylome and gene expression analysis by Dr. Garcia in our lab (e.g., CLEC11A, AHNAK, TMEM109). The final aim was to assess properties of MDS vs. healthy restoration by measuring hematopoietic progenitors. However, we were unable to definitively conclude whether any degree of rescue to normal phenotype occurred due to the rare hematopoietic repopulating stem cell frequency. The next step would be to transplant the GSK3β^{KO} cells overexpressing GSK3β into immunodeficient mice and assess the phenotype and morphology properties of engrafting cells to determine if HSC biology is restored upon GSK3β overexpression in GSK3β^{KO} cells derived from *in vitro* cultures.

5.0 Bibliography

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