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ARAM V. CHOBANIAN & EDWARD AVEDISIAN SCHOOL OF MEDICINE

Thesis

MATURING HEMATOPOIETIC PROGENITORS DERIVED FROM IPSCS TO OPTIMIZE HUMAN MODELS OF MDS

by

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B.S., American University, 2021

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2023

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There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after."

J.R.R. Tolkien, The Hobbit

DEDICATION

I would like to dedicate this work to my patient husband, Noah, my two dogs, Milo and Felix, and all family that supported me throughout this process. I would also like to dedicate this to my grandfather, John E. Ultmann, who set the groundwork for my curiosity regarding hematology and oncology from a very young age, and my grandmother Ruth Ultmann for constantly reminding me that he would have been proud.

ACKNOWLEDGMENTS

Noah: Thank you for being the most patient, loving, generous husband I could have ever asked for. Thank you for doing the dishes, laundry, and house chores over the past two years so I could focus on academics. Thanks for putting up with my migraines, stomach aches, backaches, etc., and always knowing the solution is that I'm most likely dehydrated, hungry, or just need a good walk outside with you and the dogs.

Dr. Offner: Thank you for the unconditional and unwavering support you have given me throughout MAMS. Thank you for not letting me give up, even though I begged you. Thank you for understanding my love of short and long dogs, Rothy's, and science.

Dr. Rowe (Grant): Thank you for your willingness to hire me as your lab manager and research assistant, teach me about stem cell biology and blood disorders, and answer all my questions even when they probably don't make sense. Thank you for reminding me that failure is not the worst thing to happen and, in some cases, the best way to learn and improve. Thank you for being my mentor and someone I can look to for advice.

The Rowe Lab: Thank you for teaching me new skills and techniques in the lab, always being able to help me with math (especially dilution calculations), and for always being willing to problem solve whenever I have hit a roadblock. I look forward to another year with you all!

Brianna, Seli, Ben, and Priscilla: Thank you for your unwavering support over the last two years. Thank you for all the laughs, sugar, and coffee. I am so lucky to have you all in my life.

MATURING HEMATOPOIETIC PROGENITORS DERIVED FROM IPSCS TO OPTIMIZE HUMAN MODELS OF MDS

SARA ROSE ULTMANN FIERSTEIN

ABSTRACT

Myelodysplastic syndromes (MDS) encompass a heterogeneous group of age-related hematopoietic disorders characterized by ineffective and incomplete hematopoiesis leading to an increased risk of acute myeloid leukemia (AML). The development of accurate and easily used in vitro models is crucial for understanding the pathogenesis of MDS and identifying potential therapeutic targets. Induced pluripotent stem cells (iPSCs) can be used to study MDS due to their ability to differentiate into any cell type depending on the environment. The main limitation is that the blood progenitors produced by iPSCs are of a fetal state, which hinders modeling of MDS, a disease of older adulthood. This study aimed to optimize the maturation state of blood progenitors derived from iPSCs by induction of the micro-RNA let-7, which, we hypothesize will increase the maturation and adult phenotypic state of hematopoietic progenitors.

iPSC lines were generated from healthy controls and samples containing the SRSF2 mutation, a common mutation in MDS, containing a doxycycline-inducible, stabilized let-7 transgene. A stepwise differentiation efficiently drove the iPSCs toward hematopoietic progenitors and, subsequently, other mature lineages. The hematopoietic progenitors were characterized by assessing the expression of specific cell surface markers and functional properties using flow cytometry, colony-forming assays, and multilineage differentiation abilities. These findings demonstrate the potential of using

iPSC engineering to create a novel model for MDS and other age-biased disorders by inducing let-7 expression in iPSCs and, when differentiating them, exposing them to doxycycline to promote an adult cell phenotype. This approach offers a valuable potential tool for elucidating the molecular mechanisms underlying these disorders and exploring potential therapeutic interventions.

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LIST OF ABBREVIATIONS

ALL..Acute Lymphocytic Leukemia

AML..Acute Myelogenous Leukemia

HbA..Adult Hemoglobin

ANOVA..Analysis of Variance

BMM..Bone Marrow Microenvironment

CLL..Chronic Lymphocytic Leukemia

CML..Chronic Myelogenous Leukemia

CFU..Colony-Forming Unit

DOX..Doxycycline

EV..Empty Vector

HbF..Fetal Hemoglobin

HSPCs..Hematopoietic Stem and Progenitor Cells

HPCs..Hematopoietic Progenitor Cells

HSCs..Hematopoietic Stem Cells

iPSCs..induced Pluripotent Stem Cells

LSCs..Leukemia Stem Cells

MPPs..Multipotent Progenitors

MDS..Myelodysplastic Syndrome

q-PCR..quantitative Polymerase Chain Reaction

RBCs..Red Blood Cells

CHAPTER ONE

Introduction

Overview of Adult Hematopoiesis

Hematopoiesis is an ongoing process that occurs throughout an individual's life to ensure that the body has an adequate supply of blood cells to meet daily demands and respond to increased needs caused by injuries or illnesses (Jagannathan-Bogdan & Zon, 2013). This process is tightly regulated by stochastic and instructive mechanisms that work in unison. This chapter provides an overview of the significant discoveries that have contributed to our understanding of the hematopoietic system's function in health and disease.

Adult hematopoiesis is dependent on a limited number of hematopoietic stem cells (HSCs) that possess two critical properties - multipotency and self-renewal (Park & Bejar, 2020). HSCs have the capacity to differentiate into all adult blood cell lineages, and they can uniquely divide to produce new HSC progeny without undergoing differentiation (self-renewal). This process is crucial for human patients' survival and serial transplantation assays in lethally irradiated mouse recipients (Bock et al., 1995; Rieger & Schroeder, 2012). HSCs typically remain in a low metabolic quiescence state to maintain their numbers throughout adulthood. However, they can be activated to proliferate and differentiate into more cells in response to various stresses, such as infections, inflammation, aging, and transplantation of bone marrow (Singh et al., 2020).

The conventional model of adult hematopoiesis involves the differentiation of HSCs into multipotent progenitors (MPPs), which subsequently differentiate into either myeloid or lymphoid lineage-committed progenitors. The microenvironment and expressed cytokines, growth factors, and genes are primary regulators of this process (Park & Bejar, 2020). Once the HSCs differentiate into their respective progenitor cells, they lose their ability to self-renew. However, they can still differentiate into more than one cell type if it corresponds to their newly dedicated lineage.

The recent emergence of single-cell sequencing and lineage tracing techniques has allowed for the identification of significant molecular and functional heterogeneity within hematopoietic stem cell (HSC) subpopulations. The current models of hematopoiesis describe the HSCs and hematopoietic progenitor cells (HSPCs) undergoing gradual and continuous lineage commitment. However, the HSC clones do not differentiate in a balanced manner, leading to biases toward specific hematopoietic lineages (Yamamato, 2018; Hass, 2018).

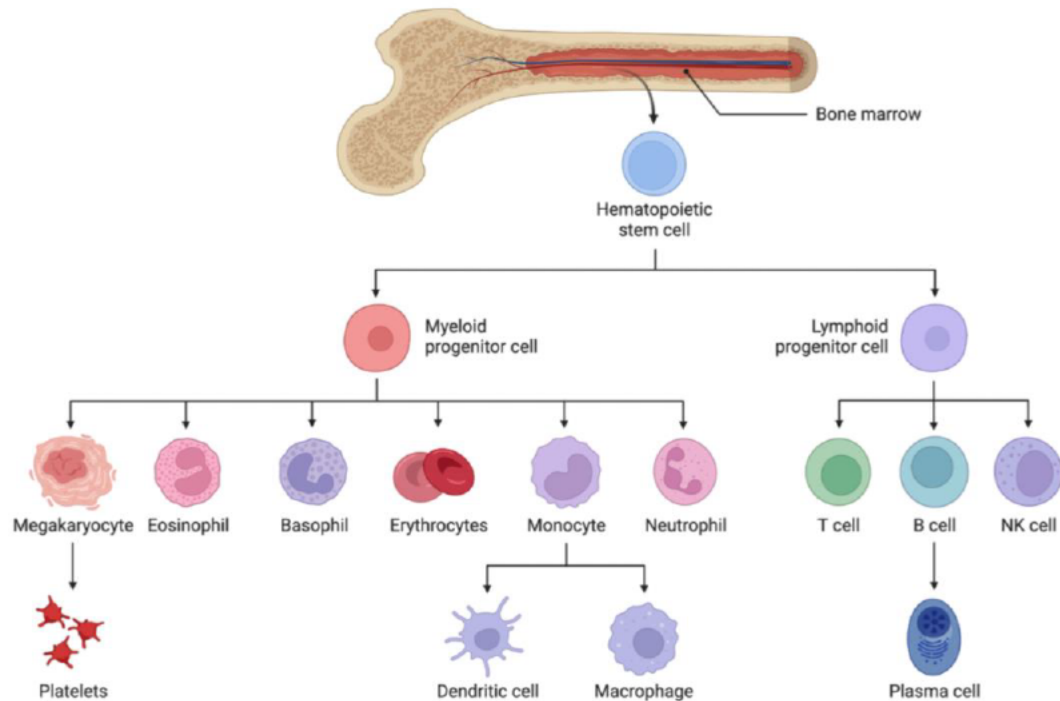


Figure 1. Lineages derived during hematopoiesis.
Starting in the bone marrow, where HSCs develop and remain quiescent until needed, and the hematopoietic stem cell can differentiate into either myeloid or lineage-derived cells. The cell cannot reverse lineages once a lineage has been chosen. Myeloid progenitor cells and lineage is discussed more thoroughly through this thesis as the disorders discussed are myeloid-based (Figure made on Bio render).

Throughout an individual's life, hematopoiesis can undergo changes in its function, location, ability to self-renew and differentiate, and level of quiescence. The bone marrow microenvironment (BMM) and the aging process play crucial roles in determining the development and health of the different cell lines, including myeloid, lymphoid, and erythroid (which are primarily generated through adulthood due to the body's constant need for red blood cells). However, the differentiation into other lineages

occurs in smaller proportions than erythroid cells, and the ratios of cell differentiation and lineage bias ratios can change daily based on metabolic needs.

Hematopoietic stem and progenitor cells (HSPCs) differentiate into erythroid cells more frequently than other lineages in prenatal life due to the developing body's consistent demand for red blood cells (RBCs) in the hypoxic uterus. However, smaller proportions of other lineages are also generated. The ratio of cell differentiation and lineage bias changes daily to meet metabolic demands. As time passes, HSCs are more likely to give rise to myeloid progenitor cells postnatally. With aging, mutations and aberrant myeloid progenitor cells accumulate, leading to hematopoietic-related disorders. A significant observation is that over time, HSCs are more likely to differentiate into myeloid progenitor cells, and as aging progresses, the generation of mutations and improper myeloid progenitor cells can lead to hematopoietic-related diseases (Li, 2011).

By abnormally high or low levels of circulating blood cells or their progenitors, normal hematopoiesis can fail in various ways. Hematopoietic failures can lead to a range of disorders, including anemia, thrombocytopenia, polycythemia, and leukopenia. Anemia occurs when there is a reduction in red blood cell (RBC) production, which can result from various reasons such as blood loss due to injury or conditions like ulcers or cancer, insufficient production of RBCs due to nutritional deficiencies (such as iron, vitamin B12, or folate deficiency), bone marrow disorders, certain medications, or increased sequestration of RBCs into the spleen or other organs. Thrombocytopenia is caused by a deficiency in the number of platelets available for clotting responses, which can result from increased destruction or insufficient synthesis of megakaryocytes.

Leukopenia refers to a reduction in the number of circulating leukocytes. These disorders can have severe consequences, and thus, it is crucial to understand how to prevent and treat hematopoietic failures.

Hematopoietic-related, non-malignant disorders such as sickle cell anemia, Fanconi anemia, and beta-thalassemia can also be caused by hereditary and genetic mutations. These diseases are attributed to polymorphisms in the genes that are essential for hemoglobin formation, thus compromising RBCs' capacity to transport oxygen, or a decline in RBCs development and distribution through the BM.

Aberrant clonal growths of blood cells can develop, leading to leukemias and blood-related cancers. Many of these disorders are caused by the unregulated growth of immature WBCs in the bone marrow (BM). This influx of immature and unregulated cells crowds out healthy cells from the BM microenvironment, eventually developing into potentially fatal diseases and associated symptoms. Acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia, acute myelogenous leukemia (AML), and chronic myelogenous leukemia are the four most frequent types of leukemia (Rowe et al., 2016). Chemotherapy, targeted treatment, and stem cell transplantation, Chimeric antigen receptor T cell therapy, are available treatment regimens; however, the most optimal treatment method is still being elucidated.

Overview of myelodysplastic syndrome (MDS)

A heterogeneous clonal disease, MDS is characterized by systemic reduction of mature RBCs (cytopenia) and generally hypercellular BM abnormalities. MDS was previously classified as a disease with minimal malignant potential because most patients

did not develop leukemia or other associated malignancies. MDS is now classified as a malignant disease due to how it affects hematopoiesis and its association with extreme clonal abnormalities and a propensity to develop into AML (Brandwein et al., 1990).

MDS can evolve into AML, a fast-growing malignancy of BM cells affecting around 30% of patients with pre-diagnosed MDS. Pre-cancer/pre-leukemia was a term used in the past (Aujla et al., 2018) but no longer applies to MDS due to its malignant qualities. In the early 2000s, it was one of the most prevalent hematologic malignancies in adults. MDS can develop in a wide age range from childhood to advanced age, particularly for those who present with an inherited BM failure syndrome (Locatelli et al., 1995).

Locatelli et al. (1995) have emphasized the significance of the bone marrow microenvironment (BMM) in comprehending the development of myelodysplastic syndrome (MDS) and the possibility of cytopenia in patients with MDS and acute myeloid leukemia (AML). The BMM, which consists of various cell types, cytokines, and matrix metalloproteinases, acts as a dynamic support system for all cell types and is fundamental in promoting hematopoiesis (Ries et al., 1999). It is essential to understand the involvement of BMM in signaling pathways leading to chemotherapeutic resistance in patients with MDS, which results in poor treatment outcomes for this disorder and, potentially, AML (Agarwal & Bhatia, 2015).

MDS is a disease with a complex genetic basis that can be caused by genetic aberrations or environmental factors. The age of onset is a significant factor in determining the type of mutation(s) involved in the disorder. Previous studies between

2000 and 2005 identified RUNX1, TP53, and NRAS as genes affecting MDS development, but they provided minimal information about the disorder. With the advent of new technologies and genomics, researchers have identified driver mutations as the primary cause of MDS, and the number of driver mutations correlates with disease severity. These mutations affect critical biological mechanisms such as RNA splicing, DNA methylation, transcription, chromatin modification, DNA repair, apoptosis, and cell signaling. Understanding these genes and mechanisms could shed light on the causes of MDS and why it is an age-related disorder (Haferlach et al., 2014; Ogawa, 2019).

Pathway/functions	Driver genes
DNA Methylation	DNMT3A, TET2, IDHI,* IDH2,* and WTI
Chromatin modification	EZH2, SUZI2, ED, JARID2, ASXL1, KMT2, KDM6A, ARID2, PHF6, and ATRX
RNA Splicing	SF3B1, SRSF2, U2AFI, UZAF2, ZRSR2, SF1, PRPF8, LUC7L2
Cohesin complex	STAG2, RAD21, SMC3, and SMCIA (PDS5B, CTCF, NIPBL, and ESCO2)
Transcription	RUNXI,.+ETV6,+GATA2,+IRF), CEBPA, BCOR, BCORLI, NCOR2, and CUXI
Cytokine receptor/Tyrosine kinase	FLT3, KIT, JAK2, and MPL. CALR, and CSF3R
RAS Signaling	PTANTI, NFI, NRAS, KRAS, and CBL (RITi and BRAA)
Other signaling	GNAS, GNBI, FBWX7, and PTEN
Checkpoint/Cell Cycle	TP53 and CDKNZA
DNA repair	ATM, BRCC3, and FANCL
Others	NPMI, SETBPI, and DDX41t

Table 1: Major driver mutations that lead to MDS development.
These pathways are all tightly regulated to ensure minimal stochastic changes. When knocked out, paired driver genes cause dysregulation of these tightly controlled pathways, commonly leading to increased clonal abnormalities. Table derived from MDS Genetics Ogawa, 2019.

According to Ogawa (2019), a significant number of patients (more than 80–85%) with MDS have a driver mutation in the spliceosome, a complex that controls RNA splicing and mRNA development. These mutations often occur early in the course of the disease and can be considered as the initiating events for MDS development (Sperling et al., 2017). One of the most potent driver mutations in MDS is SRSF2, which is commonly

found in the exonic splicing enhancer region of the pre-mRNA strand. Rozema (2017) explained that mutations in this region can disrupt the cell's ability to maintain the required exon, resulting in incorrect removal of the exon. The consequences of SRSF2 mutations include an increased risk of associated hematologic disorders, such as AML and concurrent BM failure, as well as a myeloid bias in hematopoietic development (Smeets et al., 2018).

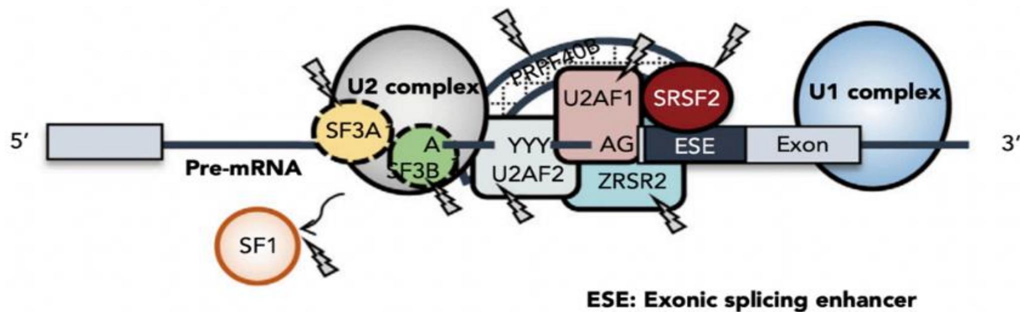


Figure 2. Mechanism of action of spliceosome-derived mutations.
The specific mutation of interest is SRSF2 which sits directly on top of the exon splicing enhancer. When SRSF2 is, mutated exons are accidentally sliced out when they should be retained (Source: Haferlach et al., 2014).

Despite extensive studies on the epidemiology of MDS mutations referenced above, human models that capture the hallmarks of the disease are lacking. There is an urgent need to develop better models for therapeutic discovery.

Generation of phenotypically adult-like HSCs from induced pluripotent stem cell (iPSC) lines to improve MDS modeling

The utilization of induced pluripotent stem cells (iPSCs) is crucial for the advancement of research in the field of hematopoietic diseases and hematologic disorders. These cells retain cellular memory and location from which they are derived, making them a valuable tool for disease modeling. Initially, cells differentiated from iPSCs were derived from somatic tissue fibroblasts and retained a fetal-like phenotype during differentiation, which was useful for disease modeling in childhood diseases or other somatic mutations. However, to expand the use of iPSCs in studying adult diseases, researchers must modify the model to yield cells that appear more adult-like to better represent disease progression (Chang et al., 2018; Studer et al., 2015).

Owing to the fact that aging is a tightly controlled system, a number of mechanisms have been explored to establish an adult-like iPSC model. Even though prior studies used external stress factors to induce aging events in the cells, it was not clear whether or not this model would be optimal. This is because a major external stress factor ended up altering mitochondrial function through oxidative stress (Chang et al., 2018; Zhang et al., 2015). Subsequently, researchers went on to explore other diseases that increased cellular aging caused by gene knockouts or protein mutation. Hutchinson-Guilford progeria syndrome, commonly known as progeria, is characterized by swift aging due to a single gene mutation; it was referenced as a model for possible inducible aging of iPSCs.

In the pursuit of creating more physiologically relevant models of disease using iPSCs, researchers have explored the use of an aged iPSC model. Agarwal et al. (2010), Liu et al. (2011), and Zhang et al. (2011) demonstrated that an aged iPSC model could be generated by introducing the point mutation that causes progeria into healthy iPSC cell lines. These studies showed that the model was capable of generating phenotypically mature cells in various differentiation environments. However, functional issues were observed, including reduced cell viability and increased spontaneous apoptosis, which were attributed to possible downstream effects caused by progeria mutations and other unknown factors associated with the rare disease (Liu et al., 2011; Zhang et al., 2011).

Despite these challenges, the aged iPSC model has been utilized for neurological research associated with aging (Miller et al., 2013). However, it is not recommended for other disease models due to the model's limitations and challenges, including the potential impact of progeria mutations on the model's functionality. Future research is needed to improve the aged iPSC model to overcome these challenges and provide a more accurate representation of age-related disease progression.

The lack of an appropriate model for studying age-related hematologic disorders with induced pluripotent stem cells (iPSCs) has been a challenge in research. The primary objective of this project was to develop a stable iPSC line using a lentivirus that induces endogenous micro-RNAs expressed naturally during normal development and aging. The aim was to create iPSCs that mimic an adult phenotype rather than a fetal one, enabling researchers to study mature-onset adult diseases such as myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) in a patient-comparable model. However,

previous models that induced cellular stress knocked out age-related genes and altered telomere sizes, which had limitations and disadvantages. Therefore, a new approach was attempted to generate adult-like iPSCs.

Lin28 is an RNA-binding protein that selectively inhibits let-7 miRNA maturation in embryonic stem cells, but not other miRNA families related to aging (Zhang et al., 2016). The lin28b/let-7 axis is a well-known mechanism primarily understood from lin28b's ability to produce and maintain fetal-like cell ontogeny (Rowe et al., 2016). The development of the hematopoietic stem and progenitor cell (HSPC) compartment is regulated from gestation into adulthood by the heterochronic lin28b-let-7-Cbx2-Erg axis (Wang et al., 2022). During normal development and maturation into adulthood, lin28b is repressed, allowing the release of let-7 miRNAs to initiate hematopoiesis of adult-like cells.

In other model organisms, overexpression of lin28 maintains a more fetal-like phenotype and self-renewal of HSCs than let-7 overexpressed cells (Stolla et al., 2019; Wang et al., 2022). This finding has contributed to the creation of models for studying pediatric and fetal-related diseases, as well as adult-related disorders in animal models. For diseases such as MDS, which can develop during childhood and manifest in adulthood, it is essential to have a model that can phenotypically show the effects in fetal and adult scenarios and their relationship.

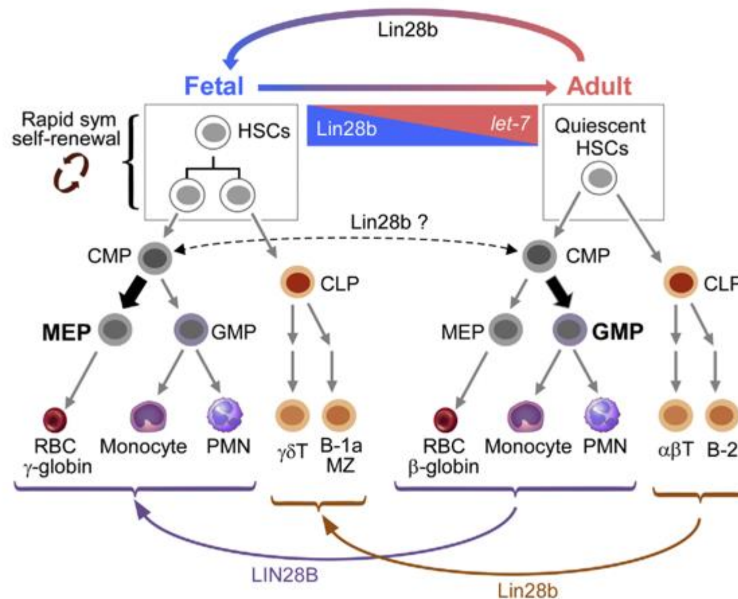


Figure 3. The Relationship between Lin28b and Let-7 Determines Fetal vs. Adult Stem Cells.
Lin28b and let-7 interact in a double-negative feedback loop. When lin28b expression is high, let-7 expression is repressed, allowing cells to maintain their fetal phenotype and renew and replenish rapidly. When let-7 is expressed, lin28b is repressed, which is thermodynamically unfavorable, generating more adult-like cells (Source: Rowe et al., 2016).

HSCs engineering/differentiation from induced pluripotent stem cells (iPSCs)

Stem cells are an important tool for regenerative medicine and disease modeling, and hematopoietic stem cells (HSCs) and induced pluripotent stem cells (iPSCs) are two primary types with different applications. HSCs are responsible for generating all blood and platelet cell types and have critical roles in the immune system. HSCs are characterized by cell surface markers such as CD34, CD38, and CD45, and can be isolated from bone marrow, peripheral blood, or umbilical cord blood (Xu et al., 2018). iPSCs, on the other hand, are embryonic-like pluripotent cells that can differentiate into

any cell or tissue type under the right conditions (Chang et al., 2018). iPSCs are generated by reprogramming adult cells using specific factors such as Oct4, Sox2, Klf4, and c-Myc (Nielsen et al., 2014).

To fully harness the potential of HSCs and iPSCs, it is essential to understand their engineering and differentiation characteristics, including their isolation and differentiation strategies. HSCs can differentiate into different types of blood cells, including red blood cells, white blood cells, and platelets, making them useful in treating blood-related diseases (Nielsen et al., 2014).

In contrast, iPSCs have broader applications, including the development of organoids and muscle cells, which are useful in regenerative medicine. iPSCs are adult cells reprogrammed to mirror embryonic stem cells (Takahashi & Yamanaka, 2006). They are induced in vitro to overexpress genes and transcription factors utilized by embryonic stem cells, including Oct3/4, Sox2, Klf4, and c-Myc. When overexpressed, these genes allow iPSCs to maintain their stemness and regenerative capabilities (Qi & Pei, 2007).

The fate of HSCs and iPSCs is determined by the environment and the presence of cytokines, proteins, and transcription factors. Understanding how these factors influence differentiation is crucial to generate specific cell types for therapeutic purposes. With further research, these stem cell types hold significant potential for disease modeling, drug discovery, and regenerative medicine. By contrast, it is possible to generate iPSCs from adult cells via several viral transduction methods, including retroviral and lentiviral transduction.

HSCs have the ability to differentiate into various types of blood cells through the use of specific cytokines and growth factors, which activate the differentiation process into RBCs, platelets, and WBCs. Meanwhile, iPSCs can also be differentiated into HSCs using methods that identify cells expressing HSC-specific markers. These engineered HSCs have various potential applications, such as drug discovery, gene therapy, and regenerative medicine. The differentiation protocols for iPSCs involve exposure to different growth factors and signaling molecules to replicate the developmental cues that cells experience in vivo. However, there are still challenges to be addressed when engineering and differentiating HSCs from iPSCs, such as enhancing the protocols for generating functional HSCs and ensuring that these cells are safe and effective for clinical use (Rowe & Daley, 2019).

Fetal-to-adult hemoglobin switching

Fetal to-adult hemoglobin switching transpires during the course of human development and is critical for hematopoiesis. The backdrop for this is the fact that RBCs contain the protein hemoglobin responsible for transporting oxygen and carbon dioxide. Fetal-to-adult hemoglobin enables the creation of only adult hemoglobin throughout development. As two primary forms of hemoglobin, adult hemoglobin (HbA) and fetal hemoglobin (HbF) are based on their chemical makeup and stage of development. HbF contains two gamma chains while HbA contains alpha and beta chains. Throughout normal development, gamma chains are degraded and replaced by alpha and beta chains, eventually resulting in the predominance of adult hemoglobin.

The fetal form of hemoglobin carries oxygen from the mother to the fetus and remains in the fetus until birth. The gamma-globin chain facilitates oxygen transport from the maternal supply to the fetal tissue since its chemical properties yield a stronger affinity for oxygen in comparison to adult hemoglobin. Gamma production stops at birth, while beta production commences and persists until the attainment of maturity.

According to Noh et al. (2009), lin28b controls the expression of HbF in erythroblasts derived from cord blood and adult CD34+ cells. This regulation does not affect the final maturation and enucleation process of these cells. In adults, lin28b is an upstream regulator of multiple genes associated with a fetal erythroid phenotype. When lin28b is present, it suppresses the let-7 family of miRNAs, which further inhibits BCL11A, a transcription factor that prevents gamma-globin gene expression, thereby promoting the production of fetal erythrocytes (Noh et al., 2009; Yuan et al., 2012). Conversely, if lin28b is absent or inhibited by increased let-7 expression, it leads to an increase in BCL11A expression, leading to the repression of the gamma-globin gene and the expression of adult hemoglobin (Yuan et al., 2012).

It is not surprising that cancer can disrupt the transition from fetal to adult hemoglobin, leading to either an excess of HbF or HbA depending on the cancer type. Chronic myelogenous leukemia is associated with increased HbF production due to enhanced gamma-globin gene activity (Katsumura et al., 2017), while AML is associated with an overabundance of HbA due to increased alpha globin gene activity. Cancer-related changes in HSC populations can lead to abnormal gamma production, resulting in reduced beta production and an increase in gamma concentration to compensate. Cancers

that use this mechanism can become resistant to traditional medications targeting beta conditionally expressed proteins (Sunami et al., 2022). Understanding the interaction between gamma and beta could help develop personalized treatments for these tumors.

Across the span of human development, the transition from fetal-to-adult hemoglobin has significant ramifications for hematologic diseases and therapeutic approaches. To clarify the significance of this transition, scientists are investigating the processes underlying the roles and features of fetal hemoglobin (gamma) and adult hemoglobin (beta). In this thesis, one of the objectives was to determine whether edited iPSCs would be able to retain an adult phenotype during differentiation, establishing adult-like erythrocytes that may have been utilized to examine adult blood diseases. According to our hypothesis, when differentiated, a mature HSC model would exhibit adult hemoglobin (beta). This contrasts with HSCs derived from the unedited or empty vector iPSC line that retains its embryonic phenotype and exhibit fetal hemoglobin (gamma).

Overview of AML

Acute leukemias are a type of blood cancer that can originate from various progenitor lineages, resulting in different oncogenic mutations. This biological diversity leads to varying chemotherapeutic sensitivities, risk of disease recurrence, and mortality rates. The two most common types of acute leukemia are ALL and AML, but they differ significantly in pathophysiology, prognosis, and age distribution. ALL is more prevalent in children, with generally better outcomes compared to adults, where the disease is rare

and associated with lower survival rates. On the other hand, AML is the most prevalent form of adult leukemia, accounting for approximately 80% of cases.

AML is a complex disease that can manifest in different forms depending on various factors such as age, symptomatology, related somatic mutations, and family history. It arises from mutations in genes that regulate hematopoiesis, resulting in the uncontrolled proliferation of undifferentiated myeloid cells. This uncontrolled cell division leads to clonal expansion of blast cells in the peripheral blood and bone marrow, disrupting the normal erythropoiesis process, and leading to bone marrow failure. In contrast to ALL, AML is a more aggressive disease, with lower overall survival rates, making it a significant challenge in clinical management. Understanding the underlying genetic and biological mechanisms responsible for AML development and progression could help develop more effective, targeted therapies for this complex disease (Jaiswal & Ebert, 2019).

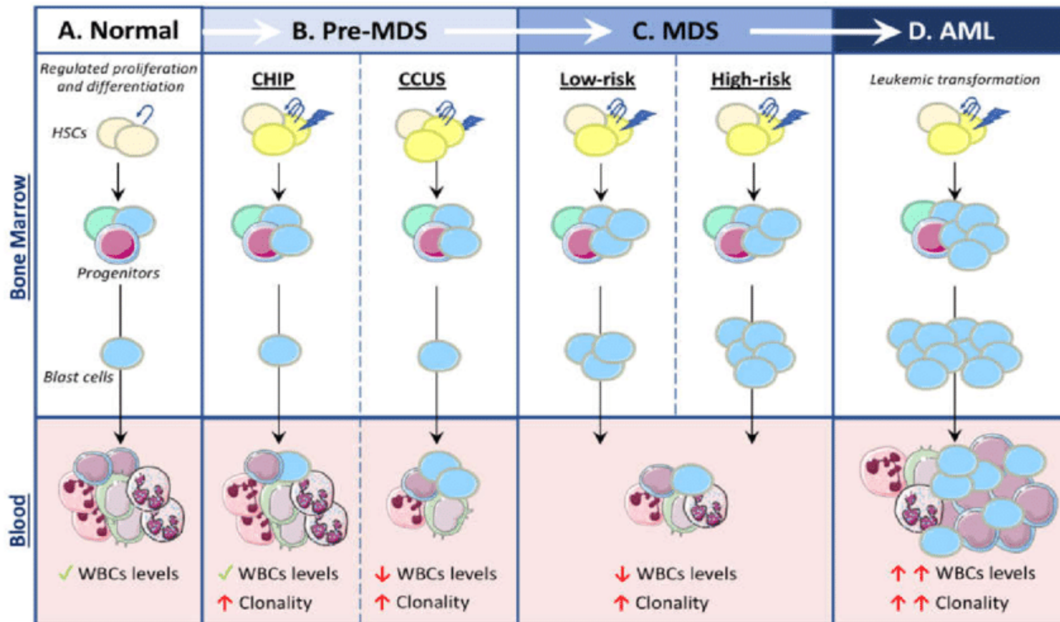


Figure 4: Acute Leukemogenesis in Humans
This figure shows the progression from normal hematopoiesis to clonal hematopoiesis to the eventual development of acute myeloid leukemia. (A) Normal/healthy progression of hematopoiesis in which hematopoietic stem cells self-renew and develop into progenitor cells. Progenitor cells develop into immature blood cells (blast cells), then mature into white blood cells (WBCs). (B) Clonal hematopoiesis of Indeterminate potential arises when an HSC develops a somatic mutation that confers a proliferative advantage over healthy cells, which culminates in forming a mutant rapidly diving clone. (C) MDS is usually caused by a genetic aberration linked to increased mutations in the patient's BM. (D) AML is the final stage disease involved in this improper hematopoietic pathway. AML causes abnormal BM growth, dysplasia, and pathological WBC production Source: Veiga et al., 2021.

Recent therapeutic advances for AML have resulted in a rise in survival rates of 15% for patients over 60 years and 40% for those younger than 60 years but above 40 years (Vakiti & Mewawalla, 2022). However, despite these advances, the overall survival for middle-aged and geriatric patients is alarmingly low, with recurrence occurring in 50-60% of patients (Thol & Ganser, 2020; Medeiros et al., 2019). Although MDS is a predominant risk factor for AML, hematologic disorders such as polycythemia vera,

thrombocythemia, and aplastic anemia significantly contribute to the occurrence of the disease (Vakiti & Mewawalla, 2022). Environmental factors such as exposure to benzene and toluene in the workplace, smoking, and family history also play a significant role in the development of the disease (Maleki Behzad et al., 2021).

Despite technological and therapeutic advancements, therapeutic options remain limited due to the stochastic nature and ever-changing disease mutations. Leukemia stem cells (LSCs), which retain stem cell characteristics such as self-renewal and the capacity to remain quiescent during treatment, contribute to relapse with drug resistance (Chopra & Bohlander, 2019; George et al., 2016). Studies have shown that LSCs are associated with poor outcomes, highlighting the need for a better understanding of LSCs and their derived cells to develop effective treatments and improve outcomes.

The current focus of research is on the impact of aging on hematopoiesis, with a hypothesis suggesting that multiple mutations accumulate in slowly dividing HSCs in the BM over time, ultimately leading to the development of AML or MDS (Chopra & Bohlander, 2019). AML is considered a highly heterogeneous disease due to the various ways in which it can develop, which makes treatment more complicated. To optimize treatment options and gain insights into the genetic components affected by these mutations, it is crucial to understand the impact of leukemia stem cells on the development of HSCs, MPPs, and progenitor cells over time (Chopra & Bohlander, (2019).

Summary

As there continues to be an increase in age-related diseases such as MDS, it becomes increasingly important to develop new models that can investigate the underlying molecular mechanisms of these conditions.

The primary distinction between fetal and adult hematopoiesis is the location in which the processes occur, and the specific cell types produced. Fetal hematopoiesis occurs in the liver and spleen and results in the production of red blood cells and immune cells necessary for fetal development. Adult hematopoiesis occurs in the bone marrow and produces a variety of blood cells required for normal body function, tissue repair and regulation of immune cells. In subsequent studies, this model may prove to be very beneficial in helping researchers comprehend MDS better and develop efficacious treatments for the adult population.

Currently, all iPSC models yield development of fetal cells due to their embryonic nature. This creates a problem when attempting to study disorders that have very different functions at different points in time. The way hematopoiesis occurs in the fetal/embryonic state is much different than the adult state and therefore using a fetal model to study an adult disorder is counterintuitive.

The overarching goal and hypothesis of this thesis is that by using inducing the expression of let-7, a microRNA, that is known for its role in maturation, in iPSCs, the creation of a phenotypically adult iPSC model can be used to create HPCs that also appear adult like and are a better model to study age biased disorders such as MDS.

Specific aims

1. Generation of stable, non-leaky doxycycline-inducible, puromycin-resistant, PKLO-Tet-on-let-7g plasmid and corresponding lentivirus expressing let-7g and an empty vector (EV), respectively.
2. Generation of an edited iPSC model in two IPS lines. C23 was generated from a healthy donor, and C13 contains the SRSF2 mutation, a common genetic aberration seen in MDS.
3. Determine whether the edited iPSC models can successfully differentiate into hematopoietic stem cells and other lineage-derived cells and maintain an adult phenotype instead of a fetal phenotype.

CHAPTER TWO

Methods

iPSC Culture Maintenance

To maintain human induced pluripotent stem cells (iPSCs) in culture, a base media called mTeSR1 was used along with a 5X supplement (Stem Cell Technologies) and 1% penicillin/streptomycin. The cells were plated on Matrigel-coated plates that were prepared 2-24 hours before cell plating, and the media was changed every day. When the cells reached about 70% confluency, they were detached from the plate by washing with 1x Dulbecco's phosphate-buffered saline (DPBS) without Mg2+ or Ca2+ and detached with ReLeSR™ (Stem Cell Technologies) to remove any differentiated iPSCs. After five minutes of incubation at 37 °C, the Matrigel was dissolved, and the cells were washed, collected, and centrifuged for five minutes at 1200 rpm. Fresh media was used to resuspend the pellet, and the cells were re-plated on fresh Matrigel-coated wells. If the iPSCs were being dethawed, a ROCK inhibitor-Y-27632 dihydrochloride (Tocris Bioscience) was added at a 4x volume for four days post-thaw. The cells were maintained in a gas ejection incubator with 37 °C, 5% CO2, and relative humidity of about 95% maintained by deionized water. The cells were regularly tested for mycoplasma biweekly to ensure that there was no contamination

Human embryonic kidney (HEK-293T) cell culture

Due to their high transfectability and the presence of the SV40 T-antigen, HEK-293T cells (CRL-1573, ATTC) are a stable line of cells allowing for virus generation.

HEK-293T cells were maintained in 1x Dulbecco's Modified Eagle Medium that contained 10% fetal bovine serum in addition to 1% penicillin/streptomycin solution. To ensure there was no contamination, cells were tested for mycoplasma biweekly. In addition to the fact that media was changed biweekly, cells were split using 0.05% trypsin to detach them from the plate.

Total RNA isolation from cultured cells

The adherent cells were washed with DPBS, and then TRIzol™ Reagent (Cat# 15596026, Thermo Fisher) was added to each well. The wells were scraped using a cell scraper, and the TRIzol containing the cells was vortexed and incubated for five minutes at room temperature (25°C). Pure chloroform was added to each sample to enhance phase separation of the RNA, DNA, and cellular debris. The samples were again vortexed, incubated at room temperature for five minutes, and then centrifuged at 12,000 x g for 15 minutes at 4°C. Samples were checked for proper RNA concentration and stored at –80 °C. The clear aqueous layer that contained the RNA was collected into a fresh tube and precipitated with isopropanol. The samples were incubated in the isopropanol overnight at –20°C or at room temperature for ten minutes. After incubation, the samples were spun at 12,000 x g, 4°C for 15 minutes, and the supernatants were aspirated to preserve the pellets. The pellets were resuspended in 75% ethanol to wash any remaining phenol or isopropanol from the sample, and then centrifuged at 7,500 x g, 4°C for ten minutes. After aspirating the supernatants, tubes containing the sample were inverted for vie minutes in order to allow alcohol to evaporate to reduce the amount of alcohol

contamination. The RNA pellet was resuspended in 15–40 µL of Ambion® DEPC-treated water.

TaqMan™ microRNA assays

To perform reverse transcription and cDNA synthesis, isolated RNA samples were standardized to 10 ng input using a TaqMan™ MicroRNA Reverse Transcription Kit (Invitrogen, ThermoFisher Applied Biosystems™, 4366596) following the manufacturer's protocol. For cDNA synthesis, 10 ng of total RNA was mixed with a reaction mixture containing 0.15 µL of 100 mM dNTPs (with dTTP), 1.00 µL of MultiScribe Reverse Transcriptase, 1.50 µL of 10X Reverse Transcription Buffer, 0.19 µL of RNase Inhibitor, and 4.16 µL of nuclease-free water, for a total of 7 µL of the reaction mixture. A final volume of 5 µL was aliquoted per sample, which was combined with 3 µL of specific miRNA 5✕ RT Primer, and the tubes were vortexed and centrifuged. Reverse transcription was carried out with a thermal cycler under the following conditions: 16 °C for 30 minutes, 42 °C for 30 minutes, 85 °C for five minutes, and then held at 4° C. The resulting cDNA sample (5 µL) was mixed with TaqMan Fast Advanced Master Mix (Invitrogen, ThermoFisher Applied Biosystems, 4444554) in MicroAmp Optical 96-well reaction plates (Invitrogen, ThermoFisher Applied Biosystems™, N8010560) at a ratio of 1:3, respectively. RT-qPCR was conducted on an Applied Biosystems QuantStudio 6 thermocycler using specific NFQ-MGB TaqMan Advanced miRNA assay probes (Invitrogen, ThermoFisher Applied Biosystems, A25576) for each miRNA present. The final copy number of miRNA per 10 ng RNA was

quantified and normalized to determine the fold-change expression compared to the negative control.

Isolation of plasmid DNA

To obtain E. coli Stbl-2 cells, a single colony was selected from a low-density LB-agar plate and grown overnight in 5 ml of LB medium with ampicillin (amp) in a shaker incubator. The culture was then used to inoculate a larger 150-mL volume of LB+amp, which was incubated overnight. The cells were harvested by centrifugation, and plasmid DNA isolation was performed using a ZymoPURE II Plasmid Maxiprep Kit. The pellets were resuspended in 14 mL of ZymoPURE PI containing RNase A, vortexed, and then mixed with ZymoPURE P2 (Blue). The solution was incubated at room temperature until transparent, purple, and viscous. Next, ZymoPURE P3 (Yellow) solution was added, and the mixture was inverted until it appeared yellow. The sample was then loaded into a ZymoPURE Syringe Filter-X and incubated for 10 minutes until a yellow precipitate was fully floating and the sample was clear. The clear lysate was collected in a new 50-mL conical tube by removing the Luer Lock plug from the bottom of the syringe filter apparatus and expelling the lysate fluidly using the plunger. Around 35 milliliters of the clear lysate was obtained and then mixed with 14 milliliters of ZymoPURE™ Binding Buffer by inverting the tube eight times. Next, Zymo-Spin™ V-PX Columns were connected to a conventional vacuum-based manifold, and the samples were added to it. The samples then underwent several washing steps before being eluted with 400 µl of ZymoPURE™ Elution Buffer and centrifuged at a speed equal to or greater than 16,000 x g for 1 minute into a clean microcentrifuge.

Generation of Lentivirus in HEK293T cells

HEK293T cells were seeded in six-well plates at a density of 1x106 cells per well and cultured in media without antibiotics 24 hours before transfection. To prepare the transfection mixture, 1.6 µg of vector plasmid (let-7g or EV), 0.7 µg of Pax2, and 1.6 µg of pCMV-VSV-G (Addgene Plasmid #8454) were added to 500 µL of Opti-MEM Reduced Serum Medium (Thermofisher, 31985062) along with 5 µL of Lipofectamine™ LTX Reagent. All the components were mixed in a 15-mL conical tube and incubated at room temperature for 15 minutes. The transfection mixture was then added dropwise to the HEK293T cells and incubated for 6–12 hours at 37 °C, 5% CO2, and 5% O2. After the incubation, the transfection mixture was removed and replaced with fresh DMEM supplemented with 10% FBS, albeit without the presence of antibiotics. The media was collected 48–72 hours post-transfection, filtered through a 0.22-µm syringe filter to remove cell debris and particles, and stored at -80 °C.

Viral Transduction of iPSC with puromycin Selection

To prepare for viral iPSC transduction, the cells were seeded at medium density on Matrigel-coated six-well plates in antibiotic-free mTeSR™1 media and incubated overnight at 37 °C and 5% CO2. On the first day of viral transduction, the iPSCs were washed gently twice with DPBS to avoid disrupting the colonies. Due to the low titer volume of lentivirus, 1 mL of viral suspension was added dropwise to the cells, followed

by 1 mL of antibiotic-free media, and 2 µL of polybrene (6 mg/mL) per well. The plate was incubated overnight at 37 °C and 5% CO2.

The next day, the media+virus was removed, and traditional media was added. The cells were allowed to express protein for 24-48 hours before starting puromycin selection. Spinfection, or viral infection by means of centrifugation, was carried out at 25°C by wrapping the plate with parafilm and subjecting it to 950 g for 30 minutes The parafilm was removed following spinfection, after which the plate was incubated overnight in a humidified incubator at 37 °C and 5% CO2. The next day, after the media+virus was removed, the cells were left to express protein for 24-48 hours before commencing puromycin selection. On day four, puromycin was added to designated wells at 2 µ/well daily. Puromycin was added to the culture to maintain the homogeneity of gene-expressing cells even after the selection.

iPSC to HSPC differentiation assay: STEMCELL kit

In order to induce differentiation of iPSCs, the STEMdiff™ Hematopoietic Kit (05310, STEMCELL Technologies, Inc.) was used for 12 days. A day before the differentiation process, iPSCs were split and counted. 76 colonies were plated per sample in a 24-well dish coated with Matrigel (Corning) and left to attach overnight. On day 0 of differentiation, medium A (STEMdiff™ Hematopoietic Basal Medium Containing Supplement A) was added. On the following day, a half medium change was done with medium A, along with WNT/β-catenin agonist CHIR99021 (SML1046, Sigma) and activin/nodal/TGFβ antagonist SB431542 (S4317, Sigma). On day 3 of differentiation, the supernatant was gently removed, and hematopoietic differentiation medium B was

added, followed by half medium changes on days 5, 7, and 10. Hematopoietic cells (HPCs) were collected on day 12 from the supernatant to derive cells into blood cell lineages.

Semi- Solid Colony Forming Assay

The human samples were cultured using semi-solid assays following the manufacturer's instructions from Stem Cell Technologies. Cells that had undergone a 12-day differentiation from iPSC to HSCs were collected, washed, and resuspended in DPBS. The cells were then counted, and numbers sufficient for two 35-mm plates were added to 15-ml tubes. MethoCult™ SF H4636 from Stem Cell Technologies was added to 50-mL conical tubes and mixed. Then, 1.5 mL of the mixture was dispensed into 35-mm culture dishes using a syringe and a blunt needle. The cells were incubated for 14 days in a humidified incubator at 37 °C and 5% CO2. The colonies were counted using an inverted microscope and gridded scoring dishes. The MethoCult™ SF H4636 consisted of Methylcellulose in Iscove's Modified Dulbecco's Medium (IMDM), bovine serum albumin, 2-mercaptoethanol, recombinant human (rh) insulin, human transferrin (iron-saturated), supplements, rh stem cell factor (SCF), rh interleukin 3 (IL-3), rh granulocyte colony-stimulating factor, and rh granulocyte-macrophage colony-stimulating factor.

Erythrocyte differentiation assay

To initiate erythroid differentiation, HSCs were subjected to a three-stage culture system. Base media, consisting of IMDM with 2% human AB plasma, 3% human AB

serum, 1% penicillin/streptomycin, 3 U/mL heparin, and 10 μg/mL insulin, was used throughout the process. During phase I (days 0–7), the base media was supplemented with 10 mg/mL of human holo-transferrin, as well as 1 ng/mL each of erythropoietin (EPO), stem cell factor (SCF), and interleukin-3 (IL-3). For phase II (days 7–12), the base media contained only 10 mg/mL of human holo-transferrin, EPO, and SCF. In phase III (day 12 onwards), the primary cell cultures were supplemented with 1 mg/mL of human holo-transferrin and EPO. Flow cytometry was used to monitor the presence of hemoglobin during every phase change.

Intracellular Hb staining

The study involved collecting cells from an in vitro erythroid differentiation assay on days 10, 20, and 30 to determine the presence of fetal versus adult hemoglobin and how it changed when exposed to doxycycline versus not exposed. The purpose of administering doxycycline to certain lines was to test for let-7 induction. The cells collected from the differentiation plate ranged from 1 x 106–5 x 106 and were washed twice with DPBS before being fixed in 1 mL of 0.01% paraformaldehyde (PFA) for 10 minutes at room temperature. After fixing, the cells were gently vortexed, then centrifuged at 600 g for 5 min at room temperature, and the supernatants were aspirated. The cells were then permeabilized with 200 μL of 0.1% Triton X-100 in phosphate-buffered saline for 5 minutes at room temperature, followed by 800 μL of flow buffer (DPBS+ bovine serum albumin) to stabilize the sample. Samples were spun for 15 minutes at 600 g, and the supernatants were removed. To each sample, 100 μL of flow buffer containing 1 μL of fetal hemoglobin monoclonal antibody (HBF-1), APC, and 1

μL of hemoglobin β antibody (37-8) FITC: sc-21757 FITC was added. Cells were gently resuspended and incubated at room temperature in the dark for 20–30 minutes. After staining, the cells were washed, centrifuged, and resuspended in a standard flow buffer for flow cytometry analysis. HUDEP 1 cells were used for positive control for APC+/FITC-, and HUDEP2 cells were used as a positive control for APC-/FITC+. Unstained cells were used for flow cytometry gating of live versus dead cell

Flow Cytometry

The cells were analyzed using the BD LSRFortessa™ Cell Analyzer (BD FACSDiva, BD Biosciences) equipped with four lasers targeting blue, red, violet, and ultraviolet. BD FACSuite software (BD Biosciences) was used for data acquisition. Forward scatter (FSC) and side scatter (SSC) were used to exclude debris and select single cells. Various fluorescent anti-human antibodies were used, including CD34-APC (Biolegend, San Diego, CA), CD45-PE-Cy7 (Biolegend, San Diego, CA), fetal hemoglobin monoclonal antibody (HBF-1), APC (Thermo Fisher, Catalog # MHFH05), and hemoglobin β antibody (37-8) FITC (Santa Cruz, Catalog # sc-21757 FITC). In some experiments, Sytox Blue stain (Thermo Fisher, S34857) was used to distinguish between living and dead cells in culture when the cells were not fixed. Unstained cells and cells stained with isotype control antibodies were used as negative controls. The data were analyzed using FlowJo software (TreeStar).

Statistical analysis

The statistical significance for overall expression levels of miRNA in RT-qPCR, colony presence in semi-solid colony-forming assays, and mean fluorescence intensity vs. total intensity in flow cytometry were determined at $p \leq 0.05$ (), $p \leq 0.01$(), and $p \leq 0.001$(). Two-way ANOVA with post hoc multiple comparisons such as the Tukey test was used to determine sample interactions. Data points were expressed as the mean ± standard deviation of three independent experiments performed in triplicate. Microsoft Excel, GraphPad Prism version 9.0, and Flow Jo 10.4.2 Software (USA) were used for data analysis, subsequent statistical tests, and graphing. Flow cytometric data analysis was performed using Flow Jo 10.4.2 Software (USA).

CHAPTER THREE

Results

Let-7g is upregulated in iPSC

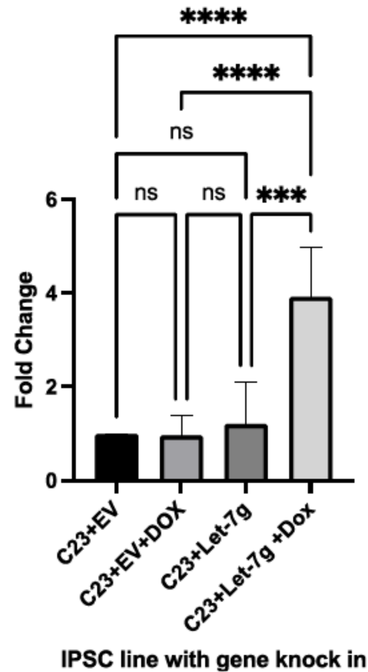
Before proceeding to the next step, we conducted experiments stepwise to ensure every component worked to evaluate the ability to create a phenotypically adult iPSC cell line to model age-biased disorders. When possible, experiments were performed in triplicate and all statistical analyses were carried out at an alpha of 0.05 or below. Blinding of sample information was performed, when needed, to allow for an unbiased visual quantification.

To create and validate a model, the first step involved determining if lentivirus and puromycin selection were sufficient to produce iPSC lines containing the desired genes. The hypothesis was that after transduction and selection, iPSCs with let-7g would exhibit significant overexpression when co-cultured with doxycycline (dox), but no effect would be seen in cells infected with an EV, even when exposed to dox. If successful, the iPSCs containing let-7g or EV would be differentiated into HSCs to investigate whether let-7g's presence when exposed to dox caused the development of adult-like HSCs instead of fetal-like HSCs.

The stable C23 cell line, derived from a healthy patient, was initially selected for the experiments due to its lack of mutations that could affect the study's outcomes. C23 cells yield fetal-like hematopoietic progenitors. The C13 cell line, on the other hand, was selected as it possesses a point mutation in the *SRSF2* gene which is recurrently mutated in MDS. Both cells lines containing the corresponding gene insertion, let-7g and EV,

were exposed to dox for 24 hours, and their expression was measured. The dox-regulated expression mechanism, which relies on the tetracycline-controlled transcriptional pathway, was utilized to regulate gene expression. This mechanism involves placing the target gene downstream of a promoter that includes a tetO sequence. The TetR protein binds to the tetO sequence, inhibiting miRNA production. However, when dox is added to the culture medium, it binds to TetR, causing a conformational shift that frees the tetO sequence, allowing the transcription of microRNAs. The addition of dox to C23+EV did not result in a significant difference compared to C23+EV+dox (Figure 5A). On the other hand, the expression of let-7g increased four-fold when dox was added to C23+Let-7. The comparison of C23+EV and C23+EV+dox, used as internal controls, showed a four-fold increase in let-7 gene expression.

A.



B.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
C23+EV vs. C23+EV+DOX	0.03727	-1.466 to 1.541	No	ns	0.9999
C23+EV vs. C23+Let-7g	-0.2098	-1.713 to 1.294	No	ns	0.9802
C23+EV vs. C23+Let-7g +Dox	-2.923	-4.427 to -1.419	Yes	****	<0.0001
C23+EV+DOX vs. C23+Let-7g	-0.247	-1.751 to 1.257	No	ns	0.9684
C23+EV+DOX vs. C23+Let-7g +Dox	-2.96	-4.464 to -1.457	Yes	****	<0.0001
C23+Let-7g vs. C23+Let-7g +Dox	-2.713	-4.217 to -1.209	Yes	***	0.0002

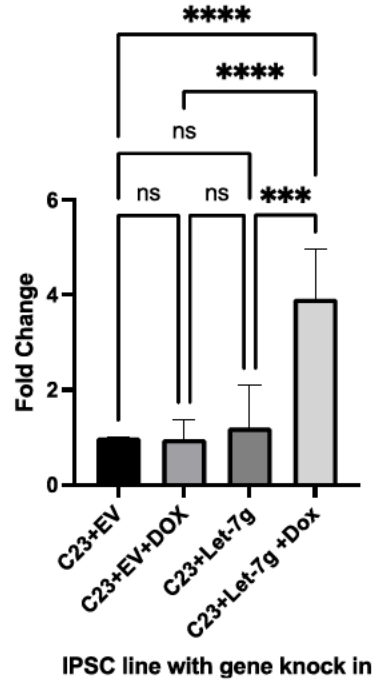
Figure 5. Expression of let-7g in C23 line +/- 24-hour doxycycline exposure normalized to U6.

(A) qPCR data from N = 3 pooled groups C23+EV and C23+EV+Dox were used as internal controls for overall expression comparison. The comparison was generally focused on C23+let-7 and C23+let-7+dox to determine % induction. (B) Two-way analysis of variance (ANOVA) and posthoc Tukey statistical test to determine the interaction between all groups and possible treatments. Statistical pairwise determined at (**) = 0.01, (***) =0.0002, (****) =<0.0001.

To confirm statistical significance within each population (N = 3 independent experiments), a two-way ANOVA and Tukey tests of multiple comparisons were carried out. The results revealed that C23+Let-7 vs. C23+Let-7+Dox had a p-value of 0.0002, indicating significant differences between the samples, similar to the fold-changes observed in the qPCR analysis. Additionally, significant p-values at <0.0001 were observed between sample combinations C23+EV compared to C23+let-7g+dox and C23+EV+Dox compared to C23+let-7g+dox, indicating that the backbone carrying let-7 was present and active. (Figure 5B).

To determine how let-7 expression was altered in a diseased/mutated cell line due to the SRSF2 mutation, the same experiment was performed in the C13 iPSC line. The C13 family showed an increased fold-change of approximately 4X when dox was added to C13+let 7 as compared to when it was not (Figure 6A). Similarly, all EV control samples compared to let-7+dox showed an increased fold-change. The two-way ANOVA and Tukey test conducted on the C13 IPS family generated p-values of 0.0001, indicating significant differences between the two groups. These results were more significant than when comparing C13+let 7 to C13+Let-7+Dox. (Figure 6B).

A.



B.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
C13+EV+DOX vs. C13+EV	0.1799	-0.7875 to 1.147	No	ns	0.9552
C13+Let-7 vs. C13+EV	0.2969	-0.6705 to 1.264	No	ns	0.8317
C13+Let-7+dox vs. C13+EV	3.713	2.745 to 4.680	Yes	****	<0.0001
C13+Let-7 vs. C13+EV+DOX	0.117	-0.8503 to 1.084	No	ns	0.9869
C13+Let-7+dox vs. C13+EV+DOX	3.533	2.566 to 4.500	Yes	****	<0.0001
C13+Let-7+dox vs. C13+Let-7	3.416	2.449 to 4.383	Yes	****	<0.0001

Figure 6. Expression of let-7g in C13 line +/- 24-hour doxycycline exposure normalized to U6.
(A) qPCR data from N = 3 pooled groups C13+EV and C13+EV+Dox were used as internal controls for overall expression comparisons. The comparison was focused on C13+let-7 and C13+let-7+dox to determine % induction. Unknown changes were anticipated due to the cell line. Mutations. (B) Two-way analysis of variance (ANOVA) and posthoc Tukey statistical test to determine the interaction between all groups and possible treatments. Statistical pairwise determined at (**) = 0.01, (***) =0.0002, (****) =<0.0001.

The increased expression of let-7g in iPSCs upon the addition of dox confirmed successful transduction of the pLKO-Tet-On Backbone carrying the let-7 gene and the effectiveness of puromycin selection, as well as ruling out non-specific effects of doxycycline on let-7g expression.

Differentiation of altered iPSC to HPC's

To investigate defective hematopoiesis, it is crucial to study healthy cells at an optimal point of differentiation. In this study, iPSCs were differentiated into HPCs over 12 days by forming a feeder layer and monitoring the ratio of attached cells (iPSCs) to floating cells (HSCs) as a qualitative measure of differentiation progress (Figure 7). Dox was added on day 9 to induce let-7g expression in the vector and increase its expression in HSCs without interfering with the differentiation process. In later experiments, dox was added on day 6 to improve cell line differentiation (Figure 8).

Morphological changes were observed in the cells starting around day 5, coinciding with the addition of medium B to the culture. On day 9, the number of floating cells had doubled in most wells, prompting the addition of dox (Figure 7B). Dox was also added on day 11. By day 12, differentiation into HPCs was considered complete as the floating fraction had increased almost four-fold from the starting amount (Figure 7C).

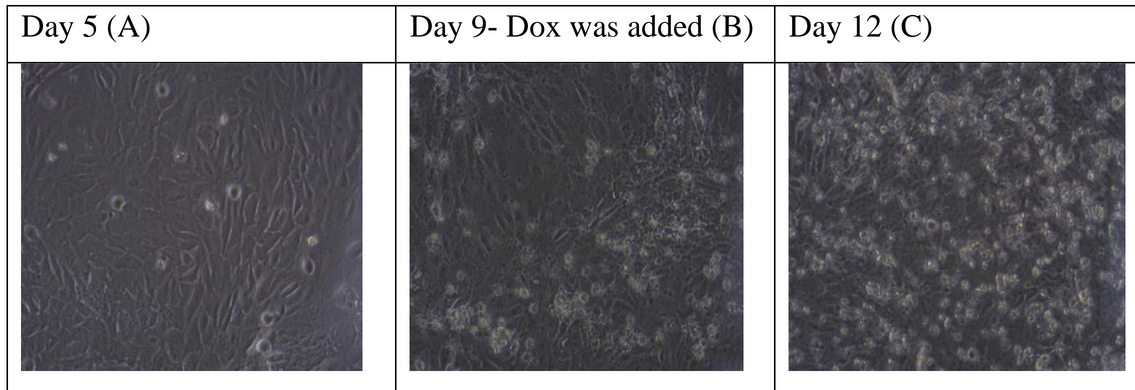


Figure 7. Morphological observations of iPSC differentiating into HPCs.
The adherent fraction is the iPSC line (C13 or C23). Emerging floating fraction that appears illuminated is differentiating HPCs. Once the experiment is switched to media B, there is a noticeable increase in HPCs.

The HPCs were collected after 12 days of differentiation, and experiments were conducted to assess their quality and determine which properties from the iPSCs were retained or lost during the differentiation process (Figure 7). The goal of these experiments was to evaluate whether HSCs with let-7 and let-7+dox exhibited improved adult phenotypes of HSCs.

To determine whether there were any changes in phenotype, the percentage of cells fully differentiated into HSCs that were CD34+ and CD45+ was determined through surface protein immunophenotyping. To ensure proper gating and identify where positive signals should occur if cells differentiated appropriately, FITC signals were collected using FITC+ single-cell beads. APC signals were collected with APC+ single-cell beads.

Treatment with dox had a minimal effect on the differentiation of the C23+EV line, but differentiation was adequate for the remaining C23 family members. In contrast,

C13 exhibited significantly reduced differentiation. C13+let-7+dox exhibited a substantial increase in mature HPC differentiation similar to C23+Let-7+dox.

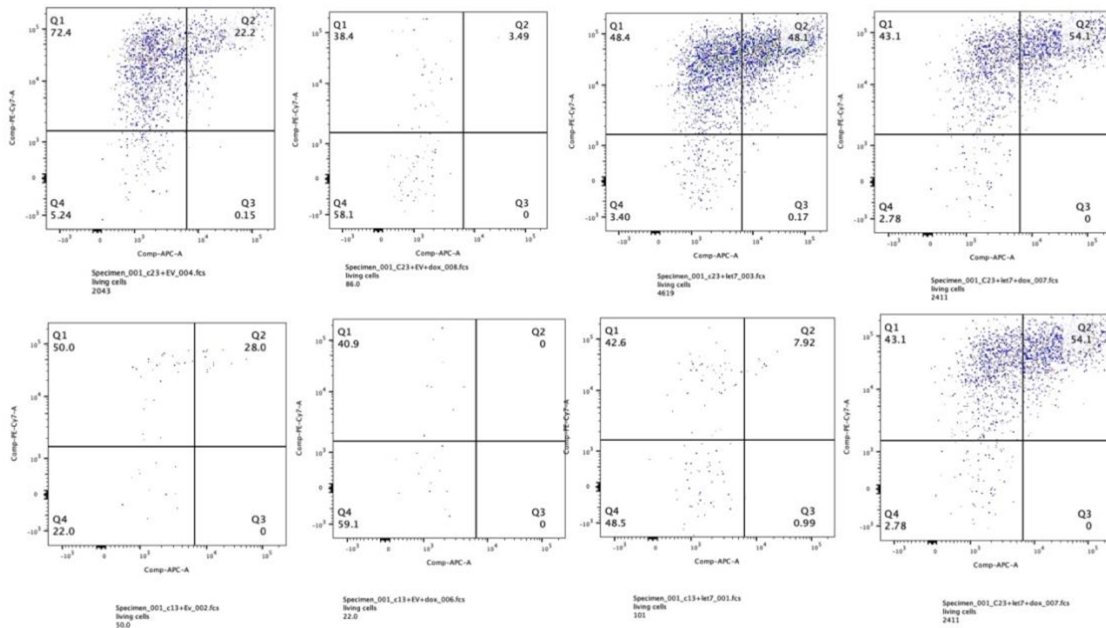


Figure 8. FACS data to determine the completeness of iPSC-to-HPC differentiation after 12 days.
Q1: CD45+ CD34-, Q2: CD45+ CD34+, Q3: CD45- CD34+, Q4: CD45- CD34-. Total MFI was calculated, and each quadrant was compared respectively. Comp-APC-A was stained for CD45+, and PE-Cy7-A (Bio Legend) was used for CD34+.

The C23 line was chosen for the remaining experiments because C13 did not differentiate efficiently. To investigate whether there was a lineage preference or bias when let-7g was overexpressed and whether dox had an independent effect, all HSCs from C23 were subjected to a colony-forming unit (CFU) assay.

To investigate whether the presence of miRNA let-7g affects lineage differentiation and whether induction by doxycycline has any influence, a CFU assay was

conducted. As lin28b fully represses let-7, it was unclear whether the presence of the gene would have any impact, or if overexpression was necessary to skew lineage differentiation. The results showed a decrease in overall colony production, especially granulocyte and erythroid colonies, when comparing C23+EV to C23+EV+Dox, indicating that doxycycline might have an independent effect, apart from gene addition. Comparing C23+EV to C23+Let-7, there was a decrease in colony number, but an increase in granulocyte production and a slight decrease in monocyte colony production. On the other hand, comparing C23+Let-7 and C23+Let-7+Dox, there was a decrease in granulocyte colony production, but significant increases in monocyte, granulocyte–monocyte, and erythroid production.

Due to the lack of sufficient data for statistical analysis, it was not possible to determine an overall lineage bias. However, when observing the lineage differentiation of cells in the presence of let-7g induced by dox, there was an increased tendency for HSCs to differentiate into monocytes, granulocyte-monocytes, or erythroid cells, and a decreased tendency to differentiate into granulocytes or a colony of all cell types combined (CFU-GEMM – this assay is known to be the progenitor cells for granulocyte, erythrocyte).

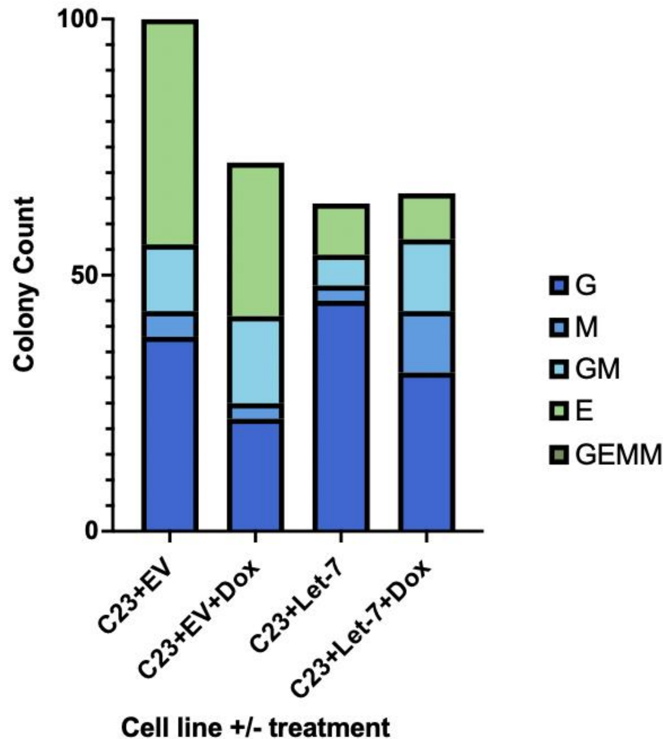


Figure 9. CFU assay of C23 HSCs post-differentiation.
HSCs derived from C23 iPSC were cultured in methylcellulose with cytokines for 15 days. Tx groups with dox had dox added to their methylcellulose. After 15 days in culture, colony morphology was scored visually, and mean counts were collected. CFU-M indicates monocyte lineage, CFU-G, granulocyte; CFU-GM, granulocyte–monocyte, CFU-E erythrocyte. Finally, CFU-GEMM, a combination of all population groups.

Determining if Induction of let-7 in differentiating Erythrocytes

The study also performed another lineage assay to evaluate the expression of fetal and adult hemoglobin (hb) in differentiating erythrocytes. This involved fixing and permeabilizing the cells, staining them with antibodies, and analyzing them using FACS. However, since C13 did not differentiate well into HPCs, it was expected that it would

also perform poorly in erythrocyte differentiation. For single expression controls, HUDEP 2 cells were used due to their known for their expression of adult hemoglobin and HUDEP 1 cells, which express fetal hemoglobin exclusively (Kurita et al., 2013)

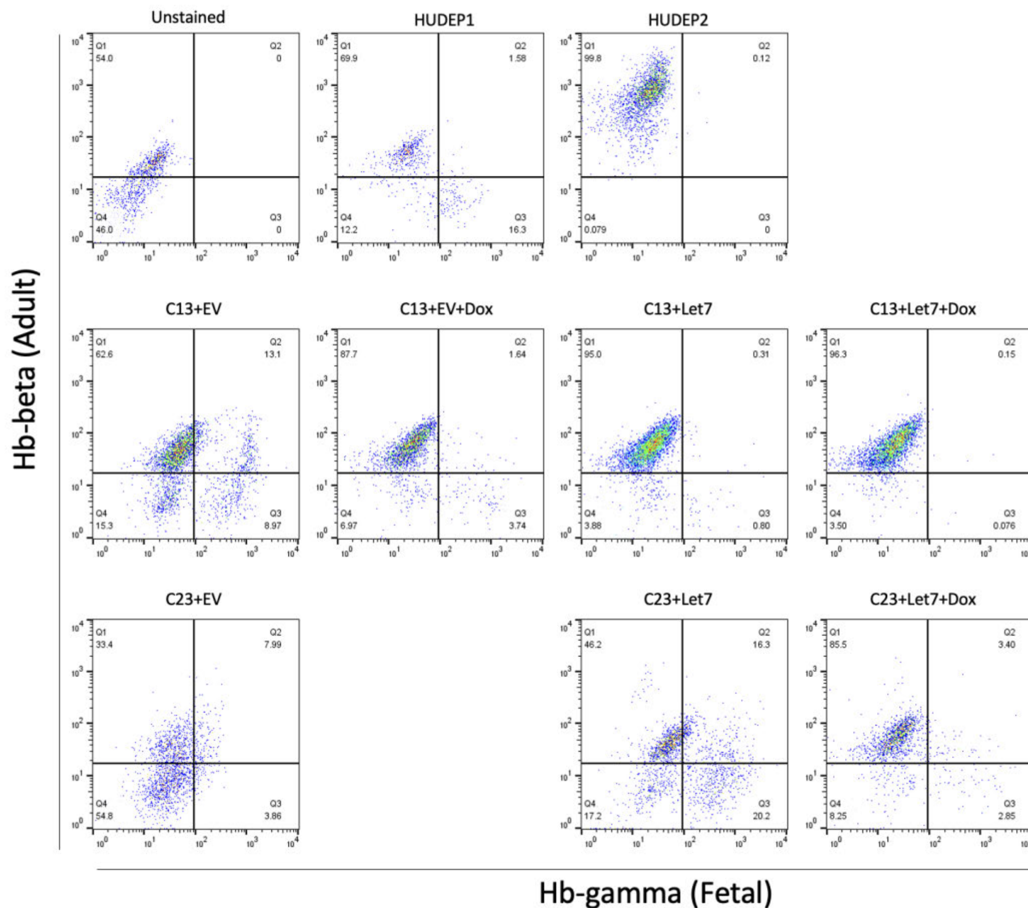


Figure 10. Intracellular Staining and Flow Cytometry to Determine the Presence of Fetal vs. Adult Hemoglobin.
In this figure, we illustrate the experimental approach used to distinguish between fetal and adult hemoglobin in the cells of interest. Intracellular staining of hemoglobin is performed, followed by analysis through flow cytometry to quantify the presence of fetal and adult hemoglobin. This method allows for the identification and characterization of different cell populations based on their hemoglobin expression profiles.

It can be seen in Figure 10, that in the C13 family the cells stained expressed majorly fetal hemoglobin even when let-7 was induced during erythrocyte differentiation. In the C23 family while one sample was missing from FACS analysis there was some adult hemoglobin seen when let-7 was induced. It appeared throughout the erythrocyte differentiation process that the cells did not respond well to the continual admission of doxycycline.

CHAPTER 4

Discussion

This thesis discussed the possibility of developing a new iPSC model that can produce HSCs with adult phenotypes, and how this model is crucial for advancing research on myelodysplastic syndrome (MDS). Even though the let-7g miRNA plays a significant role in regulating various biological processes such as cell differentiation, proliferation, and apoptosis, its synthesis is curtailed by lin28b, which is upregulated in several human malignancies, including acute myeloid leukemia (AML). AML is characterized by the accumulation of immature myeloid cells in the blood and bone marrow, leading to impaired hematopoiesis and differentiation capabilities.

The relationship between aging and the increase in clonal hematopoiesis, which can lead to the development of MDS, is a complex and multifactorial process involving various genetic and epigenetic alterations that accumulate over time. While genetic changes can occur at any age, the frequency of these mutations increases with age, resulting in mutations in HSCs that contribute to MDS development. Additionally, aging has an impact on the BMM, which plays a vital role in the regulation of HSCs. The BMM, consisting of stromal cells, cytokines, and extracellular matrices, provides signals essential for HSC maintenance and differentiation. Age-related changes in BMM composition and function can lead to alterations in HSC homeostasis, which may contribute to MDS development. Studying the mechanisms behind age-related changes in MDS can provide insight into the disease's pathophysiology and identify potential treatment targets.

In order to make progress in the field, it is important to establish a scientific model that is comparable to those used in clinical settings. There are still numerous steps to take before determining whether overexpression of let-7g is sufficient to create a new iPSC model that can generate adult-like HSCs when treated with doxycycline. Introducing an iPSC model that produces HSCs and lineage cells with adult characteristics would be significant for studying age-biased diseases that can occur at any time but become more prevalent in adults. If let-7g expression is induced by adding doxycycline at different stages of development, fetal-like models can be used to investigate how current treatment standards for conditions like MDS impact these models compared to fetal-like models. If doxycycline was added to induce let-7g expression in iPSC, HSC, MPP, and progenitor stages, it would be possible to conduct research using adult-like models to evaluate the effectiveness of current treatments for age-biased diseases, such as MDS, compared to fetal-like models.

The purpose of this thesis was to investigate whether iPSCs that were transduced with the PKLO-Tet-on plasmid, containing the let-7 gene insert, a dox-inducible element, and a puromycin-resistant insert, could differentiate into HSCs that maintained an adult phenotype when further differentiated. The initial qPCR experiment indicated that the transduction of C13 and C23 (two iPSC lines) was successful due to the increased expression of let-7g when dox was introduced (as shown in Figures 5 and 6). However, the fold-change observed experimentally, which was approximately 4-fold increase in let-7g miRNA expression when dox was added, was lower than the anticipated 10-11-fold increase. This could be attributed to the selection of a colony with weaker let-7 expression

during the generation of the let-7g plasmid for lentiviral production, or because dox may have significant or unknown interactions with iPSCs. Nonetheless, these lines were utilized for subsequent experiments to investigate if any effects could be observed

Nevertheless, further examination confirmed that some samples had successfully differentiated into HPCs. The 34+/45+ flow cytometry was performed to determine whether samples had differentiated into HSCs over the 12-day differentiation period. The C23 family showed more complete differentiation than the C13 family, which contains SRSF2, one of the most common MDS-causing mutations. Since this mutation and other downstream mutations may be present, this line was expected to struggle to differentiate. Figure 8 showed that Q2 was CD34+/CD45+, indicating that differentiation into HSC was complete and that the cell type was an actual HPC. By contrast, Q1 demonstrated CD45+CD34-, indicating incomplete differentiation into HSCs and less mature cell types. In MDS, there is some indication that CD45+CD34+ cells can indicate committed myeloid progenitors, which would explain the observed increase in myeloid bias (Ogata et al., 2004).

The results indicate the need to improve the efficiency and comprehensiveness of differentiating the iPSC lines into HSCs. To achieve better differentiation, we may need to explore alternative approaches such as extending the differentiation period beyond day 12 of the 2D-differentiation assay, possibly up to day 21. Additionally, the impact of adding dox on differentiation requires further investigation.

This thesis aims to introduce a novel model for myelodysplastic syndrome (MDS) that may more accurately resemble an adult state. The existing in vitro models that appear

fetal fail to fully imitate the intricate biology of human MDS while modeling adult disorders. Our newly designed let-7-edited model overcomes this obstacle by providing an accurate representation of the disease in adults without imposing additional stress on cells that could interfere with hematopoiesis. The model was created using iPSCs derived from either a healthy donor (C23) or iPSCs containing the SRSF2 mutation commonly observed in MDS (C13). By subjecting these cells to specific conditions, they were differentiated into hematopoietic lineages, resulting in a functional model for MDS and AML testing.

The new model developed in this thesis could offer several advantages over existing models used to study MDS if further studies are done. One major limitation of current in vitro models is their inability to fully recapitulate the complexity of the disease in adult humans due to iPSCs innate expression of fetal cells. By using iPSCs derived from both healthy donors and those with the SRSF2 mutation commonly found in MDS, the new model was able to generate a more adult-like environment for studying the disease.

The increased complexity and more adult phenotype of the new model could provide several benefits in the future once a better understanding of the effects of let-7 induction and independent effects of doxycycline is better understood. Some of these benefits could include the potential to identify new therapeutic targets and develop more effective treatments. Additionally, the model could be used to investigate the molecular mechanisms of MDS and gain a deeper understanding of how the disease progresses to AML.

Future studies and experiments will look towards elucidating a better differentiation protocol from iPSC to HPC in order to get a more robust differentiation. Other experiments

such as differentiation of the HPCs into macrophages and the continual differentiation into erythrocytes will be done to determine if the induction of let-7 does yield an adult phenotype.

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