Characterizing the Impact of the RNA Demethylase ALKBH5 on Hematopoietic Stem and Progenitor Cells

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Abstract

The RNA demethylase, ALKBH5, is a known prognostic factor in various diseases as well as critical in maintaining the leukemia initiating cells in acute myeloid leukemia. However, its role in normal hematopoiesis is unclear to date. Herein, I investigated the role of ALKBH5 in the context of human umbilical cord blood (CB) derived hematopoietic stem and progenitor cell (HSPC) self-renewal and differentiation in *ex-vivo* cultures and *in-vivo* systems. ALKBH5 expression was reduced in CB HSPCs using RNA interference (RNAi) strategy to interrogate the kinetic, phenotypic, and functional contribution of ALKBH5 in *ex-vivo* and *in-vivo* models of normal hematopoiesis. Deficiency of ALKBH5 led to higher (2.1-fold, $P<0.05$) expansion in the CD34+CD45RA- cell population compared to scramble control. Alternatively, hematopoietic stem cell enriched (eHSC, CD34+CD45RA-CD90+CD49f+) population was diminished ($P<0.05$) in ALKBH5 deficient samples. Functional capacity to produce colonies in colony forming assays was reduced by 1.5 times ($P<0.05$) in ALKBH5 deficient samples. Finally, *in-vivo* mice transplantation data show reduced long-term engraftment (3-times lower platelet concentration in peripheral blood, $P<0.05$) as well as altered human multi-lineage chimerism formation in the recipient of ALKBH5 reduced HSPCs. Thus, my experimental results demonstrate hitherto unexplored roles of ALKBH5 in regulating normal function of HSPCs. These findings underscore the need to further investigate ALKBH5 to improve our understanding of stem cell biology and to develop targeted therapies for diseases like leukemia.

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List of Abbreviations

AA2P - L-Ascorbic acid 2-phosphate

AD - Alzheimer's disease

AHR - Aryl hydrocarbon receptor

ALKBH5 - AlkB homolog 5

AML - Acute myeloid leukemia

ANOVA - Analysis of variance

BFU-E - Burst forming unit- erythroid

BMI1 - B lymphoma Mo-MLV insertion region 1 homolog

CAR - CXCL12-abundant reticular cell

CB - Umbilical cord blood

CD - Cluster of Differentiation

cDNA - complementary DNA

CFU - Colony forming unit

CFU-GEMM - Colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte

CFU-GM - Colony forming unit- granulocyte, macrophage

CLP - Common lymphoid progenitor

CMP - Common myeloid progenitor

CT -Threshold cycle

CXCL12 - C-X-C motif chemokine ligand 12

CXCR4 - C-X-C chemokine receptor type 4

DEG - Differentially expressed gene

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic Acid

EC - Endothelial cell

eHSC - HSC enriched cell population

ETV6 - ETS Variant 6

EZH2 - Enhancer of Zeste Homolog 2

FACS - Fluorescence activated cell sorting

FBS - Fetal bovine serum

FBS - Fetal bovine serum

FLT3L - FMS-like tyrosine kinase 3 ligand

FTO - Fat mass and obesity associated

G2S - Synthesis and gap 2 phases

G-CSF - Granulocyte colony-stimulating factor

GFP - Green fluorescent protein

GMP - Granulocyte/macrophage progenitor

GVHD - Graft-versus-host disease

HDAC - Histone deacetylase

HIF - Hypoxia-inducible factor

HLA - Human leukocyte antigen

HMGA2 - High-mobility group AT-hook 2

HRP - Horseradish peroxidase

HSC - Hematopoietic stem cell

HSPC - Hematopoietic stem and progenitor cell

IGF2BP - Insulin-like growth factor 2 mRNA-binding protein

IgG - Immunoglobulin G

IMDM - Iscove's modified Dulbecco's medium

IT-HSC - Intermediate term hematopoietic stem cell

KD - Knockdown

KD1 - ALKBH5 shRNA 1

KD2 - ALKBH5 shRNA 2

LDL - Low-density lipoprotein

LDS - Lithium dodecyl sulfate

LIC - Leukemia initiating cell

LMPP - Lymphoid-primed multipotent progenitor

LSD1 - Lysine-specific histone demethylase 1A

LT-HSC - Long term hematopoietic stem cell

m6A - N6-methyladenosine

$m6A_m$ - N6, 2'-O-dimethyladenosine

MEP - Megakaryocyte/erythrocyte progenitor

METTL3 - Methyltransferase-like 3

MK - Megakaryocyte

MNC - Mononuclear cell

MOI - Multiplicity of infection

MPP – Multipotent progenitor

mRNA - Messenger ribonucleic acid

MSC - Mesenchymal stromal cell

NFATc1 - Nuclear factor of activated T-cells, cytoplasmic 1

NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells

PBS - Phosphate-buffered saline

Pen-Strep - Penicillin-streptomycin

PFA - Paraformaldehyde

PMN - Polymorphonuclear cells

PRDM16 - PR/SET Domain 16

RBC - Red blood cell

RCOR1 - REST co-suppressor 1

RNA - Ribonucleic Acid

RNAi - RNA interference

RT-qPCR - Reverse Transcription quantitative Polymerase Chain Reaction

SCAC - Stem cell agonist cocktail

SCF - Stem cell factor

SCID-X1 - X-linked severe combined immunodeficiency

SCR - Scrambled shRNA

SEM - Standard error of the mean

SFEM - Serum-free expansion medium

SFM - Serum-free medium

shRNA - Short-hairpin RNA

SNS - Sympathetic nervous system

SOX17 - Sex Determining Region Y-Box 17

SR1 - StemReginin1

SRC - Skid repopulating cell

STFL - SFEM + SCF + TPO +FLT3L + LDL + Pen-Strep

ST-HSC - Short term hematopoietic stem cell

TACC3 - Transforming acidic coiled-coil containing protein 3

TBS - Tris-buffered saline

TBS-T - TBS + Tween-20

TET2 - Ten-eleven translocation 2

TPO - Thrombopoietin

Treg - Regulatory T cell

VCAM-1 - Vascular cell adhesion molecule 1

VPA - Valproic acid

WTAP - Wilms' tumor 1-associating protein

YTH - YT521-B homology

YTHDC - YTH domain containing protein

YTHDF - YTH domain family protein

Chapter 1: Introduction

1.1 Hematopoietic stem cells (HSCs): Properties & function

Hematopoietic stem cells (HSCs) are non-specialized cells with indefinite regenerative capacity that primarily reside in bone-marrow micro-environments and give rise to all types of blood cells of the organism[1]. The idea that such single precursor cell can give rise to all blood cells was introduced in early 20th century by the histologist Alexander A. Maximow[2]. Subsequently, the experimental evidence for the existence of the HSCs was presented by James Till and Ernest McCulloch through their seminal work with lethally irradiated mice in the 1960s[3,4].

HSCs possess some unique properties that set them apart from non-stem cells. HSCs in their *in-vivo* environment maintain a state of quiescence, unless activated by external signals to initiate hematopoiesis[5,6]. Active HSCs can differentiate into multiple cell lineages (Figure 1) that can repopulate the entire hematopoietic system if necessary[7,8]. However, when HSCs are not actively differentiating to progenitor cells, a portion of the stem cell population continue to self-renew throughout the lifecycle of the organism to maintain the pool of stem cells[9-11]. Another property of stem cells that plays important roles in HSC function, maintenance and stem cell transplantation therapy is their homing capacity, which is the ability of HSCs to migrate from the peripheral blood to the bone marrow[12-14].

The functions of the HSCs derive from their properties. HSCs' quiescence and self-renewal capabilities enables them to maintain an undifferentiated HSC pool throughout the lifecycle of the organism[15,16]. A portion of the undifferentiated cells leave the HSC pool regularly to participate in hematopoiesis and produce all blood cells through differentiation.

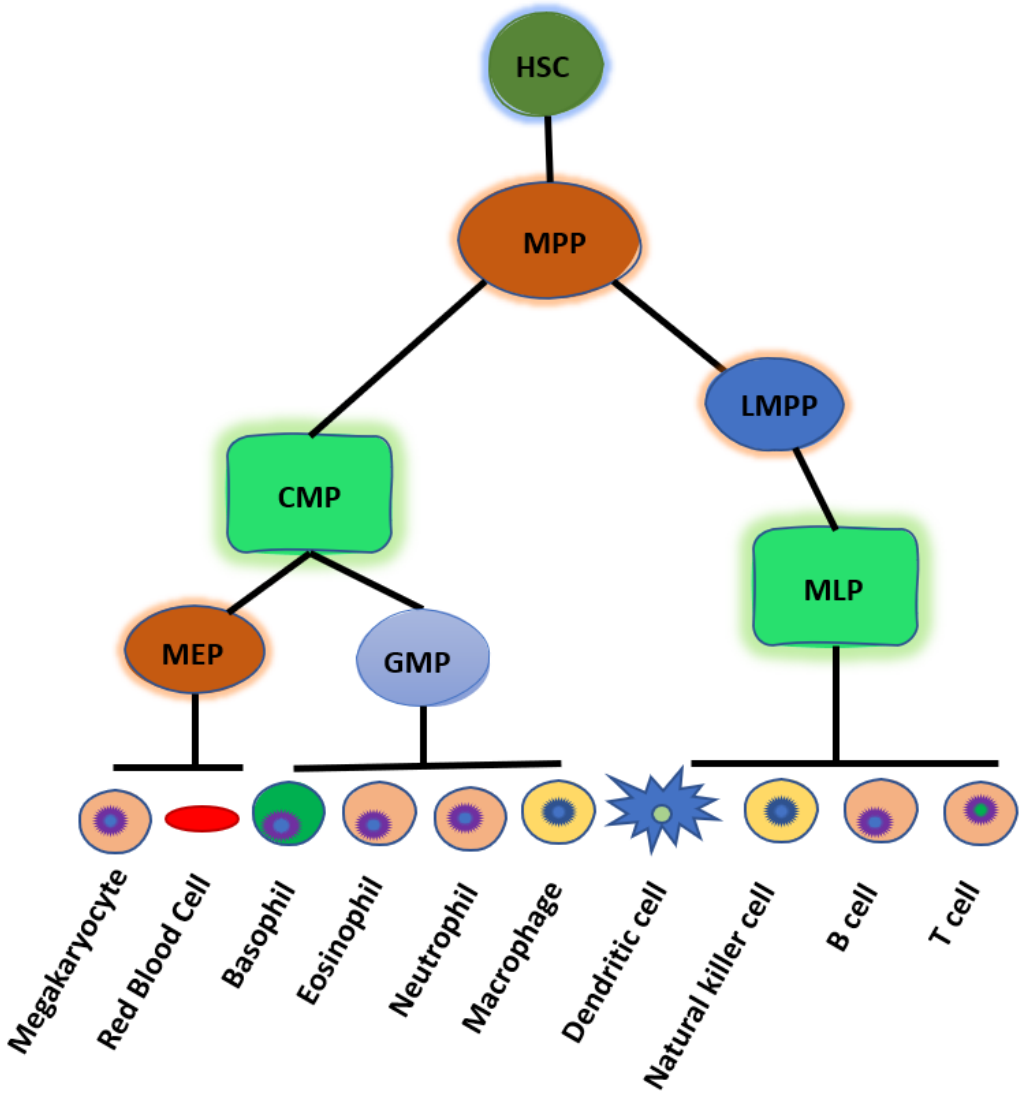


Figure 1. Hematopoietic system hierarchy. This diagram presents multipotent hematopoietic stem cells (HSCs) residing at the top of the hematopoietic hierarchy. The HSCs then differentiate into progenitors which are further restricted in their capability to give rise to different blood cells as well as their self-renewal capacities. These are multipotent progenitors (MPPs) which differentiate into common myeloid progenitors (CMPs) and lymphoid primed multipotent progenitors (LMPPs). CMPs subsequently give rise to megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs). Mature blood cells derived from MEPs are megakaryocytes and red blood cells; from GMPs are basophils, eosinophils, neutrophils, and macrophages. On the other hand, LMPP derived MLPs differentiate into natural killer (NK) cells, B cells, and T cells. Dendritic cells have been proposed to have multiple precursors including CMPs and MLPs. Diagram adapted from Zhang et al. (2018)[17].

Thus, HSCs ensure life-long generation of blood in the individuals[18,19]. Moreover, HSCs respond to stress and injury to the organism by upregulating HSC proliferation and differentiation to restore a physiological balance[20-22]. Finally, the homing aspect of HSCs contributes directly to the reconstitution of the hematopoietic and immune systems of HSC transplant recipients[23].

1.2 HSPC identification using cell surface markers

HSPCs express different cell surface markers as they progress through the differentiation process[24]. These cell surface markers are primarily proteins including phosphoglycoproteins, but can also be lipids or carbohydrates among other molecules[25-27]. While there are few different classification systems available to designate cell surface markers, the Cluster of Differentiation (CD) nomenclature is widely used to identify HSPCs. The CD nomenclature system assigns a number to each cell surface marker[28]. Single or a combination of CD markers are used to identify different cell populations within HSPCs[28,29]. CD34, a membrane glycoprotein which is widely expressed on the surface of HSPCs[30-32], has role in signal transduction and cell adhesion beside being an identifying mark[33,34]. CD45 is another glycoprotein marker which can be found on the nucleated hematopoietic cells such as monocytes, granulocytes, macrophages, B cells, and T cells[35,36]. In addition, CD45 has multiple isoforms which are expressed in varying levels in active and inactive lymphocytes[37,38]. Some other commonly used CD markers are CD38, CD90, CD19, CD49f, CD123, CD41a, and CD33[28]. A combination of cell surface marker (i.e. CD markers) to identify specific HSPC sub-populations are presented in Table 1.

Table 1. HSPC sub-population identification based on CD marker combinations. The table presents various human CD markers which can be used in combination to identify different HSPC sub-populations using flow cytometry as described by Cimato et. al. (2016)[29].

HSPC sub-population	CD marker combination
HSC enriched (eHSC)	CD34+CD45RA-CD90+CD49f+
Multipotent progenitor (MPP)	CD34+CD45RA-CD90-
Common myeloid progenitor (CMP)	CD34+CD45RA-CD123+
Granulocyte/macrophage progenitor (GMP)	CD34+CD45RA+CD123+
Megakaryocyte/erythrocyte progenitor (MEP)	CD34+CD45RA-CD123-
Lymphoid-primed multipotent progenitor (LMPP)	CD34+CD45RA+CD90-

1.3 Bone marrow niche and other sources of HSCs

The HSCs require a controlled environment to reside and function optimally. The bone marrow niche is a complex microenvironment specialized in supporting HSC maintenance, self-renewal, and differentiation[18,39-41]. This microenvironment hosts a plethora of cells including endothelial cells, mesenchymal stromal cells, and osteoblasts beside HSCs[42-47] (Figure 2). The bone marrow niche can be further subdivided into endosteal and vascular niches. These two compartments of the bone marrow niche contribute differently to the physiological regulation of HSCs in-vivo. The endosteal niche is primarily composed of osteoblasts that supports HSC quiescence through the secretion of factors like angiopoietin-1 and osteopontin[15,48,49]. Alternatively, the vascular quarter of the niche is enriched in endothelial

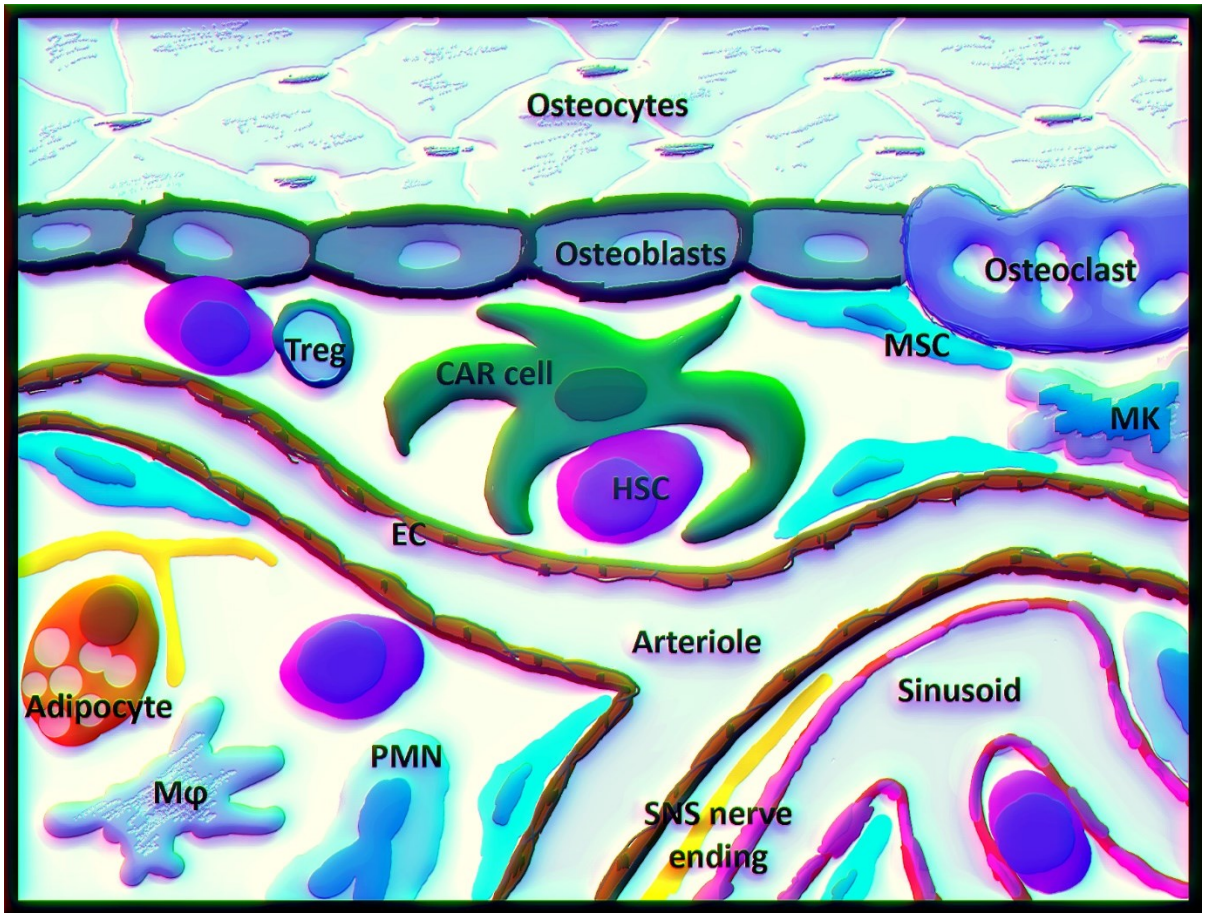


Figure 2. Bone marrow niche micro-environment for hematopoietic stem cells. Hematopoietic stem cells reside in the bone marrow supported by a heterogenous population of cells which include osteoblasts, mesenchymal stromal cells (MSCs), endothelial cells (ECs), polymorphonuclear cells (PMNs), macrophages (Mϕs), CXCL12-abundant reticular (CAR) cells, adipocytes, regulatory T cells (Tregs), osteoclasts, megakaryocytes (MKs). These cells are arranged around the bone marrow micro-vasculatures, sympathetic nervous system (SNS) nerve fibres, and sinusoids. Figure adapted from review article by Laura M. Calvi (2020)[58].

and perivascular stromal cells which contribute to HSC maintenance and self-renewal through secretion of CXCL12 factor and stem cell factor (SCF)[43,50-52]. These niche cells along with the extracellular matrix create an environment that is hypoxic, which stabilizes hypoxia-inducible factors (HIFs) controlling HSC self-renewal, quiescence, and metabolism by regulating gene expression in HSCs[53-55]. Hypoxia also ensures the protection of HSCs from oxidative stress by limiting the production of reactive oxidative species[56]. Finally, the bone marrow niche secretes various cell adhesion molecules that support the homing, adhesion, and anchoring of the HSCs in the niche[40,57]. The advantages of using bone marrow as the source of HSCs are increased engraftment success rate due to the presence of high number of hematopoietic stem and progenitor cells (HSPCs) in the niche, as well as sustained hematopoiesis in the recipient as these HSCs have long-term repopulating potential[59,60]. One major challenge in using bone marrow as HSC source is finding human leukocyte antigen (HLA) matched donor for the patients with rare HLA types[61,62]. Moreover, since HSC collection from the bone marrow is an invasive procedure, it can be painful for the donor with an added risk of infection and bleeding from the puncture site[63].

Peripheral blood (PB) can also be used as the source of the HSCs by mobilizing the HSCs residing in the bone marrow niche. Although the mechanism of HSC mobilization is not yet fully understood, growth factors like granulocyte colony-stimulating factor (G-CSF) can be used to mobilizes the bone marrow HSCs[64,65]. PB provides multiple advantages over bone marrow as a source for HSCs. For example, faster engraftment of PB derived HSCs makes PB a good candidate for a source and is associated with higher chance of successful engraftment[66]. Moreover, HSC collection from PB is less invasive and thus reduces the chance of infection, bleeding and pain in the donor[67]. However, limited mobilization of the HSCs from the BM can

be a concern in some cases[68], whereas increased chances of chronic graft-versus-host disease (GVHD) may be of concern in some other cases[69]. Despite these limitations, peripheral blood continues to be used as a source of HSCs in appropriate situations.

Umbilical cord blood (CB) is another source of HSCs that provides several advantages over bone-marrow and peripheral blood. First of the advantages is, it being non-invasive for the donor as the CB is collected from donated post-partum placenta[70]. Moreover, CB derived HSC transplantation is associated with reduced risk of GVHD[71]. However, the lower HSC dosage in the CB remains a hurdle of its use as a source of HSCs[72].

1.4 Applications of HSCs

HSC's ability to treat leukemia through bone marrow transplantation was first reported by Thomas et. al. in 1957[73], which was before the experimental demonstration of the existence of HSCs by Till and McCulloch in 1960s[3,4]. Since then, HSC transplantation has been used to treat other hematological malignancies like lymphoma and myeloma, as well as non-malignant hematological disorders like sickle cell anemia, aplastic anemia, and β-thalassemia[74-79]. Beside hematological disorders, certain inherited metabolic disorders like Hurler syndrome (mucopolysaccharidosis type I) and adrenoleukodystrophy can also be treated with HSC transplants[80-83]. More recently, encouraging outcomes have been demonstrated in HSC mediated treatment of autoimmune diseases like systemic sclerosis and multiple sclerosis[84,85]. HSCs have also been used to implement gene therapies. For example, Poletti et al (2016) used gene corrected HSCs to treat X-linked severe combined immunodeficiency (SCID-X1) patient that led to restoration of their immune function[86]. Another promising application for HSCs is their use in regenerative medicine and tissue repair, as HSCs have self-renewal properties and can differentiate to various blood cell lineages. HSC mediated treatment schemes have

demonstrated improved outcome in patients with ischemic diseases like myocardial infarction and peripheral artery disease by enhancing angiogenesis and tissue repair[87-89].

In addition to developing HSC based treatment protocols for various diseases, HSCs are also used to model different patho-physiological processes as well as to improve our understanding of HSC biology through *ex-vivo* and *in-vivo* experimentation[90-92].

1.5 *Ex-vivo* expansion of HSPCs: strategies and outcome

Various applications of HSCs and the distinct advantages and disadvantages associated with each source of HSC are driving factors for *ex-vivo* expansion of HSCs to achieve optimum cell number and properties. *Ex-vivo* expansion of HSCs refers to the process of increasing the HSC numbers in controlled laboratory environment before transplantation or use in other experimental procedure. The *ex-vivo* expansion of HSCs can help overcome some of the issues associated with stem cell transplantations, for example: GVHD due to HLA incompatibility and graft failure due to low dosage of HSCs[93,94].

Multiple *ex-vivo* expansion methods have been proposed and being improved upon by different laboratories. Some of these strategies include cytokines stimulation, co-culture systems, and independent or combined use of small molecules and growth factors. Cytokines are a group of soluble proteins or glycoproteins which perform critical roles in the maintenance, differentiation, and functions of HSCs[95-99]. Combinations of cytokines like stem cell factor (SCF), thrombopoietin (TPO), and FMS-like tyrosine kinase 3 ligand (FLT3L) have been shown to enhance the HSPC expansion in *ex-vivo* culture[100,101].

Alternatively, co-culture systems attempt to recreate approximation of physiological environment for the HSPCs in *ex-vivo* culture in the presence of other cells which support

HSPC growth and expansion. Co-culturing mesenchymal stromal cells and endothelial cells have been shown to support the stemness of the HSPCs as well as significantly improve the HSPC expansion[102,103].

Next, small molecules like StemReginin1 (SR1), UM171, valproic acid (VPA), L-ascorbic-acid 2-phosphate (AA2P) act as stem cell agonists due to their effective HSPC expansion capabilities[104-107]. SR1 is an aryl hydrocarbon receptor (AHR) antagonist and UM171 is a pyrimidoindole derivative that act by balancing the pro- and anti-inflammatory pathways and by suppressing Lysine-specific histone demethylase 1A (LSD1)/REST co-suppressor 1 (RCOR1) suppressor complex via proteasomal degradation leading to the preservation of important epigenetic marks within HSPCs[104,105,108]. VPA is another epigenetic modifier that can stimulate HSPC expansion by inhibiting histone deacetylase (HDAC)[109]. AA2P, on the other hand, enhances HSPC expansion through ten-eleven translocation 2 (TET2) mediated DNA demethylation by acting as the ligand for TET2[110].

Ex-vivo cultures of HSPC supplemented with very specific concentration of these four stem cell agonists (SR1, UM171, VPA, and AA2P) expands HSCs in a synergistic way as evidenced by increase in the skid repopulating cells (SRC) in limiting dilution assay. The combination of the agonist molecules is termed stem cell agonist cocktail (SCAC) (Table 2) and the lead SCAC X2A was able to produce a 15-fold increase in SRCs in culture over input. Comparison of single and bulk RNAseq data of X2A cultured and non-cultured HSPCs revealed that, RNA demethylase AlkB homolog 5 (ALKBH5) was among the differentially expressed genes (DEGs) which were upregulated in the SCAC X2A cultured cells[110].

Table 2. Composition of stem cell agonist cocktails. The table shows concentrations of small molecules StemReginin1 (SR1), UM171, valproic acid (VPA), and L-Ascorbic acid 2-phosphate (AA2P) in stem cell agonist cocktails (SCACs) termed X2A, X2B, SMA, SM2, and SM6.

SCACs	SR1 (nM)	UM171 (nM)	VPA (nM)	AA2P (µM)
X2A	2500	62	0.01	1000
X2B	2500	62	0.01	0.1
SMA	5023	0.35	0.502	1000
SM2	5023	125.63	0.01	1000
SM6	1000	38	0.125	250

1.6 RNA demethylases and methyl-based RNA modifications

ALKBH5 is one of the two known RNA demethylases which are associated with posttranscriptional modifications of RNA, by which gene expression can be regulated[111]. The other RNA demethylase is fat mass and obesity associated (FTO) protein. Together they are called “erasers”, as both can remove N6-methyladenosine (m6A) marks from the RNA molecules. However, FTO has the capability to remove N6, 2’-O-dimethyladenosine ($m6A_m$) as well. Among hundreds of posttranscriptional RNA modifications m6A and $m6A_m$ are the most abundant (these modifications have been reviewed in-depth by Gilbert at al (2016)[112]). While m6A was first discovered in the early 1970s in *Escherichia coli*, this modification has since been found in various species ranging from viruses, yeast to fruit flies and humans[113-117]. Consensus motifs DRACH (D = G, A or U, R = G or A, H = A, C or U) located within the 3’-untranslated regions (3’UTRs), near stop codon and within internal long exons were found to

be enriched in m6A marks[117,118]. m6A modifications are introduced to the RNAs by a group of proteins called “writer complex”, which includes methyltransferases like methyltransferase-like 3 (METTL3), METTL14, Wilms’ tumor 1-associating protein (WTAP) and few other proteins[119,120].

Another set of proteins specializing in the recognition of m6A modified sites are called “readers”. The readers include YT521-B homology (YTH) domain family proteins (YTHDF1~3)[121-123], YTH domain containing proteins (YTHDC1~2)[124,125], Insulin-like growth factor 2 mRNA-binding proteins (IGF2BP1~3)[126-128]. m6A modifications on RNA are dynamically regulated by these writers, erasers and readers. m6A modification can alter gene expression, cellular function and biological processes through modulating RNA stability. However, the stabilizing or destabilizing effect of m6A on RNA is context dependent and dictated by the reader proteins that recognize the methylation mark. For example, recognition of m6A modified RNA by the m6A reader YTHDF2 leads to destabilization of the target RNA by moving it from translatable pool to the RNA decay sites[129]. On the other hand, when the m6A methylated RNA is recognized by YTHDF1, it leads to the recruitment of the translation machinery and ultimately protein production from the m6A marked transcript[130]. The RNA demethylase ALKBH5 can regulate mRNA expression in cells via adjustment of m6A levels. Thus, the m6A marks can control the stability of the mRNA transcripts in various fashions as mentioned above (Figure 3).

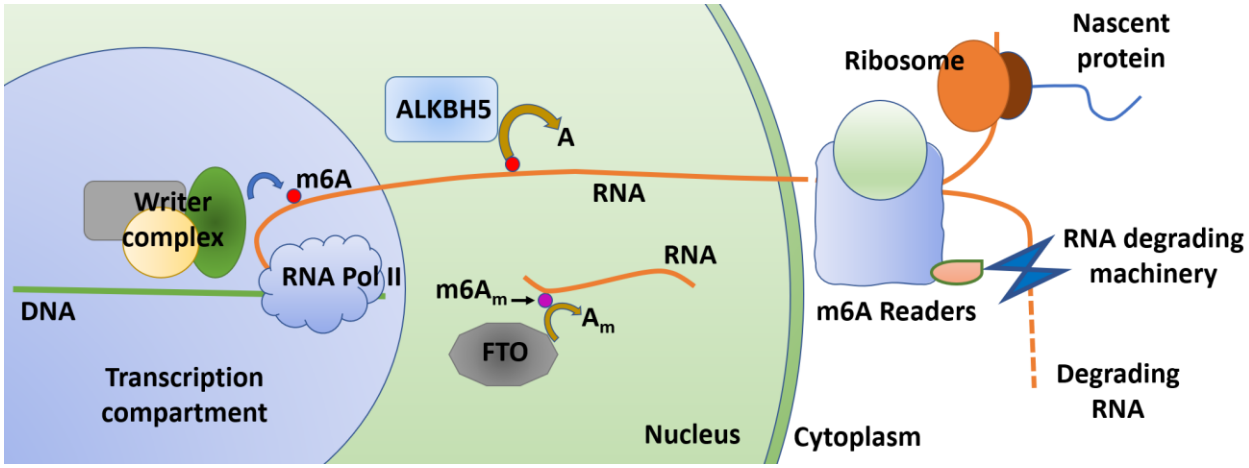


Figure 3. N6-methyladenosine modification of RNA. N6-methyladenosine (m6A) modifications on nascent RNAs are introduced by a group of proteins termed writer complex. These m6A marks can be removed by either ALKBH5 or FTO, together called m6A erasers. FTO has the additional capability to remove N6, 2'-O-dimethyladenosine ($m6A_m$) mark as well. Following m6A level adjustment by m6a erasers, RNA is transported out of the nucleus to the cytoplasm. In cytoplasm, m6A marks on RNAs are recognized by another group of proteins which are called m6A readers. m6A readers guide the RNAs toward either translation or degradation leading to increased or decreased protein expressions respectively Figure adapted from Zaccara et. al., (2019)[131].

1.7 ALKBH5 and hematopoiesis

Hematopoiesis is a process by which multipotent hematopoietic stem cells give rise to all the blood cells through a tightly controlled differentiation process. Strict control of the genetic expression in every step of differentiation ensures the normal hematopoiesis. Due to the ability of ALKBH5 to influence genetic expression through regulation of m6A levels on transcripts, ALKBH5 has the potential to impact different biological processes. Therefore, ALKBH5 has been investigated by researchers in the context of hematopoiesis to understand the extent of its impact in the physiology of hematopoiesis. Gao et al (2021) reported that ALKBH5 deficient HSPCs in mice model had a diminished ATP production but did not notice any impact in the long-term HSCs, alluding to the presence of an alternate mechanism of energy production which is not associated with ALKBH5 control[132]. In another study, Wang, et al (2020) used human CB cells to demonstrate that ALKBH5 deficient samples had reduced (albeit non-significant) colony forming potential. However, they did not find any noticeable difference in the hematopoiesis of the ALKBH5 knockout mice compared to the wild type mice[133]. Alternatively, Shen et al (2020) found a moderate decrease in the HSC population and modest increase in the progenitor and the differentiated cells in their ALKBH5 knockout mice cohort[134]. Overall, experimental data available thus far suggest a limited role of ALKBH5 in murine hematopoiesis *in-vivo* and non-significant impact on human HSPC differentiation *ex-vivo*.

1.8 ALKBH5 in human pathologies

Since its discovery in the mammalian system, ALKBH5 has been implicated in different disease processes in humans. Multiple studies have brought forth experimental evidence that ALKBH5 is closely associated with the immune system and thus play critical

roles in the pathogenesis of different diseases. For example, ALKBH5 mediated suppression of immune system via AXIN2-Wnt/DKK1 axis was found to promote colorectal tumorigenesis[135]. Li et al (2020) demonstrated that in the absence of ALKBH5 there is a decline in polymorphonuclear myeloid-derived suppressor cells which are critical in the recruitment of regulatory T cells, an important component of the tumor immune-response[136]. Moreover, ALKBH5 was found to be critical in PD-L1 mediated immune evasion in glioma[137]. Thus, ALKBH5's capability to modulate immune system contribute to its role in various diseases.

Invasiveness of malignant tumors as well as their growth and the stemness of cancer stem cells have also been shown to be regulated by ALKBH5. In acute myeloid leukemia, the self-renewal of leukemia initiating cells have been found to be dependent on ALKBH5/m6A/TACC3/MYC-p21 axis underscoring a unique technique by which malignant cells take advantage of ALKBH5[134]. Alternatively, in pancreatic ductal adenocarcinoma (PDAC), decreased ALKBH5 level is associated with poor prognosis in patients and silencing of this RNA demethylase led to enhanced proliferation, migration, and invasiveness of PDAC cells[138].

Furthermore, neurological disorders like cobalt induced Alzheimer's disease (AD) have been found to be associated with diminished level of ALKBH5. ALKBH5 deficiency can exacerbate cobalt induced nerve damage in AD[139]. In endocrinological disorder such as type 2 diabetes mellitus, ALKBH5 level was found to be elevated[140]. Thus, interrogating the correlation of ALKBH5 with different diseases has revealed complex and variable roles of ALKBH5 in disease pathogenesis, prognosis, and treatment.

1.9 Downstream targets of ALKBH5

ALKBH5 can regulate the expression of some transcripts by modulating the m6A marks. For instance, demethylation of m6A by ALKBH5 can enhance the stability of some transcripts like FOXM1[141], NANOG[142], TACC3[134], and AXL[133]. Beside impacting the stability of some transcripts directly through demethylation process, ALKBH5 can also impact the stability of some mRNAs through indirect interactions. For example, ALKBH5 can interact with m6A reader YTHDF2 to control the stability of JAK1 mRNA[143].

1.10 Rationale

ALKBH5 is an integral part of many physiological and pathological processes which makes it an interesting target for in-depth research. ALKBH5 has thus far been implicated in various disease processes and been proposed as a prognostic factor or a target for therapies. However, case-to-case variations of the impact of increased or decreased ALKBH5 levels indicate the distinct functional role of ALKBH5 in different pathological or physiological processes. The increased ALKBH5 expression in *ex-vivo* expanded CB HSPCs (Pineault/Maganti Lab data) brings up the question how ALKBH5 is modulating cellular physiology in culture conditions and if the impact extends to in-vivo systems. Understanding the impact of ALKBH5 in the context of normal hematopoiesis through experimentation with *ex-vivo* and *in-vivo* model systems will further the knowledge in stem cell biology. This knowledge may help identify appropriate targets that can be used in the treatment of different pathologies including leukemia, where ALKBH5 expression is altered.

1.11 Hypothesis and Objectives

Hypothesis: The RNA demethylase ALKBH5 reduces the self-renewal and alters the differentiation capacity of HSPCs in *ex-vivo* cultures and *in-vivo* systems.

Objective 1: Test and confirm the ALKBH5 knockdown (KD) capabilities of short-hairpin RNAs (shRNAs).

Screen shRNAs for their capabilities to reduce ALKBH5 transcript and protein levels using erythroleukemic cell line. Confirm ALKBH5 transcript reduction in CB HSPCs.

Objective 2: Track the impact of knockdown of ALKBH5 on the growth and differentiation of HSPCs in *ex-vivo* cultures.

Investigate the impact of ALKBH5 deficiency on CB HSPC expansion rate, HSPC sub-population composition, cell cycle entry status, and global m6A level using flow cytometry. Assess the stability of ALKBH5 targets in CB cells using RT-qPCR and estimate the colony forming potential of ALKBH5 reduced cells using colony forming assay.

Objective 3: Investigate the impact of ALKBH5 reduction within HSPCs *in-vivo* using xenotransplantation model.

Investigate the engraftment capacity and multilineage chimerism formation in the immunodeficient humanized mice model transplanted with ALKBH5 knocked down CB HSPCs.

Chapter 2: Materials and Methods

2.1 Cord Blood (CB) processing and CD34+ cell enrichment

CB unit collection were done in accordance with the Canadian Blood Services ethics review board and with informed written consent from mothers and the Canadian Blood Services CB for research program. CB units contained a minimum of 750 x 10^6 nucleated cells, and the mononuclear cells (MNCs) were isolated by Ficoll-Paque Plus (GE Healthcare, Pittsburgh, PA). CD34+ cells were isolated using the EasySep^{TM} Human CD34 Positive Selection Kit II (StemCell Technologies, Vancouver, BC). Isolated CD34+ cells were cryopreserved using a cryoprotectant solution containing 40% Iscove's modified Dulbecco's medium (IMDM. Thermo Fisher Scientific, Nepean, ON), 50% fetal bovine serum (FBS, Thermo Fisher Scientific, nepean , ON), and 10% dimethyl sulfoxide (DMSO, Sigma Aldrich, Oakville, ON). The Cryovials temperature was gradually brought down by placing the vials in a Mr. Frosty freezer (Thermo Fisher Scientific, Nepean, ON) in -80°C for 24 hours, before transferring them to a liquid nitrogen freezer (Thermo Fisher Scientific, Nepean, ON) until needed for cell cultures.

2.2 Small molecules and SCAC X2A

The small molecules StemReginin1 (SR1,), UM171, and valproic acid (VPA) were purchased from StemCell Technologies (Vancouver, BC) and L-ascorbic acid-2-phosphate (AA2P) from Sigma Aldrich (Oakville, ON). SR1 and UM171 were reconstituted in DMSO while VPA and AA2P were solubilized in PBS. Appropriate volumes of SR1, UM171, VPA and AA2P were mixed to prepare 50x concentration of SCAC X2A, which was aliquoted and stored at -80°C for single use.

2.3 Cell culture and transduction

CD34+ cells were cultured in 24-well plates (Corning, New York, United States) in serum-free medium (SFM) StemSpan^{TM} SFEM (StemCell Technologies, Vancouver, BC) supplemented with stem cell factor (SCF), thrombopoietin (TPO) and FMS-like tyrosine kinase 3 ligand (FLT3L), each at 100 ng/ml. All cytokines (SCF, TPO, and FLT3L) were purchased from Peprotech (Rocky Hill, NJ). The medium was also supplemented with low-density lipoprotein (LDL, Stem cell technologies, Vancouver, BC) at 10 µg/ml, 1% penicillin-streptomycin (Pen-Strep, Thermo Fisher Scientific, Nepean, ON), and 1x X2A SCAC at a concentration of 500,000 cells/ml. Cell cultures were maintained in a humidified 5% carbon dioxide (CO_2) incubator at 37°C for 14 days. On day 4, cells were concentrated by centrifugation at 300 g for 10 minutes into 100 uL and transferred to 96-well plates (Corning, New York, United States). Lentivirus transduction (supplemented with apolipoprotein E, final concentration 3ug/ml, Thermo Fisher Scientific, Nepean, ON) was performed at a multiplicity of infection (MOI) of 10, where MOI is the ratio of virus particles to target cells. Twenty-four hours post transduction, the cells were transferred to a 24-well plate. Fresh media was added on days 5, 7, and 10. Cell counts and flow-cytometric analysis were done on days 7, 10, and 14. Fold expansion was calculated for days 10 and 14 by dividing the cell concentration of the respective day by the cell concentration obtained on day 7. The fold expansion calculation was based on day 7 counts as this was the first time-point when transduction efficiency (%GFP+ cells) was assessed by flow cytometry.

2.4 Lentivirus production

HEK293T cells were seeded at a density of 2.5 million cells per 15-cm dish (Corning Falcon, Corning, NY). After 48 hours, cells were co-transfected with a 2nd-generation

lentivirus packaging plasmid psPAX2, envelop plasmid pMD2.G (Addgene, Watertown, MA), and the vector/transfer plasmid (Horizon Discovery, Cambridge, UK) in the presence of polyethylenimine (4.1 μmol/L) and NaCl (2.25 x 10^{-4} mol/L). Forty-eight hours post-transfection, the supernatant containing virus was collected and incubated with Lenti-X concentrator (Takara Bio, San Jose, CA) at a ratio of 1:3 for 6 hours at 4°C. Afterwards, the virus was pelleted by centrifuging at 1500 g at 4°C for 45 minutes. The pellet was then resuspended in a final volume of 300 μL of STFL media (SFEM + SCF + TPO +FLT3L + LDL + Pen-Strep) to prepare virus concentrate (100x). This virus concentrate was used to transduce CB cells at 10 MOI.

2.5 shRNA screen and clone selection

K562 cells were cultured for 7 days in IMDM media supplemented with 10% FBS. On day 7, cells were transduced with a shRNA expressing lentivirus concentrate at a MOI of 20 in the presence of polybrene (final concentration 5μg/mL). Seventy-two hours post-transduction, the cells were assessed with flowcytometry for transduction efficiency (%GFP+) and >95% GFP+ was achieved (Supplemental Figure 1). Cells were lysed and total RNA and protein were collected which were used for RT-qPCR and Western blotting respectively. Three shRNA clones were screened and the 2 with the highest knockdown efficiency were selected for subsequent experiments. A non-targeting scrambled shRNA (SCR) was used as control (Table 3).

Table 3 . shRNA sequences used in ALKBH5 knockdown experiments. KD1 and KD2 are the shRNA clones selected through screening, whereas SCR is non-targeting scrambled control shRNA.

shRNA	Clone sequence
ALKBH5 shRNA 1 (KD1)	TATGAGAACCTAGGTCCTG
ALKBH5 shRNA 2 (KD2)	TGAACTGGAACTTGCAGCC
Scrambled shRNA (SCR)	GTTACACGATATGTTATC

2.6 RT-qPCR

ALKBH5 knock down (KD) efficiency and ALKBH5 target stability were estimated by RT-qPCR (see Table 3 for primer sequences). One µg of RNA was isolated from respective samples and converted into cDNA using iScript^{TM} Reverse Transcription Supermix (Bio-Rad, Mississauga, ON) for RT-qPCR as per manufacturer's protocol. The cDNA was subsequently utilized for conducting RT-qPCR using a SsoAdvanced universal SYBR green supermix (Bio-Rad, Mississauga, ON). These experiments were carried out using the CFX96 touch real-time PCR detection system (Bio-Rad, Mississauga, ON).

2.7 mRNA stability estimation

mRNA stability estimation of ALKBH5 targets (AXL and TACC3) was done using a combination of transcription inhibition and RT-qPCR methods. Cells were treated with Actinomycin D (5 µg/mL, Thermo Fisher Scientific, Nepean, ON), samples were collected at multiple time points (0, 1, 3, 6 hours), and total RNA was isolated using TRIzol (Thermo Fisher Scientific, Nepean, ON). RNA was converted to cDNA, and qPCR was performed using gene specific primers for the target mRNAs and a reference gene (GAPDH). The relative expression levels at each time point were calculated using the 2^(-ΔΔCT) method. ΔΔCT represents the

difference in the threshold cycles (CT) between the target mRNA and the reference gene. The relative mRNA levels were plotted against the time points on a graph. The mRNA half-life was estimated by determining the time point at which the mRNA level decreased to 50% of its initial value. Table 4 shows the primers used in PCR experiments.

Table 4 . Primer sequences. Table lists forward and reverse primers used in ALKBH5 transcript level estimation following knockdown as well as the primers used in transcript stability assessment assay for AXL and TACC3. GAPDH was the control in all assays.

Primer	Sequence
ALKBH5 forward	5'- CCC TGC TCT GAA ACC CAA G -3'
ALKBH5 reverse	5'- GTT CTC TTC CTT GTC CAT CTC C -3'
AXL forward	5'- TTT ATG ACT ATC TGC GCC AGG -3'
AXL reverse	5'- TGT GTT CTC CAA ATC TTC CCG -3'
GAPDH forward	5'- GAA GGT GAA GGT CGG AGT C -3'
GAPDH reverse	5'- GAA GAT GGT GAT GGG ATT TC -3'
TACC3 forward	5'- AAG AAG TGG CTG CAG GCC -3'
TACC3 reverse	5'- TGG GGG TGC CCT TTT GC -3'

2.8 Western blotting

Total cell protein was extracted from harvested K562 cells using RIPA lysis buffer (Thermo Scientific, Rockford, IL) supplemented with Halt™ protease & phosphatase inhibitor single use cocktail (Thermo Scientific, Rockford, IL). Thirty µL of the protein extract was combined with 10 µL of NuPAGE™ LDS Sample Buffer (Life Technologies Corp., Carlsbad, CA) and heated for 5 minutes at 92°C. Forty µL of the sample was then loaded into a 10 well gel (Thermo Fisher Scientific, Nepean, ON) and electrophoresis was performed using a Mini

Gel Tank (Thermo Fisher Scientific, Nepean, ON). The gel was then transferred on to a methanol activated polyvinylidene difluoride membrane (Thermo Fisher Scientific, Nepean, ON) using the iBlot™ 2 Gel Transfer Device (Life Technologies Corp., Carlsbad, CA). After transfer, the membrane was blocked for 1 hour using 5% bovine serum albumin (BSA, Thermo Fisher scientific, Rockford, IL) in Tris-Buffered saline (TBS, Thermo Scientific, Rockford, IL). After blocking and washing using TBS with 0.1% Tween-20 (TBS-T, Tween-20, Sigma-Aldrich Co, St. Louis, MO), the primary antibody (ALKBH5, Cell Signaling, Danvers, MA) cocktail diluted 1:1000 with 5% BSA was added to the membrane and was incubated overnight at 4°C. The membrane was then washed with TBS-T and incubated at room temperature in the secondary antibody cocktail containing 1:10000 dilution of HRP conjugated polyclonal donkey anti-rabbit IgG (Thermo Fisher Scientific, Nepean, ON) with 5% BSA for 1 hour. Afterwards, the blot was treated with 1 mL of SuperSignal West Pico PLUS Chemiluminescent substrate (Thermo Scientific, Rockford, IL) for 5 minutes and subsequently visualized using a ChemiDoc imaging system (BioRad, Saint-Laurent, QC).

2.9 Flow cytometry

Flow cytometry analysis of all cultures was done using either Attune®/ Attune® NXT (Thermo Fisher Scientific, Nepean, Canada), Cytek® Aurora (Cytek Biosciences, Fremont, CA), or BD LSRFortessa™ (Becton, Dickinson and Company, Franklin Lakes, NJ). Cell sorting was carried out using MoFlo XDP (Beckman Coulter, Indianapolis, IN) or SH800 Cell Sorter (Sony Biotechnology, San Jose, CA). On day 0, 7, 10 and 14 of the culture, a fraction of the cells was analyzed by flow cytometry. Unless stated otherwise, all antibodies were from Becton Dickinson Pharmingen (Mississauga, Ontario, Canada). The antibodies included CD19-phycoerythrine (PE, clone HIb19), CD14-PE (clone M5E2), CD45-allophycocyanine

(APC, clone HI30), CD33-PE (clone WM53), CD34-PE (clone 581), CD90-PECy7 (clone 5E10), CD123-PerCP-Cy5.5 (clone 7G3), CD3-APC (clone UCHT1), human CD41a (GPIIb)-FITC (clone HIP8), CD49f-Alexa647 (GoH3), SYTOX AAdvanced (Thermo Fisher Scientific, Nepean, Canada), CD235a (Glycophorin-A, clone HIR2), Ki-67-Alexa Flour 488 (BioLegend, San Diego, CA), N6-methyladenosine (m6A, Novus Biologicals, Centennial, CO). Cells were stained in FACS buffer (PBS + 2% FBS) and stained with antibodies for 30 minutes on ice, washed using FACS buffer, pelleted using centrifugation at 300 g for 10 minutes, and resuspended in FACS buffer to be acquired by the flow cytometer for analysis. Compensation beads (BD™ CompBead Plus, BD Biosciences, Mississauga, ON) were used to set voltage and gating parameters for accurate fluorescence signals for all fluorophore-conjugated antibodies. Antibodies were titrated before use and fluorescent minus one (FMO) controls were used to set gates and/or quadrants.

2.10 Intracellular staining

Harvested cells were first stained with antibodies against cell surface markers using the previously described method (Section 2.8). Subsequently, the cells were fixed using 2% PFA (Thermo Fisher Scientific, Nepean, ON) and then permeabilized with 0.3% Triton (Sigma-Aldrich Co, St. Louis, MO). Following fixation and permeabilization, the cells were incubated with appropriate intracellular primary antibodies on ice for 20 minutes and washed (described above). After that, the cells were stained with a secondary antibody containing appropriate fluorophore for 30 minutes on ice and washed (described above). Finally, the cells were resuspended in FACS buffer and kept on ice until flow-cytometric analysis.

2.11 Cell cycle assay

Cells were harvested, fixed and permeabilized using the aforementioned protocol. Subsequently, the cells were treated with RNAse A (Thermo Fisher Scientific, Nepean, ON) for 10 minutes, washed (described above) and stained with SYTOX AAdvanced to assess their cell cycle state. Moreover, Ki-67 (BioLegend, San Diego, CA) staining was done to differentiate between G0 and G1 cell cycle states.

2.12 Colony forming unit assay

Ex-vivo expanded CB cells were plated in MathoCult™ Classic H4435 (StemCell Technologies, Vancouver, BC) and incubated for 14 days in a humidified incubator at 37°C and 5% CO_2. After incubation, the colonies were scored based on morphological features which included burst forming unit- erythroid (BFU-E), colony forming unit- granulocyte, macrophage (CFU-GM), and colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM).

2.13 *In-vivo* transplantation assay

All animal work was approved by the Animal care Committee of University of Ottawa. 8-week-old immunodeficient female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG, The Jackson Laboratory, Bar Harbor, ME) mice were sub-lethally irradiated (300 cGy). These mice were divided into two cohorts: control (to receive non-targeting scramble shRNA transduced cells) and ALKBH5 KD (to receive ALKBH5 KD shRNA transduced cells) group. For cell culture, 2 CB units were combined and were cultured in regular stem cell culture media (STFL). On day 0, cells were transduced with either SCR or ALKBH5 KD shRNA expressing lentivirus, and after 72 hours, flowcytometric assessment was done to estimate transduction efficiency (%GFP+). Based on the transduction efficiency, 15000 GFP+CD34+ cells were injected per

mouse via tail vein. Peripheral blood (on week 3, 10, 18) and bone marrow (on week 21) were collected to assess human cell reconstitution in both cohorts on multiple timepoint during the assay.

2.14 Human engraftment analyses

Human platelet and leukocytes were monitored in mice peripheral blood collected from the saphenous vein on weeks 3, 10, and 18 post tail vein transplantations. Murine blood was acquired using heparin coated capillary tubes and transferred into Eppendorf tubes containing heparin. A previously described single-platform protocol was followed to measure human platelet levels in samples[144]. In summary, 5 µL of blood was diluted 10-fold using PBS and stained for 20 minutes at 4°C with 2 µL of PE-conjugated human CD41a (clone HIP8, BD Biosciences, Mississauga, ON). Subsequently, the red blood cells (RBCs) were lysed by a 15-minute incubation at room temperature using 250 µL of 1X BD Pharm Lyse™ lysing buffer (BD Biosciences, Mississauga, ON). The samples were further diluted with 250 µL PBS and 50 µL of AccuCheck counting beads (Life Technologies, Frederick, MD) before acquiring 450 µL of each sample by flow cytometer for analysis. The human platelet concentration was calculated based on the dilution factor, number of CD41a+ events, and the ratio of beads concentration to bead count. Furthermore, flowcytometric analysis of leukocytes was done following staining the mouse blood samples with human anti-CD45-APC (clone HI30), CD-33-PE (clone WM53), CD-19-PE-Cy7 (HIb19) and RBC lysis. Also, long-term (LT) bone marrow (BM) engraftment analysis was done 21-week post-transplantation. BM cells were extracted from the mice hind legs and RBCs were lysed before staining with appropriate antibodies. Multiple antibody panels were built using different combination of antibodies (all purchased from BD Biosciences, Mississauga, ON): Panel A: CD45-APC (clone HI30), CD33-

PE (clone WM53); Panel B: CD45-APC (clone HI30), CD19-PE (clone Hib19); Panel C: CD45-FITC (clone HI30), CD34-PE (clone 581), CD41a-APC (clone HIP8); Panel D: CD45-FITC (clone HI30), CD14-PE (clone M5E2), CD3-APC (clone UCHT1); and Panel E: CD45-APC (clone HI30), CD56-PE (clone NCAM16.2). Antibody-stained cells were analysed using flow cytometry.

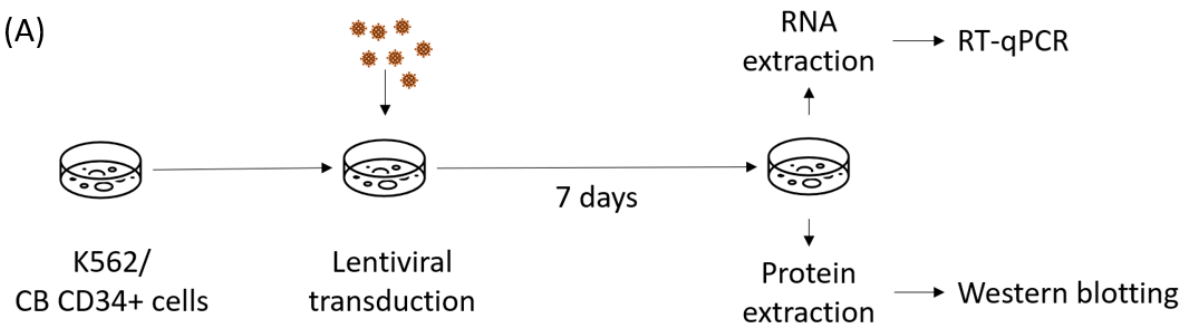
2.15 Statistical analysis

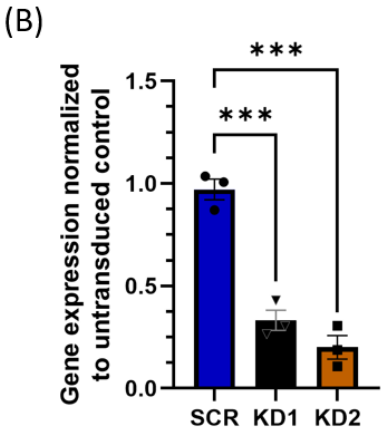
Unless otherwise stated, all results are presented as mean ± standard error of the mean (SEM). Statistical assessments were performed using GraphPad Prism v 9.0.1 (Dotmatics, Boston, MA). Statistical analysis between two groups were done using two-tailed Student's t-tests. When comparing the differences in the means between more than two groups, either one-way or two-way ANOVA were used as appropriate. A P-value below 0.05 was deemed to be statistically significant.

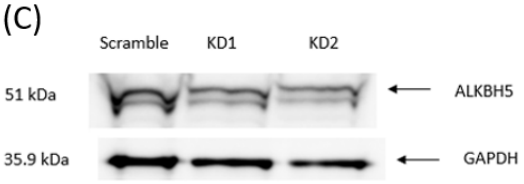
Chapter 3: Results

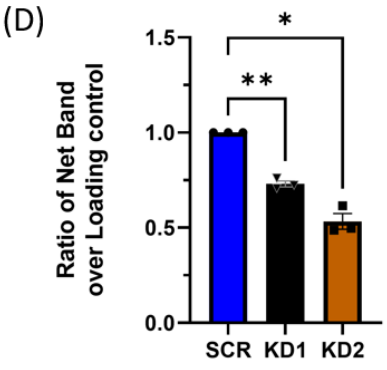
3.1 shRNA mediated ALKBH5 knockdown reduced the transcript and protein levels of ALKBH5.

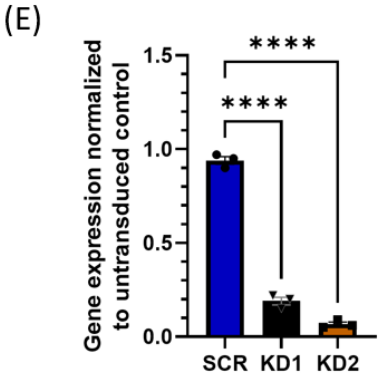
RT-qPCR analysis of transduced erythroleukemic cell-line K562 (Fig 4A) showed a 65% reduction of ALKBH5 transcript for KD1 and 79% for KD2 (P***<0.001, Fig 4B). Furthermore, Western blot analysis revealed reduction of ALKBH5 protein levels by 27% and 47% for KD1 and KD2 respectively (Fig 4C, D). Next, the ability of the selected shRNA (KD1 and KD2) clones to knock down ALKBH5 within HSPCs was evaluated. CB HSPCs were cultured within X2A and transduced using aforementioned protocol. RT-qPCR analysis revealed that KD1 and KD2 were able to reduce *ALKBH5* levels by 79% and 87% for KD1 and KD2, respectively (P****<0.0001, Fig 4E).


(A)
RNA extraction
RT-qPCR
7 days
K562/ CB CD34+ cells
Lentiviral transduction
Protein extraction
Western blotting


(B)
Gene expression normalized to untransduced control
1.5
1.0
0.5
0.0
***
***
SCR
KD1
KD2


(C)
Scramble
KD1
KD2
51 kDa
ALKBH5
35.9 kDa
GAPDH


(D)
Ratio of Net Band over Loading control
1.5
1.0
0.5
0.0
*
**
SCR
KD1
KD2


(E)
Gene expression normalized to untransduced control
1.5
1.0
0.5
0.0
****
****
SCR
KD1
KD2


SCR
KD1
KD2

Figure 4. shRNA mediated knockdown reduced ALKBH5 transcript and protein levels. (A). Erythroleukemic cell line K562 or CB HSPCs were transduced by lentivirus expressing either non-targeting scramble shRNA (SCR) or ALKBH5 knockdown capable shRNAs (KD1 and KD2) at the MOI of 20. On day 7, RNA and protein were extracted for RT-qPCR and Western blotting respectively. (B) ALKBH5 transcript levels presented for SCR, KD1 and KD2 samples. Data is normalized to un-transduced control. (C) Representative image of ALKBH5 and GAPDH protein bands from western blotting. (D) Relative ALKBH5 protein level compared to internal control GAPDH. Quantification was done using Image J software v. 1.54d. The ratio of ALKBH5 and GAPDH protein expression was normalized to the SCR control condition which was arbitrarily set to one within each experiment. To determine the statistical significances between the different conditions, a one-way ANOVA analysis was performed using the ratio of protein expression between ALKBH5 and GAPDH within each condition and experiments. Panel B-D present data obtained using K562 cells. (E) RT-qPCR data showing the ALKBH5 transcript levels in CB HSPCs cultured in X2A media and transduced with shRNA (SCR, KD1 or KD2) expressing lentivirus at the MOI of 10. Data is normalized to un-transduced control. All the graphs show mean ± SEM. One-way ANOVA was used, P*<0.05, P**<0.01, P***<0.001, P****<0.0001.

3.2 ALKBH5 deficient CB HSPCs show transient increase in growth kinetics.

To understand the impact of ALKBH5 on the cellular growth kinetics, cell concentrations were tracked during cell culture. X2A cultured CB HSPC concentration tracking (Fig 5A) for total nucleated cells (TNC), transduced CD34+ (CD34+GFP+), and transduced CD34+CD45RA- (CD34+CD45RA-GFP+) cells revealed that ALKBH5 loss led to increased cell concentration in the respective samples at day 10 timepoint of the culture. However, the concentration of the ALKBH5 deficit samples declined to similar levels as the SCR transduced cells by day 14 (Fig 5B).

Fold expansion measurement is another method to track cell growth kinetics[145-147]. Fold expansion of the queried populations on day-10 and day-14 based on day-7 count revealed that TNC (2.2-times, $P<0.05$, CD34+GFP+ (2.4-times, non-significant), and CD34+CD45RA-GFP+ populations were (2.1-times, $P<0.05$) increased in ALKBH5 deficient samples compared to SCR samples (Fig 5C).

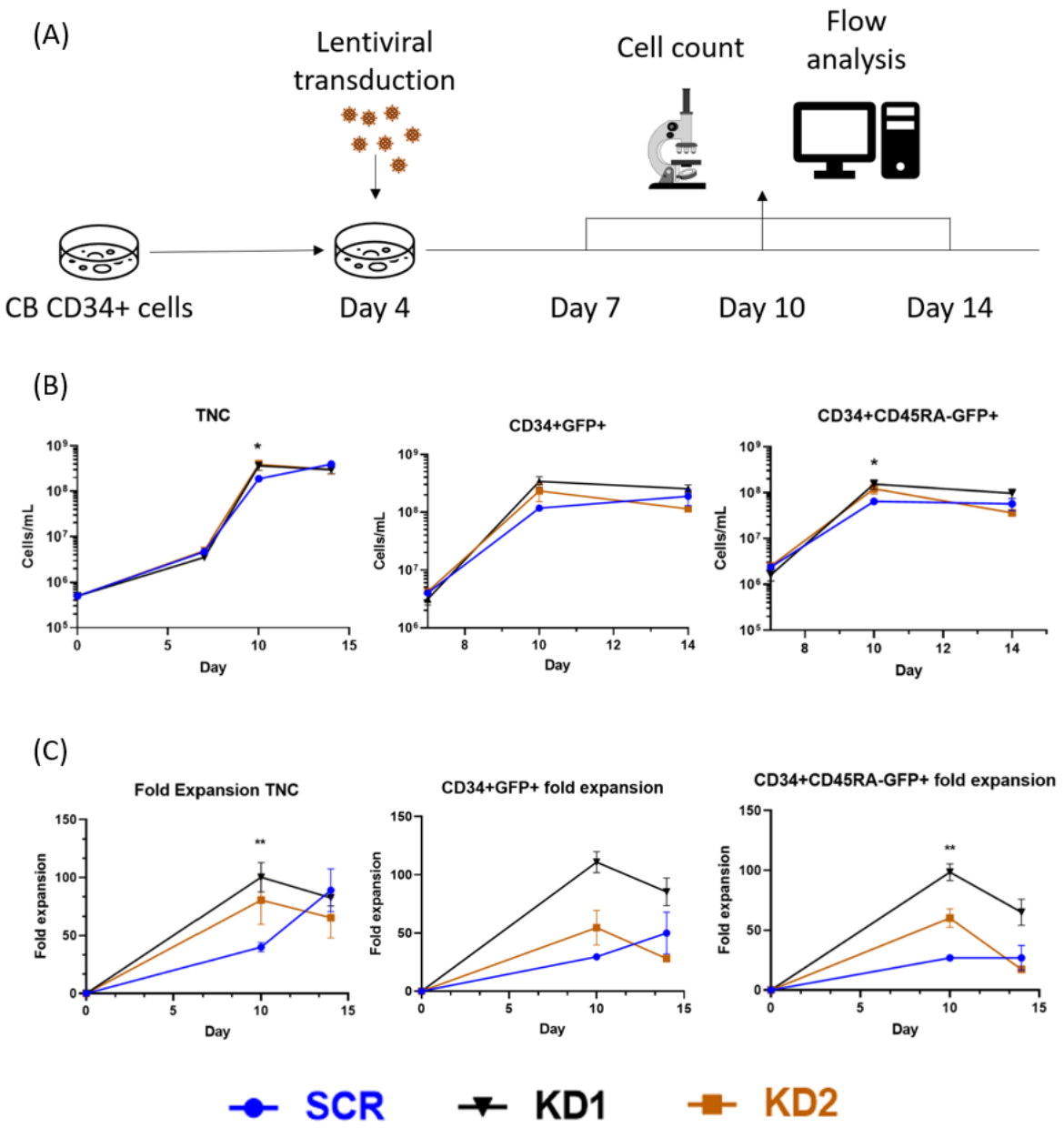
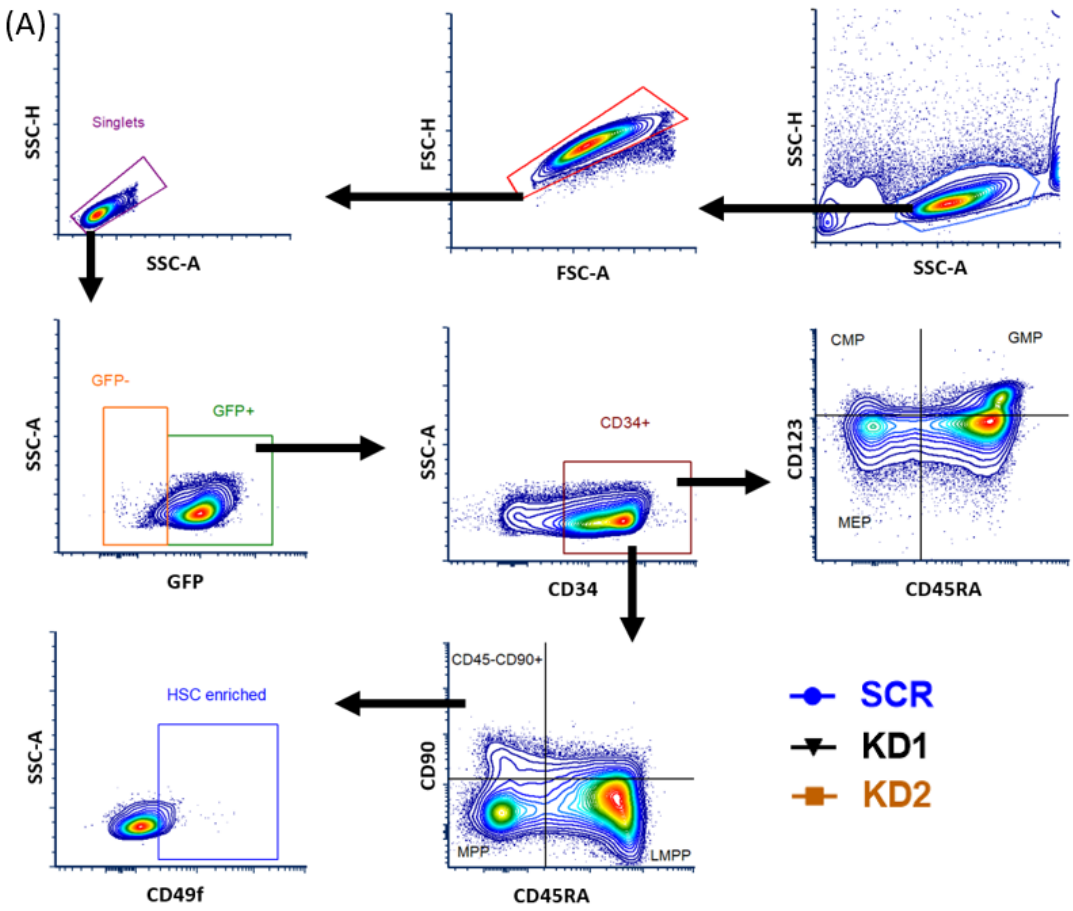


Figure 5. ALKBH5 deficiency in HSPCs led to transient increase in cell concentration and fold expansion during ex-vivo culture. (A) shRNA (SCR or KDs) transduced CB CD34+ cells were cultured stemness supportive X2A media. Either the TNC or transduced (GFP+) CD34+ or CD34+CD45RA- cells were tracked using cell count and flowcytometry. (B) Cell concentration (cells/mL) plotted for TNC, CD34+GFP+, and CD34+CD45RA-GFP+ plotted against day of culture. (C) Fold expansion of cells calculated based on day 7 cell counts presented for the aforementioned cell populations. Data is presented as mean ± SEM for 3 biological replicates. Two-way ANOVA is used; p*<0.05, p**<0.01.

3.3 ALKBH5 deficiency impacts HSPC subpopulation expansion in a variable manner.

ALKBH5 has been known to play different roles in the growth and proliferation of different cell populations[148-150]. All HSPCs despite originating from HSCs have distinct gene expression profiles that differ from each other[151]. Therefore, next I inquired how different HSPCs are impacted by ALKBH5 loss. HSPC sub-populations were identified by a combination of cell surface markers described by Cimato, et al. (2016)[29] and analyzed by flow cytometry (Fig 6A). Within X2A cultured transduced CB HSPCs, HSC enriched population (eHSC, CD34+CD45RA-CD90+CD49f+) significantly declined (KD1: $P<0.05$; KD2: $P<0.01$) by day 14 of culture in ALKBH5 reduced samples compared to SCR transduced ones (Fig 6B).

Other HSPC sub-populations assessed were megakaryocyte-erythroid progenitors (MEPs, CD34+CD45RA-CD123-), common myeloid progenitors (CMPs, CD34+CD45RA-CD123+), multipotent progenitors (MPPs, CD34+CD45RA-CD90-), lymphoid-primed multipotent progenitors (LMPP, CD34+CD45RA+CD90-), and granulocyte macrophage progenitors (GMPs, CD34+CD45RA+CD123+). On day 10 of culture, MPP proportion was higher ($P>0.42$) and MEP proportion was lower ($P>0.44$) in ALKBH5 deficient sample compared to SCR transduced samples. On day 14 of culture, LMPP proportion was higher ($P>0.40$); CMP ($P>0.65$) and GMP ($P>0.13$) proportions were lower in ALKBH5 reduced samples compared to SCR transduced samples (Fig 6C). These changes in ALKBH5 reduced HSPC sub-populations, other than eHSC, were not significant.


(A)
Singlets
SSC-H
SSC-A
FSC-H
FSC-A
SSC-H
SSC-A
GFP-
GFP+
SSC-A
GFP
CD34+
SSC-A
CD34
CMP
GMP
CD123
MEP
CD45RA
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CD45-CD90+
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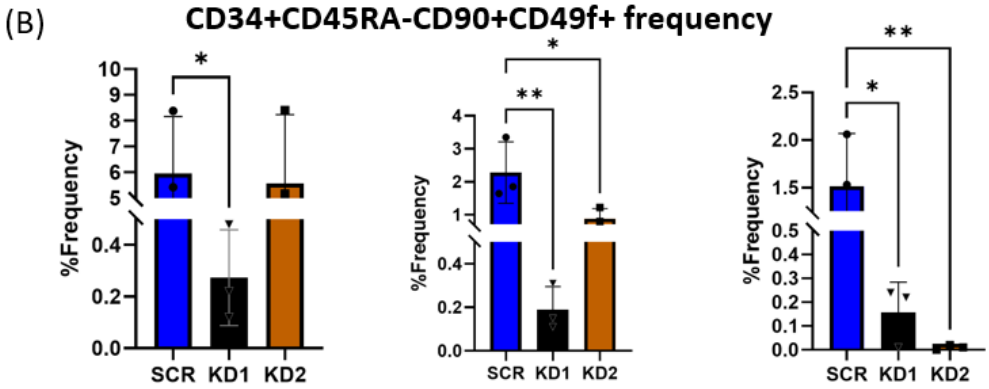
LMPP
CD45RA
SCR
KD1
KD2


(B)
CD34+CD45RA-CD90+CD49f+ frequency
%Frequency
SCR KD1 KD2
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%Frequency
SCR KD1 KD2

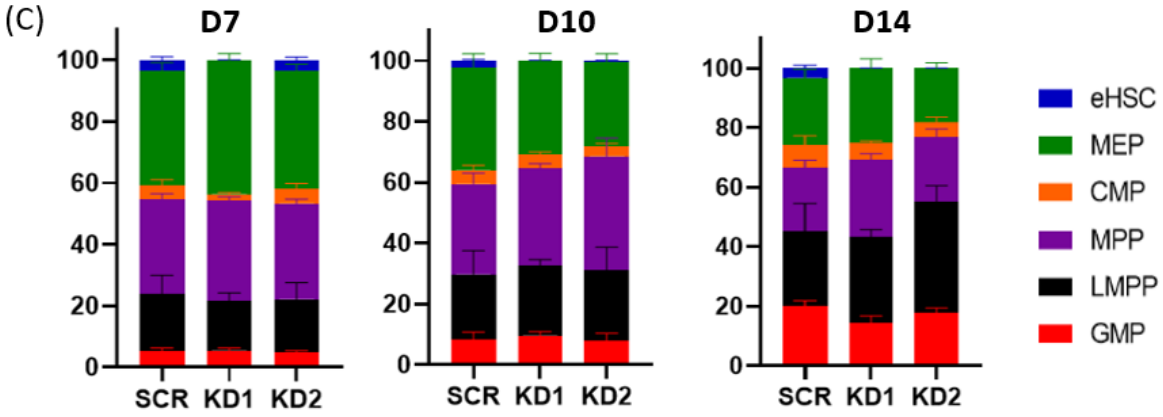
(C)
D7
D10
D14
SCR KD1 KD2
SCR KD1 KD2
SCR KD1 KD2
eHSC
MEP
CMP
MPP
LMPP
GMP

Figure 6. ALKBH5 loss impacts HSPC subpopulation fractions in *ex-vivo* culture. (A) X2A cultured CB CD34+ cells were transduced with either SCR or KD (1 or 2) shRNAs and were analyzed with flow cytometry using a combination of cell surface markers to identify different HSPC populations. Gating strategy for HSPC sub-population tracking is based on Cimato et al. (2016)[29] (B) Frequency of HSC enriched (eHSC, CD34+CD45RA-CD90+CD49f+) cell population for SCR, KD1 or KD2 transduced samples on day 7, 10, and 14 of culture. (C) Graft composition showing the proportions of eHSC, megakaryocyte-erythrocyte progenitor (MEP, CD34+CDCD45RA-CD123-), common myeloid progenitors (CMP, CD34+CD45RA-CD123+), multipotent progenitor (MPP, CD34+CD45RA-CD90-), lymphoid-primed multipotent progenitor (LMPP, CD34+CD45RA+CD90-), and granulocyte macrophage progenitor (GMP, CD34+CD45RA+CD123+) within transduced CD34+ population on day 7, 10, and 14 of culture. Mean ± SEM plotted. One-way ANOVA was done, P*<0.05, and P**<0.01.

3.4 Loss of ALKBH5 in CB cells reduced their colony forming potential.

The colony forming unit (CFU) assay was then used to investigate the impact of ALKBH5 reduction on progenitor function. CFU assay[152,153] was performed using X2A cultured day-7 CB cells (Fig 7A). Following a 14-day incubation, the burst forming unit – erythroid (BFU-E) numbers were reduced by 2.7 times ($P<0.05$) and colony forming unit - granulocyte – macrophage (CFU-GM) numbers were reduced by 1.2 times ($P>0.2$, non-significant) in ALKBH5 deficient samples. No CFU-GEMM was noted in either sample. Total CFU score (includes BFU-E and CFU-GM) was also reduced by 1.5 times ($P<0.05$) times in ALKBH5 reduced samples (Fig 7B).

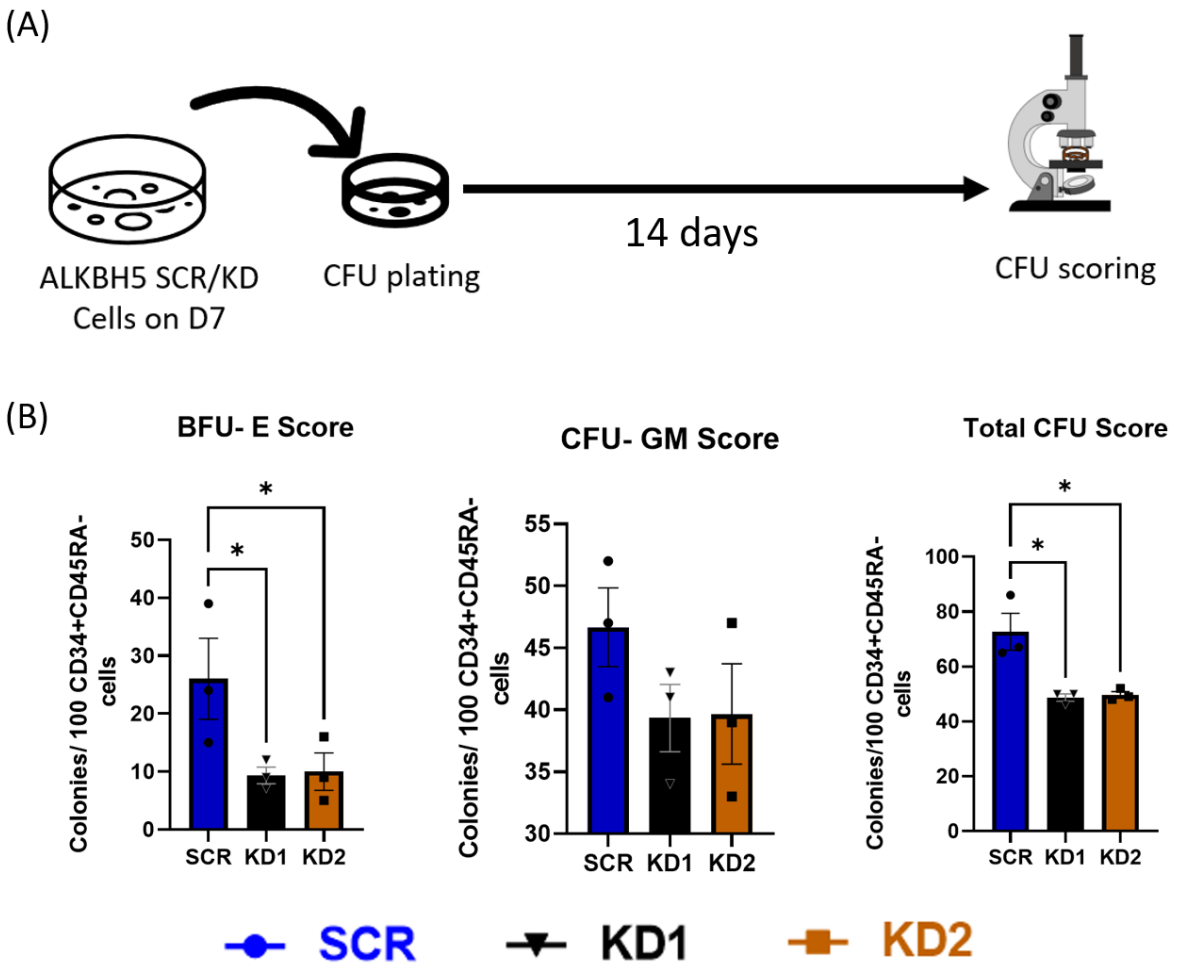

Figure 7. Colony forming potential of CB HSPCs are reduced in ALKBH5 deficient samples. (A) X2A cultured day 7 CB cells (SCR or KD transduced) were plated in methylcellulose media. The net number of TNC plated in each culture condition was adjusted so that the equivalent of 100 CD34+CD45RA- cells were present in each plate for all conditions tested. The frequency of CD34+CD45RA- cells were determined ahead of time by FACS analysis. Colonies were counted after 14 days of incubation. (B) Burst forming unit – erythroid (BFU-E), colony forming unit – granulocyte, macrophage (CFU-GM), and colony forming unit – granulocyte, erythroid, macrophage and megakaryocyte (CFU-GEMM) were counted and number of colonies per 100 plated CD34+CD45RA- cells were plotted. (Left to right) BFU-E, CFU-GM, and CFU-Total scores for SCR, KD1 and KD2 samples presented as mean ± SEM for three biological replicates. One-way ANOVA was performed, P*<0.05.

3.5 ALKBH5 deficiency within HSPCs leads to reduction in long-term engraftment.

Transplantation of HSPCs in humanized mice model (Fig 8A) can provide information on how the donor cells genetic expression or recipient microenvironment impact the engraftment success[154]. While at 3-week post-transplantation the platelet concentrations in peripheral blood were comparable ($P>0.4$), the mice cohort that received ALKBH5 deficient HSPCs (test cohort) had a higher (3.3 times, $P<0.05$) platelet concentration compared to the control cohort by week 10. However, by week 18 the test cohort had an average platelet concentration that was 3 times lower than that of the control cohort ($P<0.05$) (Fig 8B).

Tracking the CD45+ cell frequency in peripheral blood did not show noticeable differences at weeks 3 ($P>0.3$) and 10 ($P>0.6$) between the control and test cohorts. However, at week 18, CD45+ cell frequency was found to be 1.3 times lower ($P>0.4$, non-significant) in test cohort compared to the control cohort (Fig 8C).

CD34+ cell frequency tracking in the peripheral blood presented a different engraftment pattern again. Overall, CD34+ cell frequency was much lower compared to CD45+ cell frequency throughout the experiment duration. At the 3-week timepoint, the test cohort had 1.9 times higher CD34+ cell frequency compared to the control cohort ($P<0.01$). While at 10-week, the mean CD34+ frequency of the test cohort was 2.0 times lower than that of the control ($P>0.2$, non-significant); by week 18, CD34+ cell frequency in the test cohort declined significantly (1.6 times, $P<0.05$) compared to the control cohort (Fig 8D).

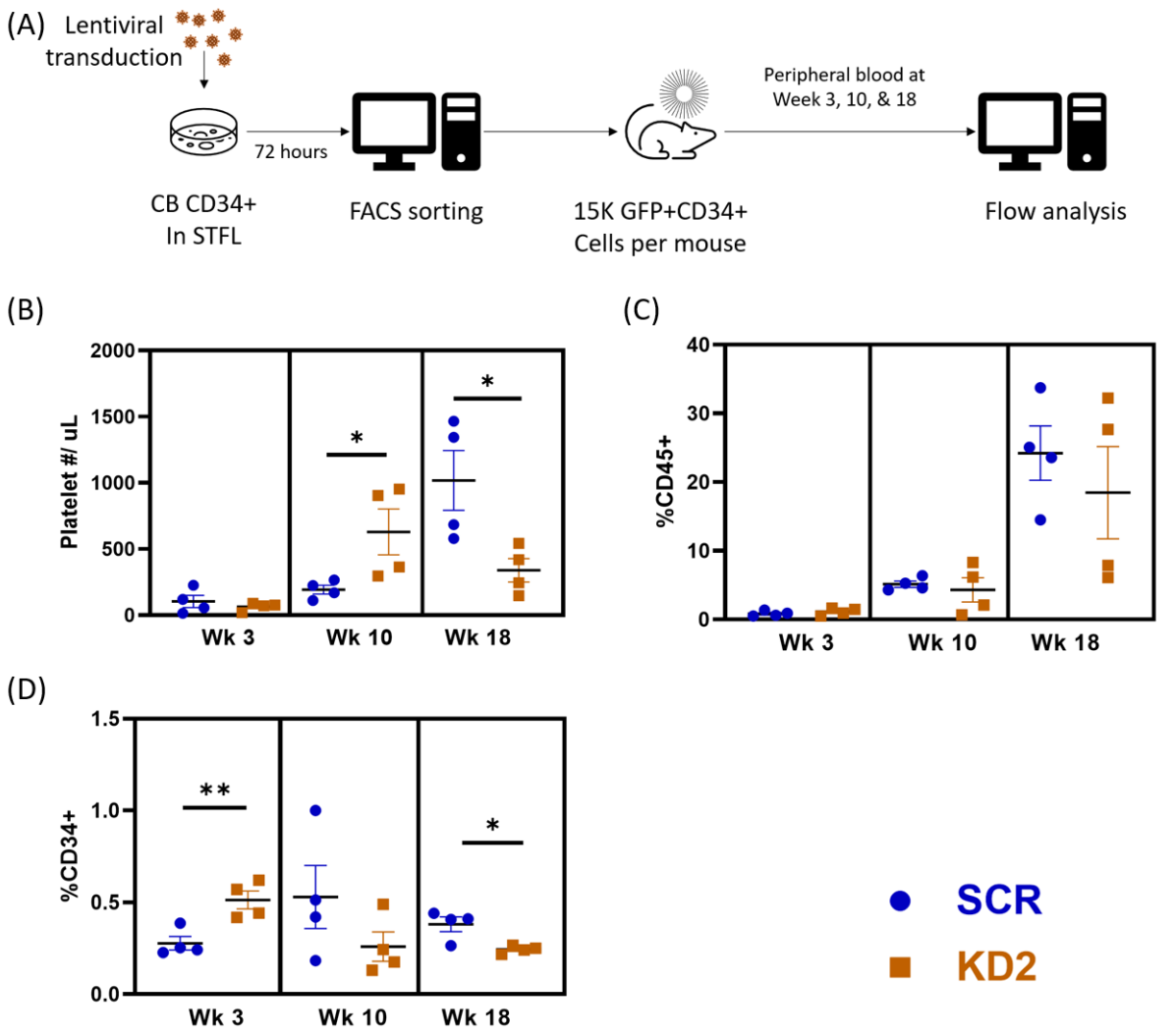

Figure 8. ALKBH5 loss in the graft led to reduced platelets, leukocytes, and CD34+ cells in murine peripheral blood. (A) Mice cohorts were injected with 15000 CD34+ cells that were either transduced with SCR shRNA expressing lentivirus (control cohort, SCR) or KD2 shRNA expressing lentivirus (test cohort, KD2). On week 3, 10, and 18 following transplantation, peripheral blood was collected and analyzed with flowcytometry. (B) Platelet concentration (CD41a+, platelet#/uL) in the murine peripheral blood. (C-D) Frequency of CD45+ (leukocytes) and CD34+ cells in murine peripheral blood respectively. Mean ± SEM presented for all values, t-test was used for statistical analysis, P*<0.05, P**<0.01 (4-5 mice per group, n=1).

3.6 ALKBH5 deficiency within HSPCs impacts multilineage chimerism *in-vivo*.

At the 21-week post-transplantation timepoint, bone-marrow analysis (Fig 9A) for human CD45 cells in the test cohort showed 1.5 times lower CD45+ cell frequency compared to the control ($P<0.05$) (Fig 9B). Subsequently, lineage specific markers were assessed in combination with human CD45 marker to estimate multi-lineage chimerism (Fig 9C-E). In the test cohort, CD45+CD14+ (monocyte) cell frequency was 1.2 times higher ($P>0.2$, non-significant) but CD45+CD33+ (myeloid biased) cell frequency was similar ($P>0.8$, non-significant) when compared to the control cohort (Fig 9C-D). On the other hand, CD45+CD19+ (B lymphocytes) cell frequency was significantly lower (1.2 times, $P<0.05$) in the test cohort compared to the control (Fig 9E). Finally, CD34+ (representing HSPCs) cell frequency was 1.8 times lower in the test cohort compared to the control ($P>0.1$, non-significant) (Fig 9F). Together these data show the impact of ALKBH5 deficiency on multilineage chimerism development in a xenotransplant model.

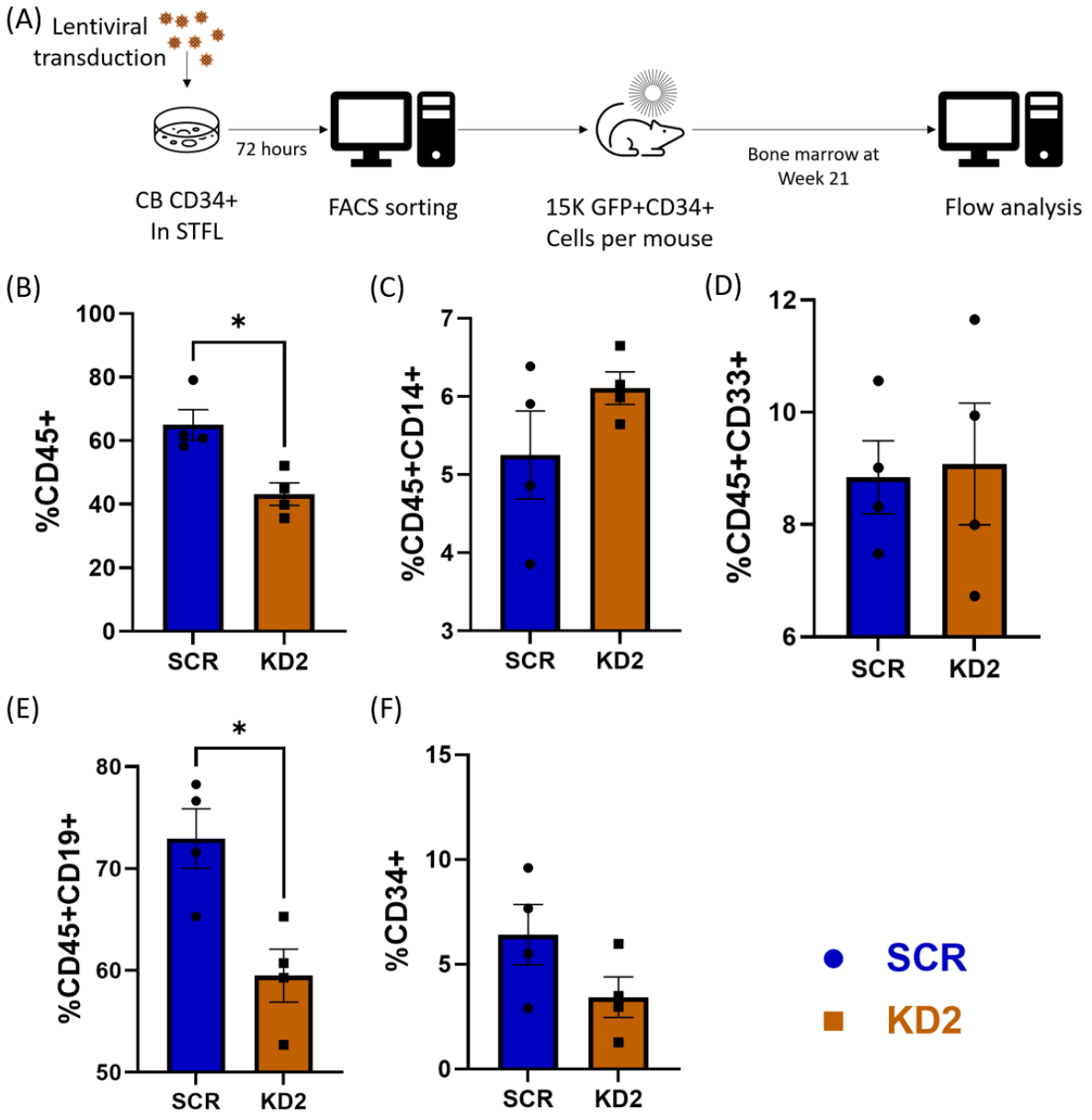

Figure 9. ALKBH5 deficient HSPC transplantation led to altered efficiency in human multi-lineage chimeric cell formation *in-vivo*. (A) Bone-marrow extracted from 21-week post transplantation mice cohorts were stained with a combination of antibodies to identify human multilineage chimerism in the control (SCR) and test (KD2) cohort. Samples were analyzed with flowcytometry. (B) Human leukocyte (CD45+) frequency. (C-E) Human multilineage populations: monocyte (CD45+CD14+), myeloid biased (CD45+CD33+), and B lymphocytes (CD45+CD19+) frequencies. (F) HSPC (CD34+) frequency. Mean ± SEM presented for all values (n=1, 4-5 mice per group), t-test was used for statistical analysis, P*<0.05.

3.7 Impact of ALKBH5 knockdown on the cell cycle entry of CB HSPCs.

Cells enter different phases of cell cycle during cell growth and proliferation[155]. Assessing the cell cycle entry status (Fig 10A) of SCR, KD1, or KD2 transduced, X2A cultured CB HSPCs on day 10 revealed no statistical difference in the percentage of cells entering G0 and G1 phase between the SCR vs KD1 or KD2 samples (G0: $P>0.1$, G1: $P>0.5$). However, notably both ALKBH5 KD (KD1 and KD2) reduced the percentage of cells in G2-S phase compared to the control though the differences were non-significant ($P>0.2$) (Fig 10B).

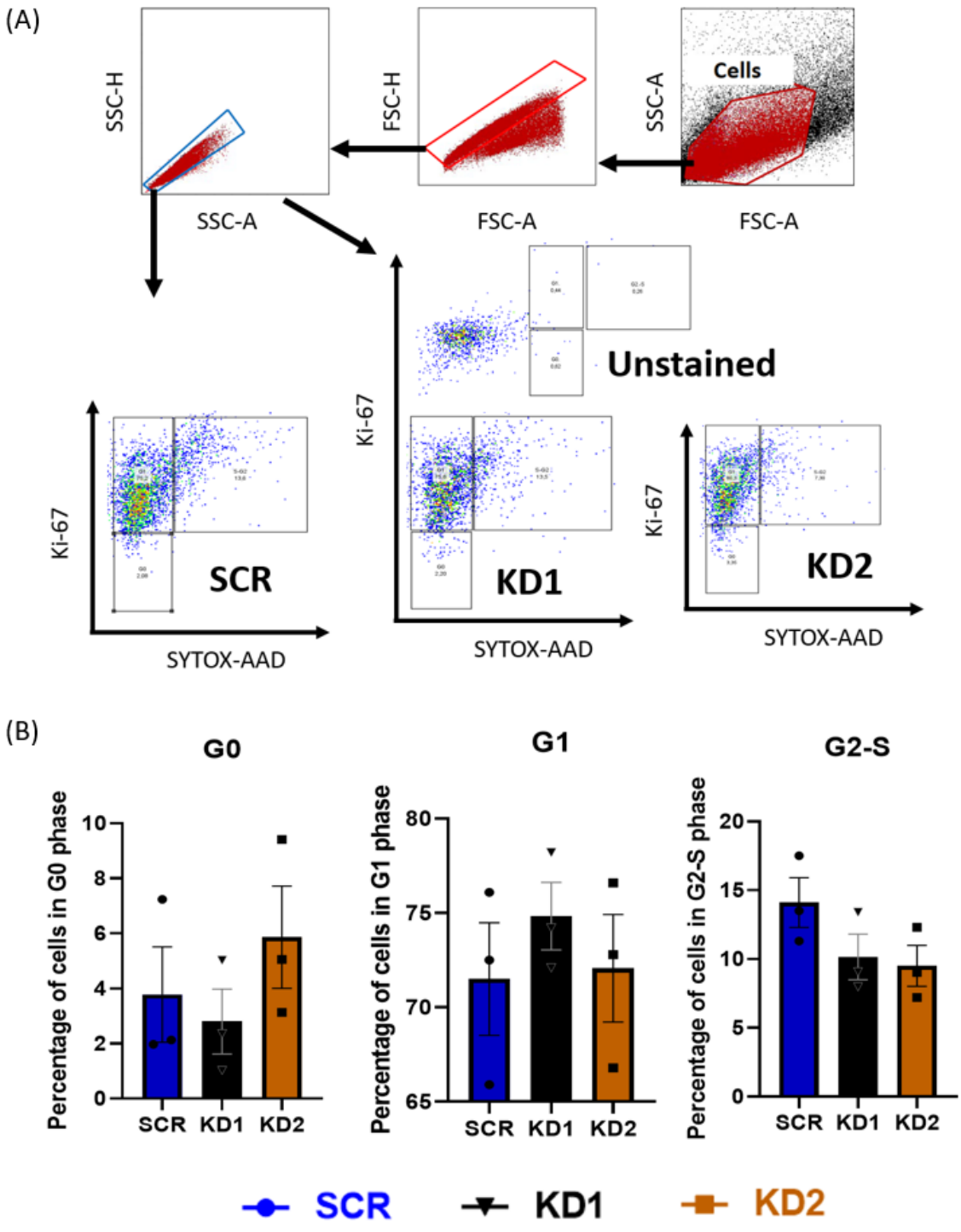

Figure 10. Cell cycle entry status of the CB HSPCs with or without ALKBH5 knockdown. (A) X2A cultured, SCR, KD1 or KD2 transduced CB HSPCs on day 10 were stained with SYTOXAadvanced dye and anti-Ki-67antibody to differentiate between different cell-cycle status. (B) Percentage of cells in G0, G1, and G2-S are plotted for each culture condition. Mean ± SEM and 3 biological samples presented.

3.8 Impact of ALKBH5 knockdown on the levels of m6A and on AXL and TACC3 transcript stability.

Finally, I investigated the impact of ALKBH5 depletion on m6A, AXL, and TACC3, three targets of this RNA demethylase[156-158]. m6A levels assessment in X2A cultured day-10 CB HSPCs revealed minimally increased levels of m6A in ALKBH5 depleted samples compared to the SCR control ($P>0.7$, non-significant) (Fig 11A). Similarly, AXL mRNA transcript level estimation using RT-qPCR following actinomycin D (5 ug/mL) treatment showed no significant difference in the degradation of AXL mRNA between the SCR control sample and KD1 or KD2 samples ($P>0.7$ at hour 3 and $P>0.8$ at hour 6) (Fig 11B). In contrast, TACC3 transcript level was found to be significantly less stable. Within ALKBH5 deficient samples, TACC3 half-life was estimated to be 1.9 times lower than the SCR control ($P<0.0001$) (Fig 11C).

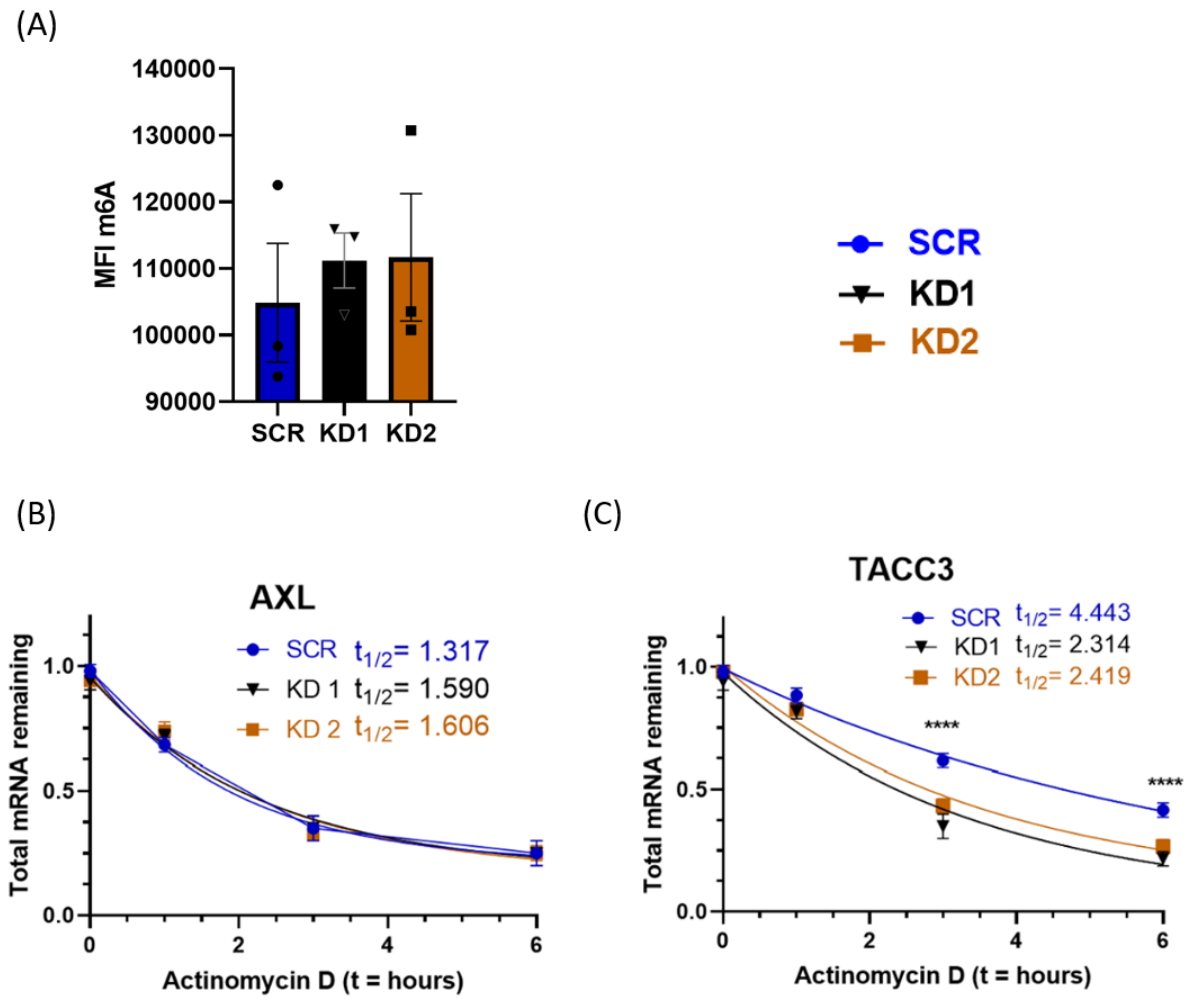

Figure 11. ALKBH5 influences the global levels of m6A RNA methylation marks and regulates the stability of TACC3 transcript. (A) m6A levels in SCR, KD1, or KD2 shRNA transduced samples. (B-C) Comparative mRNA levels AXL and TACC3 in SCR, KD1, and KD2 samples at 0, 1, 3, and 6 hours following actinomycin D (5 ug/mL). This data is representative of X2A cultured day-10 CB HSPCs of 3 biological origins. Mean ± SEM shown, two-way ANOVA used, ****P<0.0001.

Chapter 4: Discussion

The delicate balance between the HSC self-renewal and differentiation capability is a critical determinant in ensuring life-long normal hematopoiesis in an individual. This balance is also important in regulating the optimum proportions of various mature cell types and thus maintaining physiological homeostasis (this has been reviewed in-depth by Olson et al (2020)[159]). In this context, the RNA demethylase ALKBH5 may play a pivotal role, provided that ALKBH5 has an established capacity to regulate gene expression[156] and thus can impact cell function and fate decision through RNA demethylation, potentially acting as a regulator for hematopoiesis[160-162]. In this study, I have presented experimental evidence to support my initial hypothesis that proposed a critical role of ALKBH5 in HSPC self-renewal and differentiation process.

4.1 ALKBH5 is a regulator of HSPC growth kinetics.

ALKBH5 deficient HSPCs' growth kinetics tracking revealed a transient increase in cellular expansion followed by a rapid decline. My hypothesis proposed a regulatory role of ALKBH5 in HSC biology and the experimental evidence of changed growth kinetics in ALKBH5 deficient HSPCs supported this aspect of my hypothesis. The transient increase observed in cellular growth kinetics could be indicative of two cellular phenomena. The first one is, with the loss of optimum level of ALKBH5, the respective cells lost a critical regulator of cellular proliferation[156,163]. Increased growth kinetics in the deficiency of ALKBH5 indicates that ALKBH5 could be acting in a fashion that suppresses overall HSPC expansion. The second phenomenon is the rapid decline of the cellular growth in the ALKBH5 deficient samples in the final days of culture. This could be reflective of the loss of self-renewal property of HSCs which is required to maintain the HSC pool that can ensure continuous supply of

early progenitor cells that give rise to all the mature blood cells[164,165]. Experimentally observed growth kinetics change in the ALKBH5 deficient HSPCs identified an important regulatory function of ALKBH5 in HSPC proliferation as well as its potential contribution in maintaining the HSC pool.

There are reports of ALKBH5's critical role in cell proliferation and certain pathological disorders. For example, Shen, et. al., (2020) found that there is a positive correlation between ALKBH5 level and cancer stem cell renewal[158]. Another study by Wang et al. (2020) demonstrated that KDM4C-ALKBH5-AXL signaling axis preferentially promotes the self-renewal property of leukemia initiating cells and thus propagates tumorigenesis in acute myeloid leukemia[157]. Thus, these studies demonstrated the role of ALKBH5 in maintaining the stemness of LICs and proposed ALKBH5 to be a potential therapeutic target. Another study by Elcheva, et. al. (2020) highlighted the role of IGF2BP1, an RNA-binding protein, in the maintenance of self-renewal property of leukemia cells. IGF2BP1 exerts its influence on the leukemia cells through a group of downstream mediators which include MYB, HOXB4, and ALDH1A1. This study underscores the complex interconnections of the regulatory elements of cellular function given the known interactions between IGF2BP1 and ALKBH5[166]. Therefore, previously published study results corroborate my project findings in illustrating the role of ALKBH5 in HSPC growth kinetics.

4.2 ALKBH5 is important in maintaining the HSC enriched populations.

Tracking the HSC enriched population revealed a unidirectional gradual decline of this population in the deficiency of ALKBH5, which is unlike the fluctuating growth kinetics observed in tracking the total HSPC population with the same ALKBH5 deficiency. This experimental result supports my initial hypothesis that ALKBH5 has a role in HSPCs' self-

renewal and differentiation capabilities. Comparing the steady decline of the HSC enriched population to the variable rate of growth of the total HSPC population delineates ALKBH5's changeable role based on cell type[167-169]. The HSC enriched population depletion could allude to the role of ALKBH5 in maintaining the self-renewal capacity of HSCs. This was predicted based on the total HSPC growth kinetics tracking that showed a rapid decline of the ALKBH5 deficient total HSPCs in the last days of culture. However, the pattern of HSC enriched population decline correlated with total HSPC growth kinetics could also indicate a discrepancy between the self-renewal rate and differentiation rate in the deficiency of ALKBH5. It is possible that in the absence of or diminished regulation of the differentiation process by ALKBH5, the self-renewal rate of the HSCs to maintain their pool is simply not sufficient to meet the demand of the elevated differentiation rate. One interesting pattern to note when comparing the HSC population decline rate of the samples treated with different shRNAs (KD1 or KD2) is that while the frequency of this population was not the same in different samples on earlier timepoint of the experiment, the HSC enriched population were depleted by day 14 in both samples. This could potentially be due to the dose dependency of ALKBH5 in maintaining the self-renewal property of the HSCs. However, biological variability between cord blood units[170-173] as well as off-target effects of shRNA strategy[174-177] could account for some variations introduced to the depletion rate of certain populations. These experimental data present a new avenue to explore in future research, specifically how the variable eHSC depletion rate influences the early HSPC sub-populations as well as the mature blood cells in the context of ALKBH5 deficiency. HSCs reside at the top of the hematopoietic hierarchy maintaining the hematopoiesis of the organism throughout its lifetime[178,179]. Experimental evidence demonstrates that ALKBH5 has role in maintaining the HSC pool and therefore contributes to the regulation of the hematopoiesis system.

These results also link the decline of HSC enriched population with the regulation of gene expression through epigenetic modifications. My data suggest that the m6A demethylase ALKBH5 could have a potential role in maintaining the HSC pool through the regulation of stemness and differentiation. This is in line with the study by Guo et al (2022), which interrogated the histone variant H3.3, for its role in maintaining the stemness of HSCs. They found that H3.3 regulates the balance between the stemness of the HSCs and differentiation into lineage specific progenitors by controlling methylation marks like H3K27me3 and H3K9me3[180]. While there are not many studies directly exploring the relationship between ALKBH5 and the HSC stemness maintenance during healthy human hematopoiesis, quite a few studies link this RNA demethylase with cancer stem cells in different capacities. For instance, Tsuchiya et al (2022) revealed that ALKBH5 was pivotal in regulating the cell cycle phases and thus affecting stemness of the non-small-cell lung cancer cells[156]. Recent human HSC ontogeny mapping by different research groups have identified regulators of molecular mechanisms in fetal to neonatal HSCs which include HMGA2, LIN28B, EZH2, and SOX17[181-184]. These regulators are different compared to adult HSCs' which include PRDM16, BMI1, and ETV6[185-187]. While these studies do not comment directly on the role of ALKBH5, it does underscore the necessity of various regulators of hematopoiesis at different stages of lifecycle. Therefore, the potential role of ALKBH5 in the maintenance of HSC enriched population in *ex-vivo* culture condition aligns with the current knowledge and understanding of HSC biology and reveals a novel molecular mechanism for maintaining the HSC self-renewal.

4.3 Changes in ALKBH5 level is associated with phenotypic and functional changes to HSPCs.

Early HSPC subpopulations tracking revealed reduced proportions of MEPs and GMPs in the ALKBH5 deficient samples. Subsequently, ALKBH5 deficient HSPCs were found to have reduced erythroid and granulocyte-macrophage colony forming potentials. This result supports my hypothesis that ALKBH5 has an impact in HSPC differentiation. Moreover, these data demonstrate the functional change in cellular physiology through changes in colony forming potential of the ALKBH5 deficient HSPCs. My results also draw a connection between the proportion of different early progenitor sub-populations and the reduced CFU scores. Thus, both the phenotypic data obtained using flow cytometry and the functional data from the CFU assay show the impact of ALKBH5 in HSPC fate decision. It is imperative to consider that while the use of the stemness supportive SCAC platform allowed me to investigate the characteristics of the early HSPCs, this culture platform is known to favor some cellular features (related to cellular proliferation) over others[110]. Therefore, the loss of colony forming potentials of the ALKBH5 deficient HSPCs can allude towards a mechanistic aspect involving ALKBH5 on how the SCAC platform expands HSPC populations with high efficiency (15-fold increase in SRCs over input). These observations also suggest that perhaps m6A methylation may be of importance in ex-vivo expansion of HSCs when using other small molecules such as UM171, VPA, and SR1.

Phenotypic changes observed by the changes in the MEP and GMP population frequencies indicated a functional change in ALKBH5 deficient samples. This prediction was confirmed by reduced colony forming potential of the test samples, which is in line with a previous study by Li et al (2023) who found that ALKBH5 reduction led to reduced colony

forming potential as well as diminished engraftment of the ALKBH5-reduced cells in a mouse model of t(8;21) acute myeloid leukemia[188]. Both this study and the data presented in my thesis suggest a critical role of ALKBH5 in maintaining the optimum functional capacity of HSPCs. Change in the functional capacity of cells is related to cell fate decision[189,190]. A previous study by Chen et al (2021) revealed a crucial role played by ALKBH5 in the biogenesis of micro RNAs (miRNAs) through an m6A dependent manner. This ability of ALKBH5 in modifying RNAs is important in gene expression and cell fate decision[191], which is in line with altered HSPC subpopulations in ALKBH5 deficient samples in my experiments. In summary, current understanding of the role of ALKBH5 in HSPC fate decision[192] aligns with my data suggesting a change in the fate decision of ALKBH5 deficient HSPCs.

4.4 ALKBH5 contribute to the long-term engraftment potential of HSPCs.

My *in-vivo* murine peripheral blood data demonstrated reduced long-term engraftment potential of the ALKBH5 deficient HSPCs despite evidence of successful engraftment at week 3 blood analysis. This result answered another question from my hypothesis which asked if ALKBH5 had role in the engraftment potential in *in-vivo* systems. The presence of human (donor) cells[193,194] in the murine (recipient) peripheral blood at early time-point (3 weeks) post transplantation is indicative of successful homing of injected HSPCs to the murine bone marrow followed by engraftment. This implies that ALKBH5 deficiency does not impact the homing capability of the HSPCs. However, CXCR4/CXCL12 expression, which are associated with homing capabilities of HSPCs[57,195-198], can be studied in this context to further elucidate any changes in the mechanisms involved. Alternatively, the decline in the human platelets, leukocytes and HSPCs in the murine peripheral blood by week 18 following ALKBH5 deficient HSPC transplant could be due to the progressive failure of the transplanted

HSPCs to sustain the hematopoiesis for long-term, which is defined by ability to give rise to hematopoiesis beyond 16 weeks in the context of murine system[199-202]. The reduction in long-term engraftment potential could point toward multiple causes including diminished self-renewal capacity of the HSCs or impaired differentiation potential of the HSCs[179,203,204] *in-vivo*. Interestingly, the murine peripheral blood data at an intermediate point, between the evidence of successful engraftment and decline of the analyzed human cell populations, showed elevated levels of platelets and HSPCs in the test cohort. This could be indicative of elevated rate of differentiation of the transplanted HSPCs *in-vivo*[205], not unlike the transient rise in the growth kinetics observed in the *ex-vivo* culture.

The impaired long-term engraftment potential of the ALKBH5 reduced HSPCs in my experiments is consistent with previous studies that demonstrated the role of different proteins/cells in HSPC homing and their maintenance in the bone marrow niche. For example, several studies established that certain blood cells (eg VCAM-1 macrophages[206]), receptors (eg CXCR4[207]), and other interacting proteins (eg CXCL12[208]) contribute to ensure efficient homing as well as maintenance of transplanted HSPCs and subsequent differentiation to required progenitor cells[209]. A recent study by Gao et al (2021) demonstrated that ALKBH5 deficient cells displayed a competitive disadvantage at all differentiation stages except in long-term HSCs in murine system[132]. It is notable that humans and mice have a different m6A landscape which may contribute to some differences in the hematopoiesis system regulation between humans and mice[117,210]. Together these data demonstrate the potential role of ALKBH5 in regulating long-term normal hematopoiesis which expands on the current knowledge in the field of HSC biology.

4.5 ALKBH5 is a critical regulator of human multi-lineage chimerism.

Analyzing the murine bone marrow composition revealed altered human multi-lineage chimerism formation in the recipient of the ALKBH5 deficient HSPC grafts. This result aligns with my hypothesis that postulates that ALKBH5 regulates differentiation of HSPCs in *in-vivo* systems. The experimental data indicated a regulatory role of ALKBH5 in the cell fate decisions of HSPCs as evidenced by the diminished leukocytes and B lymphocytes formation in the murine bone marrow in the deficiency of ALKBH5 in the transplanted HSPCs. *In-vivo* evidence of the impact of ALKBH5 in cell fate decision further supports the significant role played by ALKBH5 in normal hematopoiesis.

Previous studies have already provided strong evidence in support of the regulatory role of ALKBH5 in cell differentiation. For instance, Yu et al (2020) found that ALKBH5 to be one of the proteins involved in the osteogenic differentiation process through NF-κB signaling[211]. Moreover, others have shown the complex yet precisely regulated nature of the hematopoiesis process involving various cellular elements besides ALKBH5. Recently Barahona de Brito et al., (2022) have identified NFATc1 to be an important regulator hematopoiesis and differentiation of HSPCs[212]. On the other hand, Lechman et al., (2015) were able to improve the long-term repopulating activity of stem cells through the introduction of exogenous micro RNA[213]. Thus, the discovery of the regulatory function of ALKBH5 in maintaining a multi-lineage population during normal hematopoiesis helps to build a more comprehensive map of cellular pathways involved.

4.6 ALKBH5 mediates Intracellular changes impacting cell cycle progression.

Assessment of some intracellular properties revealed elevated global m6A methylation in HSPC transcripts. Moreover, reduced TACC3 transcript stability and a lower percentage of

cells in G2S phase of cell cycle were observed in the ALKBH5 deficient samples. These results confirmed that ALKBH5 influences the intracellular machinery that drives cellular proliferation and thus provides some potential mechanistic insight in support of my hypothesis of the regulatory role of ALKBH5 in HSPC functions. Interpreting the results in the context of cellular growth and proliferation can help propose a sequence of events: the rise in the m6A levels is a direct consequence of ALKBH5 deficiency[214,215], as this RNA demethylase is responsible for removing the m6A marks. This global change in m6A methylation level is most likely to impact the m6A level of TACC3 transcript. The stability reduction of TACC3 transcript could be due to the recognition of this transcript by m6A reader proteins like YTHDF2 which directs the RNAs towards degradation mechanisms[216-219]. In addition, impaired cell cycle progression due to unstable TACC3 could be a contributing factor for fewer cells in the G2S phase of ALKBH5 reduced samples, as the TACC3 protein has a significant role in cell division and chromosome stability[220-223]. The results of these experiments potentially depict the impact of ALKBH5 on a portion of the intracellular mechanisms driving the cellular proliferation. As the parameters tested here are critical to the self-renewal and differentiation process of HSPCs, the changes in these parameters in ALKBH5 deficiency underscores the impact of ALKBH5 in intracellular processes.

Recent studies have found evidence of ALKBH5's influence on intracellular processes in the cellular proliferation process. Yadav et al (2022) found the m6A methylation, which is regulated by ALKBH5, to be the driving factor in cancer cell growth[224]. Moreover, Suhail et al (2015) showed that the TACC3 protein is critical in the progression of cell cycle[220]. Shen et al (2020) also demonstrated that the stability of the TACC3 transcript declines in the deficiency of ALKBH5[158]. Both, m6A and TACC3, levels change following ALKBH5 reduction

underscores the interconnectivity of intracellular mechanisms that regulate cell growth and proliferation.

4.7 Proposed mechanistic model

Current knowledge in the field and my experimental results highlights the critical role and interconnection ALKBH5 has in the regulation of normal hematopoiesis. Therefore, I propose a model (Figure 12) where inhibition of normal function of ALKBH5 by reducing the transcript dosage (using RNAi strategy) leads to impaired demethylation (of m6A) which ultimately disrupts the balance between self-renewal and differentiation of HSCs by modulating certain gene expression essential for HSC maintenance. Disruption of the balance between self-renewal and differentiation leads to rapid differentiation to progenitor cells marked by exhaustion and depletion of HSC pool.

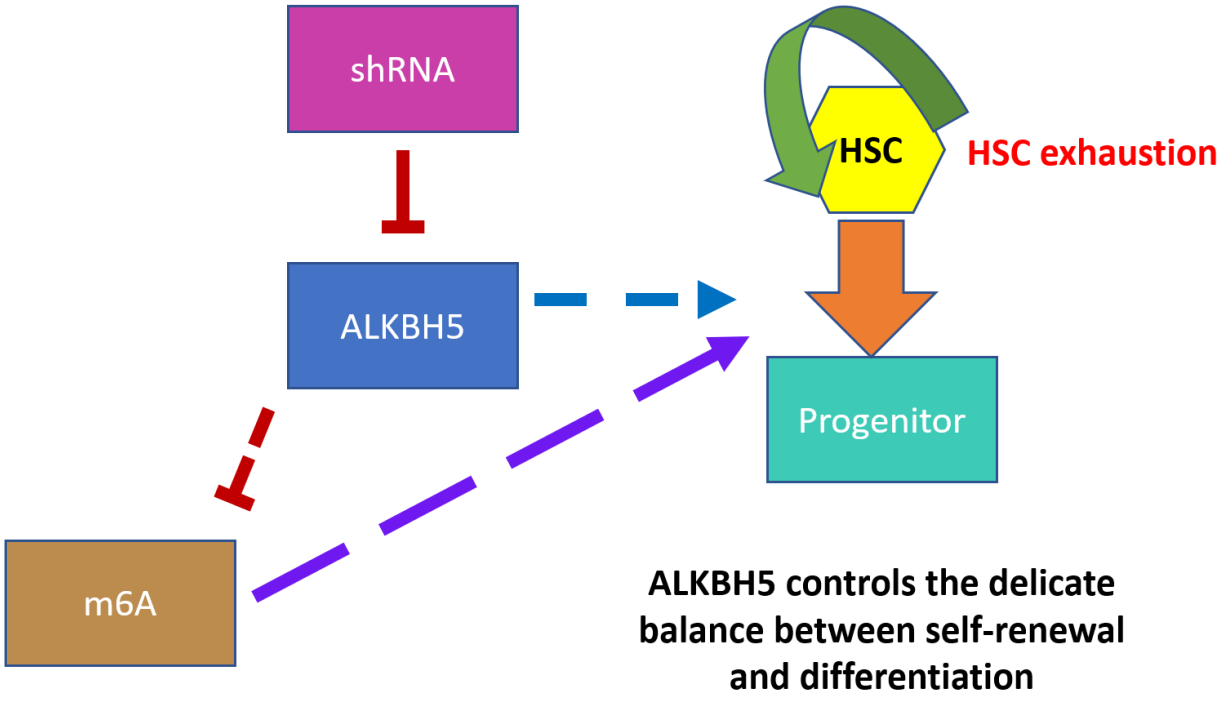

Figure 12. Proposed model of HSC self-renewal and differentiation regulation by ALKBH5. This model based on experimental findings and known properties of the model component suggests that ALKBH5 maintains (through RNA demethylation of m6A) the balance between the self-renewal and differentiation properties of HSC. Disruption (using methods like RNA interference strategies like shRNA) of this process can lead to HSC exhaustion by upregulating differentiation. m6A: N6-methyladenosine, HSC: hematopoietic stem cell.

4.8 Significance, Limitations and Future directions

The RNA demethylase ALKBH5 has been found to be an important prognostic factor in different diseases[225-228]. It has also been shown to play a critical role in the maintenance of self-renewal capacity of leukemia initiating cells[158]. Furthermore, some studies suggested a limited or dispensable role of ALKBH5 in normal hematopoiesis[157,158]. However, most of these studies reported the impact of the protein on murine HSCs. Furthermore, murine transcripts have been reported to have a different m6A landscape compared to humans[210]. To my knowledge, I demonstrated for the first time the impact of ALKBH5 within healthy human hematopoiesis both *ex-vivo* and *in-vivo*.

Studying the HSPC behavior in *ex-vivo* conditions present multiple challenges including approximation to the physiological environment, maintaining the appropriate population in the culture conditions long enough to study the time dependent impacts of any treatment. To address some of these concerns, the experiments conducted for this project took advantage of the SCAC X2A culture platform, which is efficient in maintaining early HSPC populations for longer in *ex-vivo* culture[110]. Investigating the impact of ALKBH5 within populations of different maturity levels/characteristics will require the use of different culture conditions/media[229,230]. However, using a different model system for *ex-vivo* culture may introduce some variations to the results, that could present a challenge in comparing these results. Moreover, different umbilical CB sourced HSPCs could be the source of some variability which is an inherent characteristic of biologic differences between samples. One way to overcome this limitation is to include more biological samples so the results are not skewed by outliers. Finally, while these experimental results provide a glimpse into some intracellular features like m6A methylation, cell cycle status and TACC3 stability for day 10,

more data points before and after this day will give a more comprehensive understanding for the sequence of events that occur in the deficiency of ALKBH5.

My project findings demonstrate the impact of ALKBH5 in the normal hematopoiesis through *ex-vivo* and *in-vivo* studies. Other studies have already showed the indispensable role of ALKBH5 in the maintenance of the leukemia initiating cells (LICs). Therefore, future studies could be directed towards mapping the gene regulatory network associated with ALKBH5. Identifying the potential interaction with other proteins, as well as the impact on the epi-transcriptomic landscape of relevant RNAs and metabolic pathways critical for normal HSC function could expand our understanding of the role of ALKBH5 in the context of normal HSC biology. Comparison of the normal cellular mechanism to that of leukemia could help identify downstream targets of ALKBH5 that are essential in maintaining LIC but not HSCs. This knowledge could contribute to developing targeted therapies for different types of diseases where ALKBH5 plays critical roles.

4.9 Conclusion

My findings not only provide new insights into the regulatory role of ALKBH5 in the self-renewal and differentiation of HSPCs, but also introduces new research avenues to be explored. The potential implications of furthering this work could extend from improving our understanding of stem cell biology to developing improved stem cell expansion platform to identifying therapeutic targets for various disease treatment. We are presented with complex interactions within the transcriptional network, among proteins or the components of different metabolic pathways as research in the stem cell biology field continues. The data presented here underscores the importance and complex influences of ALKBH5 in HSPC biology, further emphasizing the need for continued investigation.

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Supplemental Figure

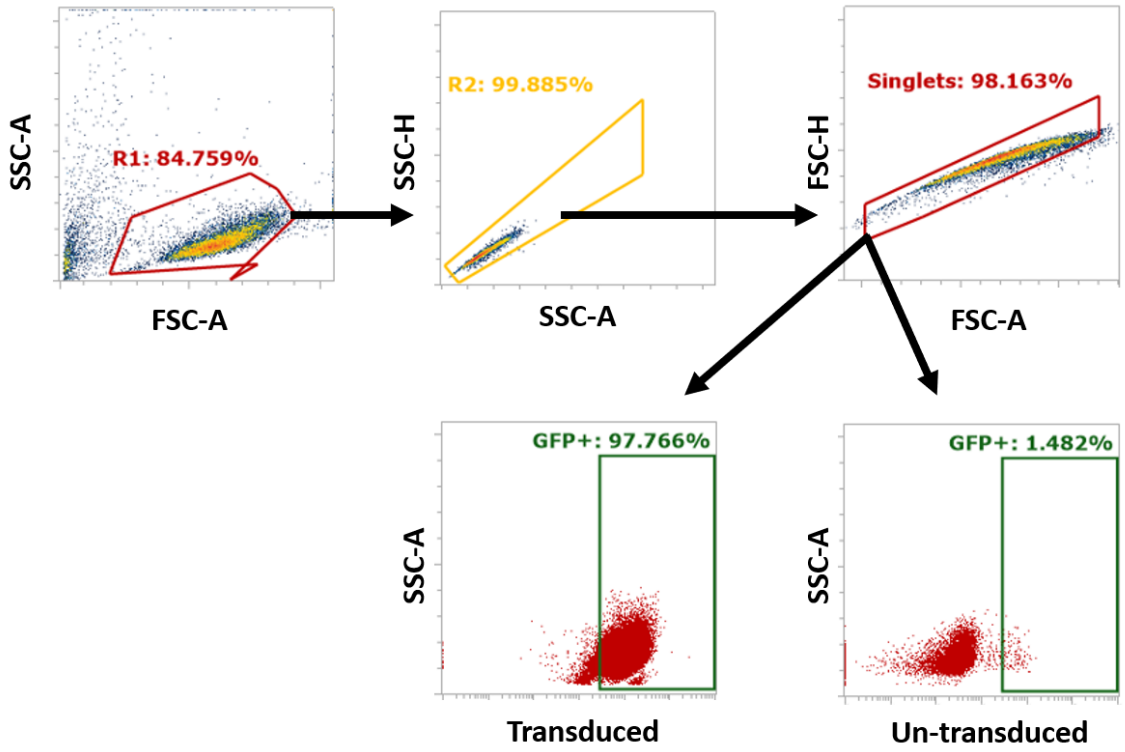

Figure 13. Transduction efficiency estimation. Transduced and un-transduced cells were analyzed using flowcytometry. Proportion of transduced cells compared to the total nucleated cells were expressed as percentage (GFP+) for transduced and un-transduced samples. Flow cytometry plots prepared in Attune^{TM} Cytometric software (Version 5.2.2302.0).