Cord Blood CD34+ Expansion Using Vitamin-C—An Epigenetic Regulator

Sakhar Almoflehi

A thesis submitted in partial fulfillment of the requirements for the Master of Science in Immunology

Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine University of Ottawa

Abstract

Vitamin-C (Vit-C) has been shown to modulate hematopoietic stem cells and leukemia stem cell frequency in-vivo. Herein, Vit-C analogue, L-ascorbic acid 2-phosphate (AA2P), was investigated as a new potential HSC expansion agonist. Cord blood CD34+ cells were expanded in cultures with or without AA2P. AA2P induced a 2-fold increase in the expansion of stem and progenitor subsets including lymphoid-primed multi-potential progenitors ($p<0.05$, $n=3$) and functional colony forming progenitors. The functional properties of AA2P grafts was evaluated with a xenotransplant model. Superior platelet levels in the periphery ($p<0.05$) and human bone marrow engraftment (median 75% hCD45+ cells for AA2P Vs. 48% for PBS control at week-22, $n=3$, $p<0.05$) was detected in AA2P cohorts Vs. control. In summary, my results demonstrate that AA2P is a new stem and progenitor expansion agonist with AA2P-expanded stem and progenitor cells capable of increased engraftment and higher platelet recovery. These findings may aid to overcome cord blood limitations; thereby, improving clinical relevance.

Acknowledgments

Firstly, I would like to thank Canadian Blood Services for providing me with the facilities and resources required to pursue my research project. I would also like to thank my thesis supervisor, Dr. Nicolas Pineault, for his valuable guidance and support throughout my master's degree. This opportunity has allowed me to work with various research tools and master invaluable techniques in the field of stem cell research.

I would also like to thank the members of the Pineault Laboratory for their support and guidance. A special thank you, to Dr. Javed Manesia for teaching me the techniques required for this project and for his continuous support and help throughout this journey. I would also like to thank Roya Pasha, Suria Jahan, and Richa Kaushal for their assistance during my time at the Pineault Laboratory.

I would like to thank my family for all of their loving support throughout this process. A very special thank you the other honours students that I had the pleasure teaching. They have made a lasting impact that got me to where I am today.

Lastly, I would like to thank my thesis advisory committee members, Dr. Caryn Ito and Dr. Lisheng Wang for their suggestions and guidance though the many committee meetings.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

5azaD — 5-aza-2-deoxycytidine

5mC — 5-methylcytosine

5hmc —5-hydroxymethycytosine

AA2P — L-Ascorbic acid 2-phosphate

AHR — Aryl hydrocarbon receptor

BFU-E — Blast forming unit–Erythroid

BM — Bone Marrow

CB — Umbilical Cord Blood

CFU — Colony forming unit

CFU-M — Colony forming unit monocyte

CFU-GM— Colony forming unit-granulocyte, macrophage

CFU-GEMM — Colony Forming Unit-granulocyte, erythroid, macrophage, megakaryocyte

CLP — Common lymphoid progenitors

CMP — Common myeloid progenitors

CpG — cytosine-phosphate-guanine dinucelotides

CXCL12 — CXC-chemokine ligand 12

CXCR4 — CXC-chemokine receptor 4

EPCR — Endothelial protein C receptor

EpcrHg — CD34+CD45RA-EpcrHigh

FACS — Fluorescent activated cell sorting

FBS — Fetal bovine serum

FLT-3 — Fms like tyrosine kinase 3

GMP — Granulocyte-monocyte progenitor

HDAC — Histone deacetylase

HPC — Hematopoietic progenitor cells

HSC — Hematopoietic stem cells

HSCT — Hematopoietic stem cell transplantation

HSPC — Hematopoietic stem and progenitor cells

IMDM — Iscove's Modified Dulbecco's Medium

LDA — Limited dilution assay

LDL — Low-density lipoprotein

LT— Long-term

LTC-IC — Long-term culture initiating cell assay

LMPP — Lymphoid-primed multipotent progenitors

Mab — Monoclonal antibodies

MEP — Megakaryocyte-erythroid progenitor

MPP — Multipotent progenitor

NSG— NOD-SCID interleukin (IL)-2Rγ

PB — Peripheral blood

PBS — Phosphate Buffered Saline

PGE2 — Prostaglandin E2

ROS — Reactive oxygen species

SCAC — Stem cell agonist cocktail

SCF — Stem cell factor

SCID — Severe combined immune deficiency

SFM — Serum free culture

SR1 — StemReginin1

ST — Short-term

TET — Ten-eleven translocation

TNC — Total nucleated cells

TPO — Thrombopoietin

TSA — Trichostatin

Vit-C — Vitamin-C

VPA — Valproic acid

Chapter 1: Introduction

1.1 Hematopoiesis—The Hematopoietic Stem Cell

A continuous process involving renewal, proliferation, differentiation, and maturation of all blood lineages, hematopoiesis is described as a cellular hierarchy where at the apex resides—the multipotent hematopoietic stem cell (HSC)[1–4]. All components of blood are derived from this common cell which produces progenies responsible for the formation, development, and specialization of all functional blood cells that are released from the bone marrow (BM) into circulation in an adult[1-2]. Within this system, only HSCs possess both multipotency and long-term self-renewal. The ability to differentiate into all functional blood cells is termed multipotency; the ability to give rise to HSC itself without differentiation is termed self-renewal—these encompass the hallmark properties of HSCs[5]. Proliferation and differentiation behaviour of HSCs are key for maintaining a balanced and lifelong blood supply.

To ensure enough mature blood cells are generated, as many are short-lived[5], more than 1 million cells are produced per second[6,7]. HSCs, which represent 0.01% of the total BM cell population[8], remain quiescent under normal conditions, but induced to proliferate under demand or conditions of stress[6]. To meet these demands, the tight regulation of HSC is required—dysregulation of these programs lead to unchecked growth of cells such as those disorders found in leukemia[9]. Control of HSC pool is maintained by asymmetrical and symmetrical division[9]. Asymmetrical division produces one stem and one differentiated[10–12]; symmetrical division produces two differentiated or two stem cells[13–16](Figure-1).

HSC self-renewal is regulated by intrinsic and extrinsic signals that are managed by localized microenvironments termed “niche”[17]. Niche support stem cell function by producing factors that act directly on stem cells[18]. In adult mammals, the BM is the specialized niche where

hematopoiesis largely takes place and is regulated by cellular vascular endothelial cells and osteoblasts[17,19](Figure-2). These cells make up structures and extracellular matrix that tightly regulate steady expansion, stemness, and differentiation[20]—maintaining the integrity and function of HSCs.

Specifically, BM endothelial cells act as a mechanical barrier that prevent entry of mature red blood cells and platelets into the BM from circulation, regulate cellular trafficking, hematopoiesis and osteogenesis[21–23]—contributing to a specialized perivascular niche where most hematopoietic stem and progenitor cells (HSPC) reside[24–27] Here, HSCs that remain quiescent have been identified to shelter close to the endosteal surface in the trabecular bone[17]. Interestingly, HSC maintenance is dependent on less permeable endosteal blood vessels that support a low reactive oxygen species (ROS) niche[28]. To reinforce this, BM arteries retain HSC populations by downregulating nitric oxide production, improving adhesion, and reducing migration[29]. However, the existence of a diverse vascular niche exists whereby quiescent HSCs must egress and home to other distinct locations for differentiation to proceed[27,28].

The release of factors to promote HSC maintenance is another important function of niche cells, most important of which include stem cell factor (SCF), CXC-chemokine ligand 12 (CXCL12/SDF-1a), and thrombopoietin (TPO). Available in both membrane-bound and soluble forms, SCF is an important growth factor that requires binding to the receptor tyrosine kinase, expressed by HSCs, for maintenance properties[30–33]. Similarly, CXCL12 activates signalling through CXC-chemokine receptor 4 (CXCR4), expressed by HSCs, to promote HSC maintenance and retention in the BM[34]—deletion of either these proteins deplete HSCs from the BM[35,36]. In addition, their role in homing properties of transplanted HSCs is integral for increased engraftment and proliferation activity[37,38]. Finally, by activating myeloproliferative leukaemia protein

signalling on HSCs, TPO is an important growth factor for megakaryocyte and platelet production, and important for HSC maintenance[39–41]. However, it is still unknown whether local or distant production of TPO is key for HSC maintenance as the liver produces the highest levels of TPO[42,43]. Overall, perivascular stromal- and endothelia-cells together express or secrete factors that promote proliferation, differentiation, and maintenance of HSCs that are necessary for long-term (LT) hematopoiesis.

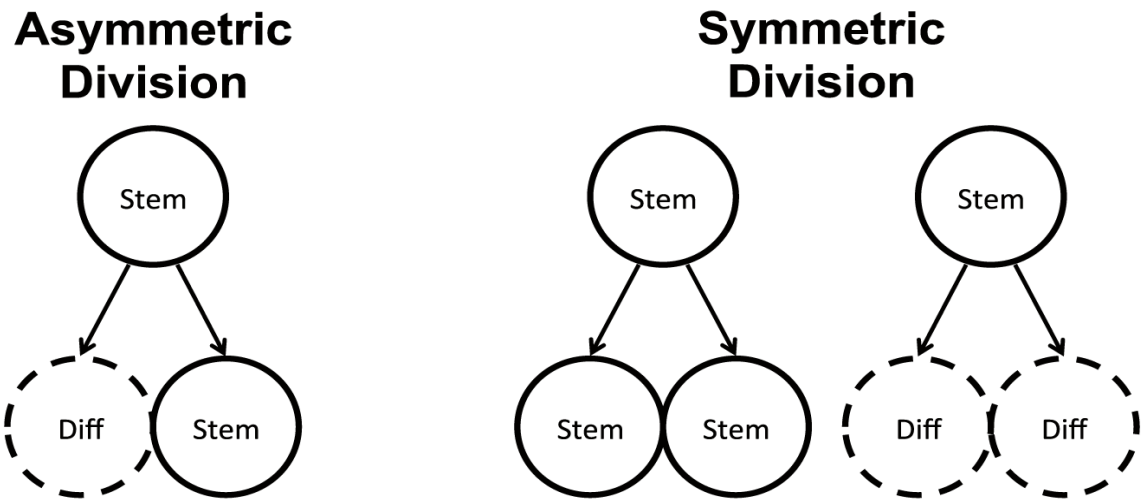

Figure 1. HSC Differentiation: Asymmetric and Symmetric. Two models of division an HSC can undergo. Model one (left): asymmetrical division where a stem cell produces one daughter and one differentiated cell. Model two (right): symmetrical division where an HSC produces two differentiated cells or two daughter cells. Figure adapted from Shahriyari et. Al[9], 2013. (Content of the legend is from my thesis).

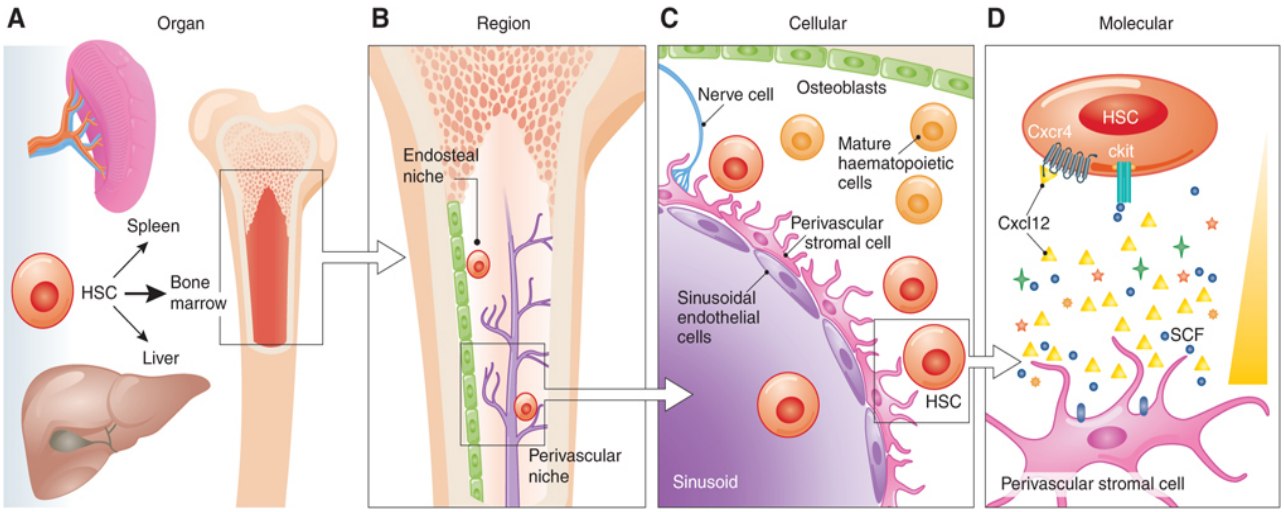

Figure 2. Bone marrow & the hematopoietic niche. Visualization of the HSC niche at increasing resolution (A) HSCs can circulate and take up residence in organs like the spleen and liver, but the majority of functional HSCs in an adult reside in the BM (B) Localization of HSCs in perivascular or endosteal regions, where they interact directly or indirectly with different kinds of cells that make-up the HSC niche (C) HSC at the cellular level (D) Niche cells release factors that maintain hematopoietic balance by favouring HSC quiescence, and self-renewal or differentiate when needed. This figure shows how HSC location is becoming increasingly more detailed with the introduction of novel tools and refined approaches. Figure adapted from Ugarte et al.[220] (Content of the legend is from my thesis).

Recent single cell study such as transplant and RNA sequencing has revealed that the HSC subpopulation is highly heterogenous (Figure-3). This HSC heterogeneity consists of varying differentiative and proliferative potential, which have been elucidated via single HSC transplantation[44,45]. Unlike LT-HSCs that replenish a lifelong supply of blood with their ability to self-renew and contribute to multilineage reconstitution, short-term (ST) HSCs can only accomplish this for up to 6 weeks[46–48]. Loss of self-renewal potential is evident in ST-HSCs developing into progenitors that become increasingly lineage restricted with further differentiation[49]. The first descendant from HSCs, the multipotent progenitor (MPP), proliferate extensively before maturing to terminally differentiated cells[6]. Here, MPPs are cells that initiate differentiation—bridge LT self-renewal of HSCs and first lineage-committed progenitors. MPPs can generate all mature blood cell types but have limited self-renewal capacity; therefore, only able to support transient hematopoiesis[46,47].

CD34, a transmembrane phosphoglycoprotein[50], is expressed on most human hematopoietic repopulating cells and progenitor populations[51–53]. Further enrichment of human HSCs within the CD34+ population can be achieved by combining other markers, such as the multi-functional transmembrane protein CD38[54,55]. Expressed in various immune cells and progenitors[56], only cells that reside in the CD38 low or negative fraction give rise to multilineage colonies and engraftment[51,57–60]. Moreover, cells positive for CD90 are highly enriched for LT culture-initiating cells and capable of repopulating immunodeficient mice[58,61]. Furthermore, an isoform of CD45 that negatively regulates certain classes of cytokine signaling, CD45RA, is important for identifying HSPC[62]. Here, HSPCs that are functionally multipotent but have incomplete self-renewal capacity are CD34+ CD38− CD45RA−CD90−[63]. Ultimately, repopulating activity can be probed using the following phenotypic markers:

CD34+CD38−CD45RA−CD90+[57,64–66]. More recently, CD49f positive cells with a CD34+CD38−CD45RA−CD90+ phenotype have been shown to be highly enriched in HSC activity, while CD34+CD38−CD45RA−CD90+CD49f− are enriched in MPP; thereby, further distinguishing HSC and MPP subsets[67]. These phenotypic profiles are tailored for primitive HSC populations that begin LT cultures, capable of repopulating immunodeficient mice, and present in human transplants[50].

Blood cells can be categorized into either the myeloid or lymphoid lineage by MPPs (CD34+CD38–CD45RA–CD90–)[68] that give rise to precursors—separable at the progenitor level[69]. Although hematopoietic progenitor cells (HPC) are CD34+, they co-express CD38 with cell surface proteins specific to lineages such as CD33 (myeloid lineage)[70] and CD19 and CD10 (B-lymphoid lineage)[71]. Of importance is the oligopotent intermediate, the common myeloid progenitor (CMP: CD34+CD38+CD45RA–CD123–)[68], capable of differentiating into granulocyte-monocyte progenitor (GMP: CD34+CD38+CD45RA+CD123+)[68] or the megakaryocyte-erythroid progenitor (MEP: CD34+ CD38+CD45RA–CD123^{-})[68,2]. GMPs can produce granulocytes (basophils, eosinophils, neutrophils, and mast cells) or monocytes (macrophages); MEPs go on to make red blood cells and platelets—all transient mature cell populations[72]. Unlike MPPs capable of multi-lineage differentiation, lymphoid-primed multi-potent progenitors (LMPP: CD34+CD38–CD45RA+CD90–)[72] that are downstream of MPPs replenish durable adaptive immune cells (B and T) and natural killer cells through the common lymphoid progenitor (CLP: CD34+ CD38– CD45RA+CD90–)[68], granulocytes, and monocytes—all but erythrocytes and megakaryocyte[72,73].

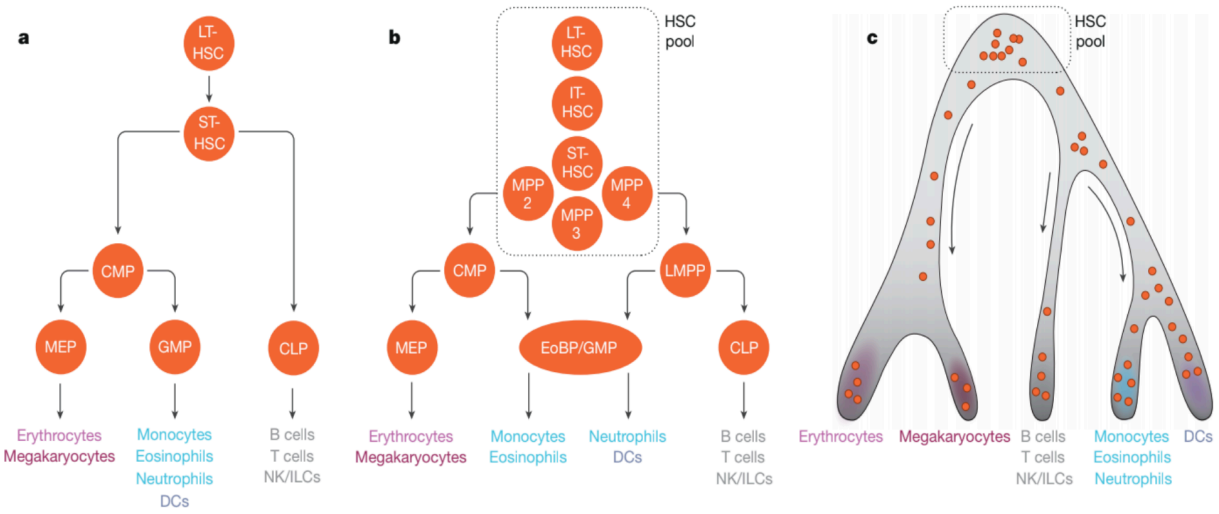

Figure 3. Hierarchical models of hematopoiesis. Changes in the understanding of HSC hierarchy and hematopoiesis. a) year 2000: homogenous HSC population, downstream of which common myeloid progenitor (CMP) and common lymphoid progenitor produce indicated cells[221] b) 2005-2015: new observations that HSC pool is heterogenous in self-renewal and differentiation. The addition of lymphoid primed multipotent progenitor (LMPP) and heterogenous granulocyte-monocyte progenitor (GMP)[222] c) 2016-present: continuum of differentiation where each red dot represents a single cell and its localization[221]. (Figure adapted from E Laurenti and B Göttgens, 2018 (Content of the legend is from my thesis).

1.2 Functional Assays Available to Track HSC and Progenitors

HSC populations can be identified based on their differential expression of multiple surface proteins[50]. Positive selection for CD34 and CD90 combined with negative selection of mature hematopoietic markers (lineage markers) enriches HSPCs[74]. Although fluorescent activated cell sorting (FACS) methods are important for revealing enriched HSC populations, identification of HSCs is determined by assays intended to measure stem cell function[74]. Functional analysis of HSPCs are important for providing the highest quality of stem cell product needed by transplant recipients. Functional properties of HPCs can be assessed using the colony forming unit (CFU) assay by examining the growth and morphological colonies of cells grown in a specialized semisolid medium[75]. Progenitors detected with this assay include burst-forming unit-erythroid (BFU-E), colony-forming unit erythroid (CFU-E), colony-forming unit monocyte (CFU-M), colony-forming unit granulocyte (CFU-G), CFUs containing granulocyte and monocyte (CFU-GM), and immature CFUs containing granulocytes, erythrocytes, monocyte, and megakaryocytes (CFU-GEMM)[44]. These enumerated colonies provide important information for measuring functional progenitors present in stem cell products; therefore, underscoring its clinical relevance[76]. More importantly, there exist a well-established positive correlation between the graft CFU content and engraftment activity, despite the fact that HSC are not detected using the CFU assay[77]. The long-term culture initiating cell assay, or LTC-IC, is another in vitro assay that can detect progenitors that are far more primitive than those detected by the CFU assay[78]. However, due to its long processing time (up to 7 weeks), this assay is not routinely used in clinic and reserved for research work.

Only transplantation experiments can truly measure HSC function. The transplantation assay is performed to evaluate HSC activity via transplantation into irradiated mice in research settings—it is the gold standard assay which requires serial transplantation to confirm engraftment

and self-renewal capabilities[79]. Human HSC 'grafts are transplanted intravenously (i.v.) or intra-femoral into immunodeficient mice such as the NOD-SCID interleukin (IL)-2Rγ (NSG)[80]. The mice carry two mutations on the NOD/ShiLtJ genetic background; severe combined immune deficiency (scid) and a complete null allele of the IL-2 receptor common gamma chain (IL2rgnull)[81]. Unlike NOD/Scid mice that prevent LT studies because they develop lymphoma, NSG mice lack B, T, NK cells, support 5-fold higher human CD34+ engraftment, and defects in lymphomagenesis allow for LT investigation[81]. While NSG mice are a viable model for reconstituting human HSPC, there are some developmental function defects that remain[81–83]. For example, differentiation of human HSC into functional myeloid cells and NK cells is limited[84,85], B-cells do not undergo optimal maturation to become memory and antibody-producing cells[86,87], and T-cell maturation is disturbed due to the lack of thymic support which follows a strong bias in B-lymphocyte differentiation[88,89,].

LT engraftment is now evaluated 4-months post transplantation since ST-HSCs and MPPs produce progenies for up to 16 weeks post-transplantation[90]. LT-HSC potential requires evaluating engraftment 4-months following transplantation or performing secondary transplantation to assess self-renewal ability of HSCs present in the primary graft[91,92]. The ability to generate serial progenies and subtypes in addition to sustaining a lifelong supply of blood components can be tested using serial transplantation assays. Serial transplantation assays are used to test the power of HSCs' ability to self-renew; therefore, testing LT potential[93]. Self-renewal capacity of HSCs inevitably declines with time, leading to the accumulation of unrepaired and damaged cells as well as mature differentiated cells[94]. Ultimately, the serial transplantation assay is great for assessing HSC exhaustion and aging, which accompany a decline in stem cell function. Finally, HSC frequency in a given graft or cell aliquots can be estimated using the limiting dilution assay[44]. A

series of dilutions of test cells are transplanted into mice and the actual frequency of HSC can be calculated by Poisson distribution[44].

1.3 Umbilical Cord Blood: The Good and The Bad

HSC transplantation (HSCT) is the most successful and final course of action available to treat patients with hematological malignancies, BM failure, severe anemia, and many other health related problems[68]. Umbilical cord blood (CB) is readily available, efficiently stored, HLA-mismatch friendly, and less associated with graft-versus-host disease [68,95]. The first CB transplant was used to treat and save the life of a child with Fanconi anemia, a fatal genetic disorder[96]. The successful transplant cured the hematological manifestation of Fanconi anemia by replacing the recipients genetically diseased blood cells[96]. 25 years later, the recipient of the first CB transplant is alive and well—now, all disorders treated with BM transplants can be successfully treated with a CB transplant[97,98]. However, CB has slow early neutrophil and platelet engraftment; reduced LT immune reconstitution or homing that is associated with faulty adhesion receptor fucosylation on HSPCs; and higher risk for graft failure[99–102]. Furthermore, a single CB unit does not contain enough cells for an adult transplant recipient[103]. Ultimately, a low number of HSPC in CB units are thought to be the cause of this problem[104].

1.4 Overcoming CB limitations: The Strategies

The main limitation of CB transplantation is the small number of HSPCs per CB unit which often leads to delayed engraftment[68]. Two independent strategies are currently under investigation to improve the engraftment and use of single CB units; increasing the homing efficiency of HSC and progenitors by priming cells to the BM and, ex vivo expansion of CB HSPCs to produce large quantities of HSC and progenitors[105–107].

Successful HSC transplantation requires transplanted cells to harbour into their niche, the BM, and engraft[108]. HSPCs normally reside in the BM to function as intended—to proliferate, differentiate, and propagate hematopoiesis[108]. However, HSPC egress from the BM and enter circulation via process of mobilization or to harbour the BM by homing[109]. Homing activity is dependent on chemoattractant CXCL12 that is expressed by endothelial cells in the BM[110,111]. CXCL12 binds and activates CXCR4 that is found on hematopoietic cells[112]. Evidently, improved homing and repopulation of HSCs in the BM is associated with increased production of CXCL12 and expression of CXCR4[113]. For example, short exposure of prostaglandin E_2 (PGE_2) to human HSCs significantly enhances homing by increasing CXCR4 mRNA and surface expression; thereby, boosting migration towards SDF-1a and homing to the BM which accompany improved engraftment[114]. These migratory properties can be examined by transwell migration assays that determine the capacity of these processes. HSPC transmigration is achieved through the use of stimulating agents, such as CXCL12, that act to direct hematopoietic migration[109]. Here, cells are positioned in the upper well and must transmigrate, bypassing micropores, towards the bottom chamber where the chemoattractant resides[108] (Figure-4). The transwell migration assay allows for reliable and cost-efficient results that are important for insight into HSPC migratory behaviour.

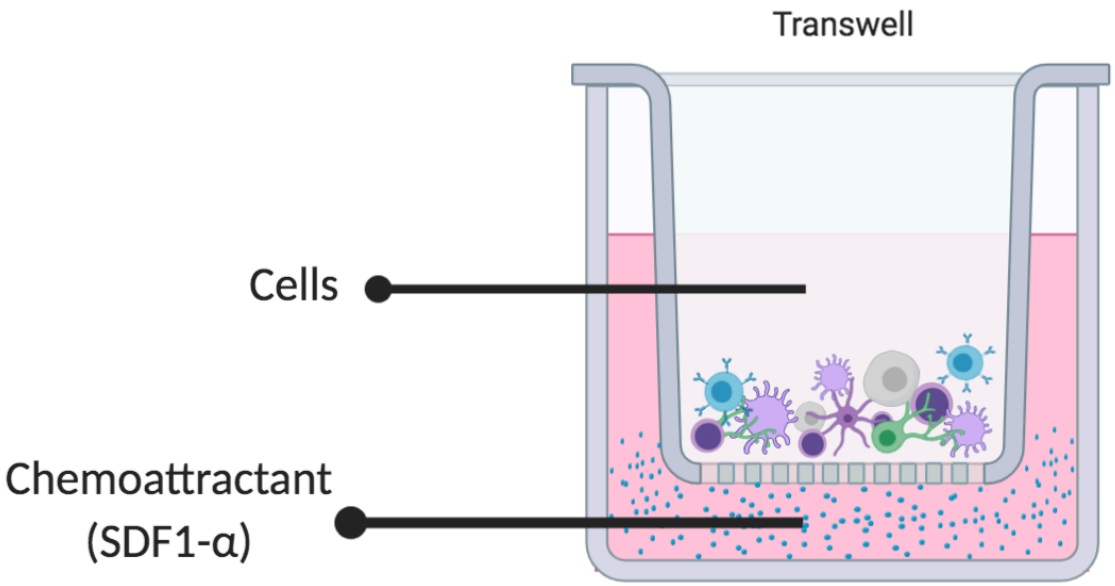

Figure 4. Transwell migration assay. Cell plated at the upper chamber must bypass 5µm pores to the lower chamber where the chemoattractant CXCL12/SDF1-a is present. The chemoattract binds cells expressing CXCR4 receptor and directionally pulls the cells towards its concentration.

This interaction regulates retention, migration, and mobilization of HSCs in hemostasis and injury[113]. Additionally, homing of HSPCs requires binding to P- and E-selectins found on the BM vasculature[115]. This binding supports transmigration of HSPCs to their respective niche; therefore, improving engraftment. CB CD34+ cells have reduced binding affinity to selectins because of reduced fucosylation of receptor such as P-selectin glycoprotein ligand-1[102]. To overcome this problem, Popat et al. treated HSPCs with guanosine diphosphate fucose and the enzyme fucosyltransferase[116]. By improved surface fucoslyation, HSPCs can better bind selectins; thus, improving HSPC homing to the BM. This strategy resulted in enhanced engraftment in patient and shorter time to neutrophil and platelet engraftments.

Conversely, the other strategy to improve clinical application of CB units is to produce a large number of HSCs and progenitors in culture for transplantation[104]. As exemplified, early acting cytokines such as SCF, fms like tyrosine kinase 3 (FLT-3), and TPO at high concentrations together promote cell cycle entry of HSC and their proliferation in individual and synergistic manner[104]. However, in recent years, it is the use of small molecules that has achieved the greater expansion of HSC and progenitors. These small molecules can collaborate with early acting cytokine and promote high cell growth along with maintenance of HSC-markers.

Here, SCF, TPO, and FLT-3 are the core cytokine mix that best support HSC growth in-vitro[58]. Removal of these cytokines result in cell death and reduced expansion, which necessitates their use in HSC cultures[106]. However, loss of CD34 and CD133, cell surface-proteins expressed by HSPCs, is followed by differentiation, and is inevitable with cultures supplemented with cytokines alone[5]—thereby, limited and transient. With that being said, expansion protocols that combine small molecule activity to modify genomic and epigenetic regulators of HSCs together with early acting cytokines is the current popular strategy[117].

1.5 Identification of Small Molecules as New Stem Cell Agonists

Ex-vivo expansion remains important for HSC-based therapy for maintenance of self-renewal and inhibition of differentiation properties[118,119]. Most HSPCs are CD34+ and enriched with ST and LT repopulating potential[120]. However, as indicated above, expansion of HSPC with engraftment activity with cytokine alone failed to move the field forward. The identification of the so-called, stem cell agonists, revolutionized the field in recent years.

1.5.1 Trichostatin A (TSA) and 5-aza-2-deoxycytidine

TSA is a histone deacetylase (HDAC) inhibitor that interacts with the catalytic site of HDACs[121]. 5-aza-2-deoxycytidine (5azaD) is a cytosine analogue that acts as a DNA methyl transferase inhibitor[122]. 5azaD is added into the DNA and covalently binds to and irreversibly blocks maintenance of methyltransferase, allowing passive demethylation when cells replicate their DNA and divide—causing demethylation of genomic DNA as a secondary event[123]. Alone and in the presence of fetal bovine serum (30%) and cytokines, these molecules have a much lower potential to expand CD34+CD90+ population: a 1.8-fold expansion with TSA and a 1.7-fold expansion with 5azaD[124]. However, CD34+ cells treated with 5azaD/TSA (ex vivo) had significantly higher acetylated histone H4, which resulted in a 5-fold of CD34+ and 12.5-fold expansion of CD34+CD90+ after 9-days when compared to control[124]. Functional quantification of these cells using NSG mice showed that not only cells expanded with 5azaD/TSA retained multilineage differentiation but showcased a 9.6-fold expansion of the absolute number of severe combined immunodeficient (SCID) repopulating cells (SRC)[124]. These results suggest that by adding 5azaD/TSA to culture conditions, a slower rate of cell division and expansion of the absolute number of SRC will be the case[124].

1.5.2 Valproic Acid (VPA)

VPA is another HDAC inhibitor. HSCs treated with VPA exhibited more immature morphologies than control after 7 days, consistent with increased G0-G1 phase of cell cycle—this accompanied increased expression of CD34+CD90+ and reduced apoptotic cells[125]. However, VPA treated cells resulted in reduced cell proliferation, which suggest that VPA prolonged the duration of the cell cycle instead of inducing cell death[125]. This evident in slower loss of CD34+ cells overtime. Although reduced cell proliferation, VPA significantly improved expansion of primitive HSCs and early subsets of CD34+CD90+ (16.1-fold), CD34+CD38- (7.2-fold), and CD34+CD133+ (16.2-fold) by week 3[125]. Primary plating of VPA treated cells showcased reduced colonies as opposed to an increased replating potential of secondary colonies when compared to control; thereby, indicating a more immature HSPC population[125]. With loss of CD34 expression in LT cultures, CD34+ mRNA was 3- to 7-fold higher in VPA treated cells than control[125]. Increased histone acetylation levels promote *HOXB4* self-renewal gene in HSCs, which can explain the prolonged CD34 mRNA by VPA[125]. Finally, transplantation of VPA-treated CB CD34+ cells showed significantly higher human CD45+ and CD45+CD34+ chimerism compared to control[126]. This can be attributed to epigenetic reprogramming of CD34+ for upregulation of *SOX2*, *OCT4*, and *NANOG* that are important pluripotent genes[126]. Therefore, these results explain the importance for targeting and modifying epigenetic regions for ex vivo expansion of HSCs.

1.5.3 StemRegenin1 (SR1)

The first small molecule identified in a chemical screen directed at finding stem cell agonists is StemRegenin 1 (SR1). SR1 is a small molecule discovered by Boitano et al. that maintains less differentiated CB precursors. CB CD34+ cells cultured with SR1 after 5 weeks resulted in a 10-fold increase in total nucleated cells (TNC) and 47-fold increase in HSPC

populations[127]. Furthermore, production of multilineage colonies using the CFU assay suggests that SR1 promotes the expansion of multipotent progenitors[127]. Transplanted SR1-expanded CB CD34+ cells into NSG mice showcase significant ST and LT engraftment, while appropriate multilineage capacity is demonstrated by distribution of human myeloid and lymphoid cells[127]. Poisson statistics reveal SRC frequency of SR1 cultured cells is 1 in 40 compared to 1 in 597 found in control[127]. Without SR1, SRC frequency is reduced by 1000-fold where most of the progenitor cells in these control cultures have lost the ability to engraft NSG mice[127]. Adding SR1 greatly improves these results as an 8-fold increase in SRC frequency is the case and accounts for an overall 17-fold increase in SRC content when compared to control[127]. SR1 accomplishes this by directly binding and inhibiting the aryl hydrocarbon receptor (AHR), which represses cytochrome P450 1B1 and the AHR repressor[127]. Importantly, removal of SR1 from culture conditions resulted in quick differentiation, which indicate SR1 activity to be reversible[127]. Similarly, expansion results using SR1 were negligible without cytokines—underscoring the importance of cytokine supplementation[117,127]. Ultimately, these results demonstrate a promising small molecule capable of promoting self-renewal and multilineage engraftment capacity for CB HSPCs.

1.5.4 Pyrimidoindole–UM171

Most expansion systems achieve progenitor expansion at the expense of LT-HSC. Fares et al. developed a delivery system that produces CB cells with repopulation properties using small molecules with potent LT-HSC potential. Of 5280 small molecules, UM729 was selected for its superior ability to expand enriched CD34+CD45RA- cells. Optimization of UM729 by structure activity relationship produced a superior analogue, UM171, with improved expansion potential on CD34+ cells[128]. The presence of UM171 in culture resulted in CD34+ frequencies similar to SR1, but proportionally higher, and a 3-fold improved engraftment potential of CD34+ macaque cells[128].

Moreover, CB CD34+ treated with UM171 produced a 13-fold higher frequency of LT-HSCs when compared to control[128]. Enriched LT-HSC populations were significantly greater in presence of UM171 when compared to SR1 and control[128]. This suggests that both SR1 and UM171 stimulate HSC proliferation, but UM171 favours the expansion of LT-HSCs, while SR1 may preferentially expand ST-HSCs. This evident in transplantation results that indicate UM171 to significantly increase HSC number (6-fold) and HSC potential in comparison to SR1[128]. Of important note, reconstitution potential of UM171 transplanted cells exhibit an increased myeloid differentiation phenotype, which is commonly found in unexpanded cells[128,129]. Although SR1 presence in UM171 treated cultures hinder the proliferative activity of expanded cells, expansion of CFU-GEMM and suppression of mature cell production, especially erythroid and megakaryocytic differentiation, was enhanced with the addition of SR1—showcasing synergistic activity to promote ex vivo expansion of progenitor subsets over mature cells[128]. Overall, UM171 treated cells upregulate surface molecules such as endothelial protein C receptor (EPCR) and transmembrane protein 183A that are important for maintaining a balance between pro- and anti-inflammatory mediators[128,130]. By doing so, UM171 activates a negative feedback loop regulated by EPCR that protects HSPCs from inflammation borne cytotoxicity[130]. Additionally, UM171 treated CB CD34+ cells are increasingly stimulated to express EPCR proteins that are found on primitive HSCs. Therefore, CB CD34+ cells treated with UM171 result in robust ex vivo expansion and LT engraftment by maintaining pro- and anti-inflammatory responses that are important for HSC survival.

1.6 Stem Cell Agonist Cocktail promote Robust HSPC Expansion

Dr. Manesia's work, in the Pineault lab, aimed to maximize the expansion of HSCs through complementary individual and synergistic activities of stem cell agonists. For this, he first screened

12 molecules and narrowed down SR1, UM171, VPA and L-Ascorbic acid 2-phosphate (AA2P) as small molecules of greater interest. Predicative models for HSC expansion were developed by design of experiments. Using response surface methodology and machine learning, we identified a series of SCACs composed of these 4 agonists at varying concentrations. A series of stem cell agonist cocktails (SCAC) X2A, X2B and SM6 composed of the small molecules at varying concentrations sustained greater expansion CB CD34+CD45RA- and CD34+CD45RA-EpcrHigh (EpcrHg), HSC-enriched cells.

The results showed that significant differences on cell growth and type of HSPC is expanded when changing the small molecule concentration. For example, SM-2 containing high concentrations of UM171 produced high expansion of EpcrHg, but low cell growth. SM-A and SM-6 maximized expansion of CD34+CD45RA- cells but had lower EpcrHg expansion partly due to lower UM171 levels. Furthermore, improved expansion of HSC-enriched cells (CD34+CD45RA-CD38-CD90+), MPPs, and LMPPs were observed when compared to serum free culture (SFM) controls, but not downstream progenitors. The best cocktail combination, X2A, produced a balanced expansion of EpcrHg and CD34+CD45RA- cells. Moreover, expansion of both HSC-enriched fractions were significantly reduced by lowering AA2P concentrations.

The activity of SCACs on the engraftment properties of expanded HSPCs was tested using the NSG transplant models. As our phenotypic results depicted, the strongest ST recovery of platelets and leukocytes were X2A-expanded HSPCs. Likewise, LT human reconstitution in BM was better for X2A- and SM-6-expanded HSPCs. With that being said, limiting dilution transplantation assay showed a net 15-fold increase in HSC number in X2A cultures over the non-expanded primary cells. In accordance with the reduction in cell expansion achieved ex vitro, a

loss in ST and LT engraftment activity was observed with X2B, which is a cocktail essentially identical to X2A but with negligible amount of AA2P.

Ultimately, phenotypic and functional analysis of SCAC-expanded HSPCs show that small molecules can act with each other to promote engraftment. AA2P can synergize with strong stem cell agonists to promote ST- and LT-engrafting HSPCs. However, whether AA2P is capable of independently expanding HSC and progenitors with high engraftment activity remains unclear—and is one objective of my research project.

1.7 Vitamin-C and Stem Cells: Antioxidant Properties

Living organisms continuously generate reactive oxygen species (ROS) from internal or external sources because of aerobic life[131]. The real problem is apparent when excessive levels and constant exposure to ROS is the case—capable of damaging cellular biomolecules like DNA, proteins, and lipids[132]. However, they are important biochemical processes that control cell proliferation and cell signaling[133]. Cellular antioxidant defenses such as catalase, peroxidase, superoxide etc. and water-soluble vitamins like vitamin-C (Vit-C) are essential for balance as they scavenge and neutralize unstable ROS products[132,134]. Nonetheless, ROS production is capable of overwhelming antioxidant defences and causing oxidative stress, which is involved in an array of diseases[132].

A cofactor for several enzymes, L-ascorbic acid or Vit-C is a water-soluble antioxidant found in human plasma and mammalian cells[135]. Humans are unable to synthesize Vit-C and must thus acquire it through diet[132]. Readily undergoing two consecutive and reversible oxidation steps to generate dehydroascorbate and ascorbate free radical, Vit-C can become an efficient electron donor involved in various redox reactions[133,132]. Furthermore, Vit-C can cooperate with glutathione to maintain a stable redox environment[136,137]. However, transient oxidative stress can be the case

with increasing intracellular Vit-C levels[138]. In this case, Vit-C can behave as an antioxidant and a prooxidant, the latter of which is needed at high concentrations in the presence of catalytic iron and copper ions to generate O_2^- and H_2O_2[139]—therefore, indicating a potential benefit of having low concentrations of intrinsic Vit-C in cells.

1.8 Epigenetic Modulation via Methylation/Demethylation

HSC behavior is regulated in part by exogenous and intrinsic mechanism, the latter of which is the result of complement transcription factors expressed in HSC[74]. Transcription factors also recruit co-factors that modulate the chromatin around target genes, to facilitate or impede transcription of specific regions of the genome—this is epigenetic regulation of gene expression[74]. Epigenetic modifications by DNA methylation is important in hematopoiesis by controlling HSC self-renewal and lineage differentiation[140]. Complications in regulating these epigenetic programs can lead to abnormal stem cell function and cellular modifications. Ten-eleven translocation (TET) family proteins act through Fe2+ and a-ketoglutarate-dependent dioxygenases to demethylate DNA[141]. By catalyzing the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethycytosine (5hmc) allows DNA demethylation by replication-dependent dilution or base excision repair[141]. Myeloid and lymphoid malignancies are common in TET-2 loss-of-function, which is associated with DNA hypermethylation, tumor development, and poor prognosis[141].

Unmethylated cytosine-phosphate-guanine dinucleotides (CpG) are targeted by DNA methyltransferase proteins and mediate the transfer of a methyl group to a cytosine[142]. DNA methylation patterns take place within regions highly conserved with repeating CpGs, which cover sites of transcriptional initiation—accounting for 60-70% of tissue specific gene promoters that include housekeeping and developmental genes[143]. Essentially, hypermethylated regions interfere with transcription factor binding thus inhibiting gene expression[144].

1.9 Stem-Cells and Metabolites

Physiological changes in metabolites and whether these changes can alter stem-cell fate and hematopoiesis, has been a central question in the recent years. Genetic changes in metabolic enzymes alter stem-cell function and cause cancer progression[145,146]. Likewise, dietary changes regulate signaling pathways that stimulate stem-cell function[147]. However, it has yet to be confirmed whether physiological or dietary changes can alter stem-cell function via changes in metabolite levels.

The most enriched metabolite *(in vivo)* in HSCs and MPPs is Vit-C, 2-20 folds higher than in downstream cells[140]. Interestingly, Vit-C depleted mice have significantly higher HSC frequency, underscoring its role as a metabolite that alters stem cell fate by modulating HSC frequency[140]. Vit-C accomplishes this by promoting and restoring TET enzymes in culture, which catalyze some steps towards unmethylated cytosine[148,141]. Early events of leukemogenesis increase HSC frequency and self-renewal that are associated with mutations that inactivate TET-2, similar to findings after Vit-C depletion[149–152]. Moreover, these abnormal self-renewal programs increase cell survival and have a myeloid differentiation bias that is observed in *TET-2* knockout cells[153]. Biochemical studies suggest that Vit-C restores iron to the Fe2+ state in the TET catalytic cycle by binding TET-2 and acting as a targeted electron donor; thereby, increasing the rate of 5mC oxidation by 8-fold[154]. Hence, promoting epigenetic remodeling by increasing 5-hydroxymethylcytosine formation, Vit-C reverses abnormal HSPC self-renewal programs that are observed in hematological cancers like leukemia[153].

However, Vit-C is unstable in solution and prone to degradation when exposed to heat, light, metal ions, and oxygen—hindering its biochemical activity[155–157]. More importantly, complicating its use in prolonged studies because of its apparent limitations as a stable molecule. AA2P, a more stable oxidation-resistant and bioactive derivative of Vit-C, has been adapted in

several studies and effectively used to grow various cells[158–163]. This exemplified in studies that investigate increase in myogenin expression, promote differentiation in muscle cells, and enhance proliferation and collagen synthesis in stellate cells and skin fibroblasts[163–166]. Thus, AA2P demonstrates great potential to substitute Vit-C as a more suitable candidate for ex vivo studies done on HSPCs.

Ultimately, our understanding of the synergetic work of TET family enzymes and Vit-C require further investigation. Addressing the mechanism by which Vit-C augments TET function, specifically TET-2, is integral for elucidating whether TET-2 is an optimal target for expansion of HSCs and other subsets.

1.10 Hypothesis and Objectives

Hypothesis: AA2P acts as a promoter of TET-family proteins to expand hematopoietic stem and progenitor cell populations in vitro

Objective 1: Define the impact of AA2P on the expansion of CB HSPC enriched cells

Define the impact of AA2P individual activity on the growth and expansion of CB CD34+ cells. Define the impact of AA2P on the expansion of HSPCs through phenotypic characterization of CB cells in culture by multicolor flow cytometry analyses. Define the functional impact of AA2P on progenitors using the CFU assay.

Objective 2: Investigate the migration activity of AA2P-expanded CD34+ CB cells

Define the impact of AA2P on CXCR4 expression by flow cytometry analysis and determine the homing properties towards SDF-1/CXCL12 using a SDF-1 transwell migration assay.

Objective 3: Impact of AA2P-expanded cells on engraftment using a transplant model

Determine whether addition of AA2P to culture improves the engraftment properties of CB expanded cells. Test the functional properties of AA2P-expanded CB CD34+ cells to reconstitute NSG mice. Peripheral blood (PB) analyses will be done on weeks 3, 10, and 18 to track ST and LT-engraftment, while also monitoring platelet and leukocyte levels. Investigate the LT human BM multilineage engraftment in control and AA2P mice cohorts.

Chapter 2: Materials and Methods

2.1 Collection and Processing of CB units and CD34+ Enrichment

UCB was collected following healthy term delivery and informed consent from mothers, in accordance with institutional approval from the Research Ethics Board of the Canadian Blood Services ahead of the study. Mononuclear cells were extracted from UCB using Ficoll-Paque Plus (GE, Pittsburgh, PA) following manufacturer instructions. CD34+ cells were isolated from mononuclear cells using EasySep™ Human CD34 Positive Selection Kit II (catalog no. 17856; StemCell Technologies) according to manufacturer guidelines. CD34+ purified cells were determined by stained monoclonal antibodies (Mab) against CD34, CD45RA, and CD38 using flow cytometry. CD34+ enriched cells were cryopreserved until use. CD34+ cells were supplemented with cryoprotectant solution containing 40% IMDM, 50% FBS and 10% DMSO. Cryovials were placed in a Mr. Frosty rate-controlled freezing container, which was then placed in a −80 °C freezer for 24 h. After 24 h, the cryovials were transferred to a liquid nitrogen Dewar flask for storage until thawed for analysis. Only ≥90% purified CD34+ cells were used for culture conditions. A total of two CB units and $6x10^4$ to $1x10^6$ CD34+ enriched cell preparations were used for this work.

2.2 CD34+ Expansion Culture

Pooled CD34+ cells (two donors) cultures were carried out in 24-well plates (Corning, New York, United States) or T-175 flasks (ThermoFisher Scientific) in serum-free expansion medium (SFM) (SFEM, StemCell Technologies) supplemented with SCF, TPO, FLT-3 each at 100 ng/ml, low-density lipoprotein (LDL) (LDL, StemCell Technologies) at 10 µg/ml and 1% Penicillin-Streptomycin (Gibco). All cytokines were purchased from Peprotech (Rocky Hill, NJ, USA). Cell cultures were maintained in humidified 5% carbon dioxide (CO_2) incubator at 37°C

for the required duration. Trypsinized CB CD34+ enriched cells were counted by hemocytometer and plated ($3.6x10^3$cells/mL) in SFM. Empirically optimized 14-day expansion protocol was followed which included replenishment of cytokines and small molecule on days: 4, 7, and 10. Medium refreshment by adding 400 µL volume of fresh medium with supplements was done on day-4. Conditions were diluted 1 in 6 with fresh media on day-7 (final volume 600 µL); and diluted 1 in 3 with fresh media on day-10 (final volume 900 µL). AA2P, obtained from SigmaAldrich, was dissolved in PBS and diluted in SFM for the intended concentration range (0.01 to 2500 µM). The control condition consisted of phosphate-buffered saline (PBS). Cell expansion was monitored on day 0, 7 and 14 by flowcytometric analysis using Attune cytometer (Thermo Fisher Scientific Inc, Nepean, Canada). Absolute number of stem and progenitor cells were determined on day 7 and 14, and net fold expansions were derived by dividing absolute numbers obtained by absolute number plated on day 0. The production was calculated by multiplying the frequency of intended population with net fold expansion of total nucleated cells. Only the optimal concentration of AA2P (500µM) was used for the rest of the investigation.

2.3 Flow Cytometry

A portion of cultured cells on days 0 (fresh), 7, and 14 were analysed for HSCs and their downstream progenitors using flow cytometry. Antibodies were purchased from Becton Dickinson Pharmingen (Mississauga, Ontario, Canada). All Mabs were used at the optimal dilution based on titration results, and the list of antibodies and combination used for different screens are detailed in the table below. Compensation beads were used to set voltages and gating parameters for accurate fluorescence signals for all fluorophore-conjugated antibodies (Thermo Fish Scientific). A fluorescence minus one control for all antibodies was used to discriminate between positive and negative signals for the gating parameters. Cells were stained in 100ul in FACS buffer (PBS+2%

FBS) and stained with fluorescence-conjugated antibodies for 30 minutes on ice, washed and resuspended in FACS buffer. A maximum of $1x10^6$ events were acquired for all samples. Flow cytometry analysis of all cultures was done using an Attune® Cytometer (Thermo Fisher Scientific).

Table 1. Antibodies used to probe for expansion of CB CD34+ cells via FACS

Antibody	Conjugate	Catalog #
CD34	PE	555822
CD38	FIT-C	555459
CD38	APC	555462
CD45RA	APC	550855
CD45RA	APC-H7	560674
CD90	PECY7	561558
CD49f	Aexa647	562473
CD49f	PERCP-cy5.5	562473
CD133	FIT-C	5190531666
CD123	PERCP-cy5.5	558714
CD45	APC	555485
CD45	FIT-C	555482
CD19	FIT-C	555412
CD33	PE	555450
CD3	APC	555335
CD14	PE	555399

2.4 Functional Progenitor Population using the Colony Forming Unit Assay

Freshly isolated CD34+ cultured CB cells (800/plate) from AA2P or control cultures were resuspended in MethoCult™ Classic H4435 (StemCell Technologies) and incubated for 2-weeks in humidified incubator at 37°C and 5% CO2. After culture, plates were scored based on morphological features which included CFU-GEMM, granulocyte, erythroid, macrophage, megakaryocyte; CFU-GM, granulocyte and monocyte; BFU-E, erythroid; CFU-G, granulocyte; CFU-M, monocyte. Finally, the sum of all different type of colonies was compiled as CFU-Total. Cumulative CFU was calculated as follows (the average number of colonies enumerated in 2 dishes × total mononuclear cell number)/input mononuclear cell number.

2.5 Chemotaxis Assay

The in vitro migratory behavior of CB $CD34^+$ cells were performed using 6.5-mm diameter, 5-µm pore transwell plates (Corning, USA). $1x10^5$ of control- or AA2P-expanded CD34+ cells, counted using Accucheck beads (Thermofisher), were suspended in 100µL of SFM and placed on the upper chamber of the transwell. Presence or absence of SDF-1a [100 ng/mL] at 650µL was added to the lower compartment. The Transwell plates were incubated at 37°C, 5% CO_2 for 4 hours. Cells that had migrated to the lower compartment were collected and stained using Mab CD34-PE, CD38-FIT-C, CXCR4-APC, CD45RA-APC-H7, CD90-PECY7, and CD49f-PERCP-cy5.5. MFI results were attained using the CXCR4 channel with the intended population. The migration percentage of populations from the $1x10^5$ was determined by flow cytometry.

2.6 Transplantation of CB Cells into NSG Mice

All mice procedures were approved by University of Ottawa Institutional Animal Care and Use Committee. Immunodeficient NOD.Cg-*Prkdc*^{scid}*Il2rgtm*^{1Wjl}/SzJ (NSG) (catalog # 005557) mice were purchased from The Jackson Laboratory. This study used sex- and age-matched mice. Following acclimation and successful breeding, sub-lethally irradiated (300cGy) 8-12 weeks old recipient mice were randomly divided for tail vein administration of: (a) saline; (b) PBS control-expanded progenies of CB CD34+ cells; and (c) AA2P-expanded progenies of CB CD34+ cells after 2-weeks of culture. The final culture equivalent to the $5x10^3$ progenies of SFM control- or AA2P-expanded CB CD34+ cells resuspended in IMDM+2%FBS were injected after 14 days. Saline control was used as a negative control. Assessment of human cell reconstitution were done using either PB samples collected via the submandibular vein at weeks 3, 10, and 18 or from the BM of sacrificed mice 22 weeks post transplantation.

2.7 Humanized Mice Engraftment Analysis

Human platelet and leucocytes were tracked in the murine blood from saphenous vein 3 to 18 weeks after transplantation at indicated time points. Murine blood was collected in heparin coated-capillary and then transferred into heparin containing tube. For platelet analysis, 5µl of blood was diluted 10 times with PBS and stained for 20 mins at 4°C with 2µl of human CD41a PE-conjugated (BD, cat# 555467). After incubation, red cells were lysed by an incubation of 15 mins at room temperature with 250 µl of 1X BD Pharm Lyse™ solution (BD). Samples were then diluted with 250ul of PBS followed by addition of 50ul of Accucheck Beads (Thermofisher). 450uL of each sample was then acquired by Attune Flowcytometer. Human platelet levels were calculated based on the dilution factor, human platelet gate, and bead concentration over bead count. For human leucocyte analyses, 40µl of PB was stained with anti-CD45-APC, CD33-PE,

and CD19-FIT-C. Post 20 minutes incubation at 4°C, 550 µL of PBS+2%FBS was added and then removed after 6 minutes of centrifusion at 250g. Finally, 50 µL of accucheck beads (Thermofisher, cat #PCB100) was added, and engraftment was quantified using frequency of CD45+ cells via FACS.

2.8 Detection of human BM Chimerism and Multilineage Engraftment

Mice hind limb long BM cells of recipient AA2P or PBS cultured progenies were flushed and resuspended in 600 µL of IMDM (StemCell Technologies) +2% FBS. Cells were treated with 2mL of pharm lyse solution (BD, cat# 555899) diluted 1:10 with distilled water, and incubated at room temperature for 10 minutes. Cells were centrifuged at 300g for 10 minutes and resuspended in 1mL IMDM+2%FBS. For analysis, $2x10^5$ was stained with various lineage-specific Mab (BD): Panel A: CD45-APC, CD19-FIT-C, CD33-PE; Panel B: CD45-FIT-C, CD3-APC, CD14-PE; and Panel C: CD45-APC, CD34-PE, CD38-FIT-C. Post 20-minute incubation at 4°C, cells were resuspended with 450 µL of PBS+2%FBS and assayed by cytometry with a maximum of $1x10^6$ acquired events.

2.9 Statistical Analysis

Results are reported as mean ± SEM for the specified *n* value shown in the figures. The significance of difference between two groups for in vitro data was determined using either the two-tailed Student *t* test or multiprobe analysis using other appropriate tests such as ANOVA (unless stated otherwise). Significant difference in 2-way ANOVA is determined between independent variables, control and AA2P, on the cell population. The significance of difference for in vivo data was determined using the unpaired t-test or 2-way ANOVA. Data processing and statistical analyses were performed with GraphPad Prism 8.0 (GraphPad Software, Inc., USA) and

Microsoft Office Excel (Microsoft, USA). A P value of less than 0.05 was considered as statistically significant.

Chapter 3: Results

3.1 Impact of AA2P on CD34+ CB cell growth

The amount of metabolite available for absorption in culture can alter cell growth. Herein, I sought to evaluate whether AA2P, its structure is shown in Figure 5A, could expand CB CD34+ cells, and if so, identify the dose needed to produce the best expansion. AA2P was screen for an optimal working concentration from a minimum of 0.01µM to the maximum 2500µM concentration. Preliminary experimental results, not presented herein, provided guidance for this range of AA2P to be tested. The ideal scenario would be to achieve reasonable expansion at a short time to have higher HSPC content. Day-7 results showed that control and AA2P were comparable in cell growth (data not shown), and so I extended the time required for growth to 2-weeks (Fig. 5A).

Enriched CD34+ CB cells were cultured in SFM supplemented with the early acting cytokines SCF, FL, TPO in the presence of increasing concentration of AA2P. Flow cytometry analysis was used to track expansion of the TNC, CD34+CD45RA-, or CD34+CD38-CD45RA- as these cell populations have been shown to be progressively enriched in progenitor and stem cell activities. The gating strategy utilized to qualitatively investigate the above-mentioned cell populations are presented in Figure-5B. The increased cell growth and expansion of AA2P on CD34+ populations are presented in Figure-5C-E. The minimum dose of 0.01µM up to 10µM of AA2P had negligible expansion when compared to control, but nascent growth was achieved at concentrations greater than 50µM. An increase in CD34+, CD34+CD45RA-, and CD34+CD45RA-CD38- is the case from 50µM to 250µM, but not always significant. Although HSPC populations are not significant within the 50µM to 250µM range, a significant difference in

TNC is evident from 100µM to 2500µM. Furthermore, a significant two-fold increase in CD34+CD45RA- and CD34+CD45RACD38- was achieved between SFM control and AA2P at 500µM, 1000µM, and 2500µM, with the best response at the 500µM. Similar effects are obtained in the CD34+CD45RA-CD38- population, 1000µM being the exception. AA2P at 500µM yielded a 2-fold (more or less) increase in TNC, CD34+CD45RA-, and CD34+CD45RA-CD38- cell populations, and no cytotoxic activity was observed within the 0.01µM to 2500µM range ($p<0.05$, n=3; Figure-5C-E). In summary, AA2P at 500µM produced the most significant expansion in the TNC, CD34+CD45RA- and CD34+CD45RA-CD38- populations. Based on these results, all further investigation of AA2P activity were conducted with AA2P at the optimal 500µM concentration.

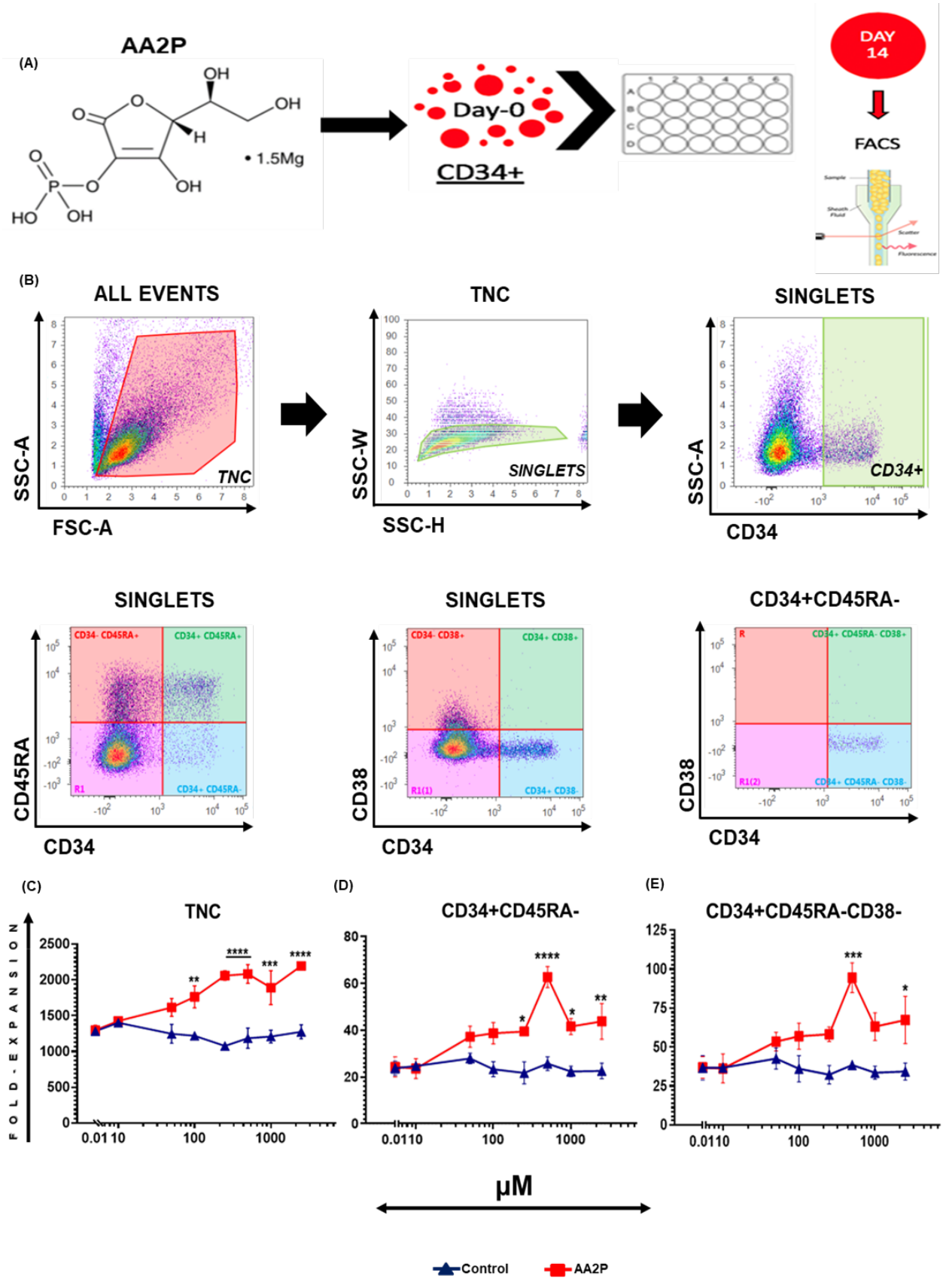
AA2P
(A)
• 1.5Mg
Day-0
CD34+
DAY 14
FACS
(B)
ALL EVENTS
TNC
SINGLETS
SSC-A
FSC-A
SSC-W
SSC-H
CD34+
CD34
SINGLETS
SINGLETS
CD34+CD45RA-
CD45RA
CD34
CD38
CD34
CD34
(C)
TNC
(D)
CD34+CD45RA-
(E)
CD34+CD45RA-CD38-
FOLD-EXPANSION
μM
Control
AA2P

Figure 5. AA2P promotes expansion of HSPC populations. AA2P was added to plated CB-derived CD34+ cells in SFM cultures. **(A)** Chemical structure of Vitamin-C analogue, AA2P, and culture procedures. The effects of AA2P on CB CD34-derived cells were measured via FACS on day-7 (no difference; not presented) and day-14. **(B)** Representative flow cytometry dot plots of CB cells after 14 days of culture. **(C-E)** Fold expansion of phenotypically defined cell subsets after 14 days in cultures with or without AA2P at varying concentrations (0.01μM-2500μM). Significant difference when compared to SFM control in **(C)** TNC and **(D-E)** HSPC enriched subpopulations (CD34+CD45RA- and CD34+CD45RA-CD38-) following treatment with AA2P. Data presents the mean ± SEM of 3 independent experiments. Significant difference is in 2-way ANOVA and is determined between independent variables, control and AA2P, on the cell population. (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

3.2 Impact of AA2P on ex vivo production of HSPC subsets in cultures

The previous results suggested that AA2P may be a new agonist capable of promoting expansion of HSPC populations. To gain further insight into this phenomenon, I tracked the production of phenotypically defined subpopulations known to be enriched in HPCs. For my work, I adapted Tarunina et al[68] and Notta et al[67] flow cytometry analyses to carry out panels of cell surface antigens that are used to identify and quantify HPCs (Figure-6A). Expanded cultures were probed for CD34+CD38- and further enriched for multipotential (MPP) or primed progenitors (LMPP) based on surface CD90 and CD45RA expression. MPPs (CD34+CD45RA-CD38-CD90-) in AA2P treated cultures were 2.5-fold higher in production than in SFM control, but the results were non-significant ($p=0.1$, Figure-6B). AA2P-expanded CD34+ cells resulted in a 82.3 ± 53.1 production of downstream LMPPs, herein defined as CD34+CD45RA+CD38-CD90- cells, compared to a 23.9 ± 16.5 in SFM control, a significant 3.4-fold difference in LMPPs in AA2P treated cultures ($p<0.001$, $n=3$; Figure-6B). Next, I sought to investigate downstream myeloid progenitors within the CD34+CD38+ fraction. These myeloid progenitors can be compartmentalized based on CD123 and CD45RA expression, and their phenotypic profiles are as follows: MEP (CD34+CD45RA-CD38+CD123-); CMP (CD34+CD45RA-CD38+CD123+); and GMP (CD34+CD45RA+CD38+CD123+). Among the myeloid progenitors, a 2.3-fold increased production was exhibited in the CMP subset ($p>0.05$, Figure-6B), while MEP and GMP were comparable to SFM control. In summary, a non-significant increase in MPP and CMP production was the case, while other progenitors remain unaffected by AA2P treatment. Only LMPPs that are marked by their CD34+ CD45RA+CD38- CD90- expression showed a significant 3.4-fold increase in production.

Furthermore, I wanted to investigate whether these expanded progenitor populations exhibited functional properties by using the CFU-assay, which is also a good indicator for graft potential[77].Post 2-weeks culture, control- or AA2P-expanded CB CD34+ cells were plated on methylcellulose that contains growth factors necessary for myeloid lineage colonies; and formed colonies were scored 14 days after initial plating (Figure-6C). Combined colonies of CFU-M; CFU-G; CFU-B; and CFU-GM (CFU-Total) in AA2P-plated CD34+ cells were 1.9-fold higher than in control ($p<.0001$, two-way ANOVA; n=3, Figure-6D). Among the CFU-Total of AA2P treated cultures, the CFU-M fraction produced the largest fold increase in net CFU number (3.9-fold, $p<0.001$), with equal contribution from both CFU-GM and BFU-E at 2.9-folds higher ($p>0.05$) than in control. Overall, AA2P-derived colonies exhibited increased CFU-Total—a significant portion of the CFU-Total was CFU-M and increased CFU-GM and BFU-E.

Finally, I needed to confirm whether the HSC population is increased by AA2P. Again, FACS was used to probe for HSCs that expressed CD34+CD45RA-CD38-CD90+. A paired t-test was performed to determine the mean difference between AA2P-treated HSCs and SFM control-HSCs. AA2P-expanded CD34+ cells resulted in a 3.9 ± 1.8 production of HSC-enriched subset compared to a 2.3 ± 0.2 in SFM-control ($p=0.16$, n=4; Figure-6E). Although CD90 is very good at segregating HSC from MPP, it cannot absolutely segregate these subsets as heterogeneity still exists among these populations[67]. CD49f is an integrin that was recently shown to provide better enrichment of HSC from MPP[67]. The addition of CD49f to CD34, CD38, CD45RA, CD90 will better compartmentalize these rare HSC populations. Again, production was calculated to determine if any difference exists among AA2P and SFM control in the HSC enriched CD34+CD45RA-CD38-CD90+CD49f+ compartment. Still, no significant difference was observed in the above-mentioned HSC compartment in AA2P-expanded CB CD34+ cells ($p>0.05$,

n=4; Figure-6F). In summary, my ex vivo results concluded that AA2P preferably increased the expansion of HPCs, specifically LMPPs, while HSC populations remained largely unchanged. However, HSC functional properties have yet to be determined; therefore, require a functional assay to successfully answer these limited qualitative analyses.

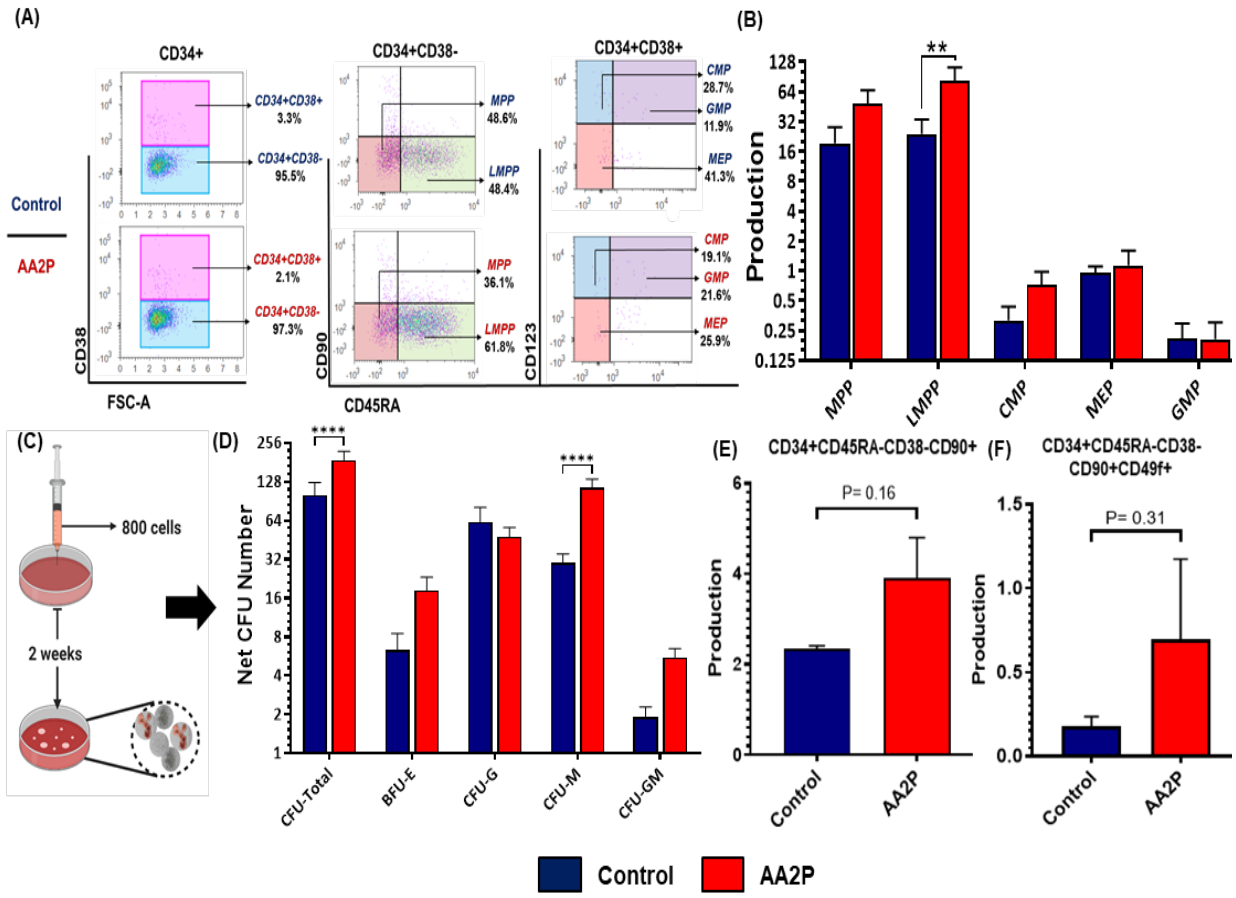

Figure 6. AA2P promotes the expansion of lymphoid primed multipotential progenitor and CFU. (A) Representative FACS profile of CB cells for the CD34+CD38- and CD34+CD38+ subsets after 14 days of culture. **(B)** The production of phenotypically defined subsets after 14 days of culture: control (blue) and AA2P (red) multipotent progenitor (MPP), lymphoid-primed multipotential progenitor (LMPP), common-myeloid progenitor (CMP), megakaryocyte-erythroid progenitor (MEP), granulocyte-monocyte progenitor (GMP). Data presents the mean ± SEM of 3 independent experiments. Significant differences were determined by 2-way ANOVA (**p<0.01) from SFM control. **(C)** CFU schematic showcasing the plating of 800 cells from control or AA2P-expanded CB cells. Colonies were scored 2-weeks after plating. **(D)** CFU-Total, total content; BFU-E, erythroid; CFU-G, granulocyte; CFU-M, monocyte; CFU-GM, granulocyte and monocyte. CFU-total and CFU-M. Data presents the mean ± SEM of 3 independent experiments. Significant differences (**p<0.01 and ****p<0.0001) is in 2-way ANOVA and determined between independent variables, control and AA2P, on the cell population. **(E)** Production of CD34+CD45RA-CD38-CD90+ per starting cell and **(F)** Production of CD34+CD45RA-CD38-CD90+CD49f+ HSC-enriched subsets per starting cell. Production quantified after 14 days of cultures. Data presents the mean ± SEM of 4 independent experiments. P values were determined by a paired t-test (p>0.05) from SFM control.

3.3 Impact of AA2P on the migration activity of CB expanded Cells

Afterwards, I wanted to determine whether AA2P would stimulate the capacity of CB progenitors to migrate towards SDF-1a since the migration activity of CB progenitors is intrinsically hindered when compared to their adult counterpart [102,116]. First, I set out to investigate if cultured CB progenitors in the presence of AA2P would increase the expression of CXCR4. This receptor and its ligand, CXCL12/SDF-1a, play a pivotal role in the homing activity of HSPCs to the BM. Furthermore, increased CXCR4 expression significantly improves homing and retention of transplanted HSC content in situ[112]. With that being said, I investigated the mean fluorescence intensity (MFI) of the FITC conjugated CXCR4 channel in cell populations gated for TNC, CD34+, CD34+CD45RA-CD38-, and CD34+CD45RA-CD38-CD90+. CXCR4 MFI results of AA2P-expanded CD34+ exceeded SFM grown cells ($p=0.053$), while other populations remained the same ($n=4$, Figure-7A).

Thereafter, expanded cells were placed in an SDF-1a transwell migration assay to examine whether expansion under AA2P could affect the chemotaxis properties of progenitors. Post 4hr incubation, media in the lower chamber containing migrated cells were quantified by cytometry. In addition to SFM control, a condition plated with cells containing no SDF-1a was used as a negative control. As expected, AA2P-expanded CD34+ cells that expressed the highest MFI for CXCR4 accompanied the highest number in migrated cells, though the difference failed to be significant. Additionally, TNC and CD34+CD45RA-CD38- of AA2P-expanded CD34+ cells showed migration activity similar to that of control. A trend of reduced migration was seen for the CD34+CD45RA-CD38-CD90+ population, but again, this was not significant ($p=0.5$, $n=4$, Figure-7B). Ultimately, MFI parameters of CXCR4 showed that cells expressing the highest CXCR4 followed an improved migratory response to SDF-1a.

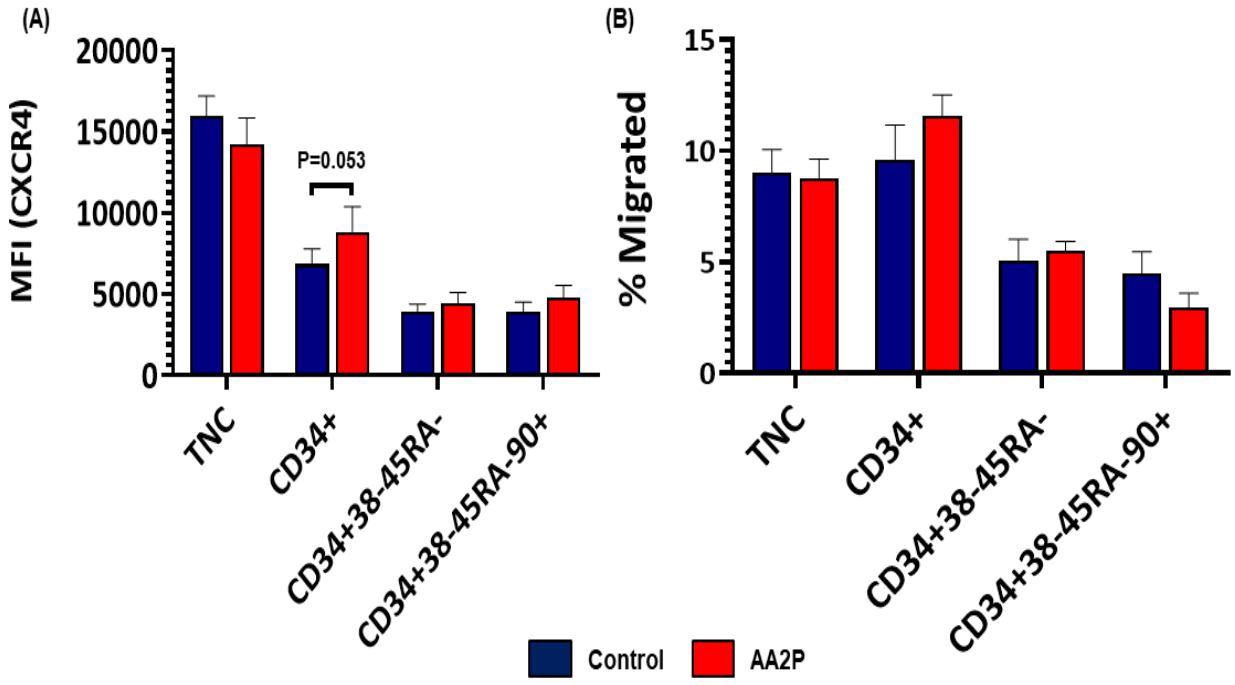

Figure 7. Impact of A22P on CXCR4 expression and on the migration CB cells. AA2P-expanded CD34+ cells were plated on the upper chamber of a transwell assay. Lower chamber contained SDF-1α (100nM). Migrated cells were quantified 4hrs post incubation using markers indicated above (CD34+, CD34+38-45RA-, CD34+38-45RA-90+). **(A)** MFI of CXCR4+ channel was quantified in addition to markers examined above. AA2P-expanded CD34+ cells showcase near significant CXCR4+ expression after 14 days (p=0.053). Data presents the mean ± SEM of 4 independent experiments. P values were determined by 2-way ANOVA (p>0.05) from SFM control. **(B)** Migratory activity of AA2P-expanded CD34+ cells exhibit no significant difference, although improved migratory activity is observed in CD34+ cells alone (p=0.3). Data presents the mean ± SEM of 4 independent experiments. Significant differences in 2-way ANOVA and is determined between independent variables, control and AA2P, on the cell population

3.4 Impact of AA2P on the engraftment properties of CB expanded HSPCs

Successful engraftment is largely dependent on HSPC content in both ST and LT engrafting stem and progenitors[104]. Transplant models are great for measuring the functional properties of HSPC to sustain early engraftment and prolonged hematopoiesis[65,167,168]. In this case, I wanted to test whether AA2P could enhance the engraftment properties of CB progenitors expanded ex vivo with early acting cytokines. The total progeny of $5x10^3$ of PBS control- and AA2P-expanded CD34+ cells were transplanted into sublethally irradiated NSG immunodeficient mice by intravenous tail injection, while a PBS saline injection was used as a negative control (Figure-8A).

Engraftment was tracked first in the periphery via PB analyses of platelets and leucocytes on weeks 3, 10, and 18 post transplantation to observe short-, intermediate, and long-term engraftment (Figure-8B). Platelets are small enucleated cells that are released by mature megakaryocyte into circulation[169]. Platelet recovery after CB transplantation is significantly delayed[170]; which therefore pushed me to monitor these cells. PB extracted from humanized mice was stained for human CD41 to account for platelets, which lack expression of the pan-hematopoietic marker CD45+. (Figure-8C). My results revealed that mice cohorts of transplanted AA2P-expanded progenitors had significantly higher human platelet levels throughout the observation periods (week 3, median of 24 and 46 for control and AA2P, platelet 10^3/mL), mid (week 10, median of 198 and 505 for control and AA2P, platelet 10^3/mL) and late (week 18, median of 178 and 765 for control and AA2P, platelet 10^3/mL) ($p<0.05$, n=3, Figure-8E).

As with platelets, neutrophil engraftment is also delayed following CB transplantation[171,172], so myeloid leucocytes were monitored using the marker combination CD45+CD33+. It is also important to measure multilineage capacity of transplanted cells as some are lineage restricted[67]; here, tracked using lymphoid CD45+CD19+. Representative FACS profile

of lineage specific CD45+ with or without CD33+ and CD19+ are presented in Figure-8D. Although improved throughout all weeks, engraftment of CD45+ with or without human CD33+ or CD19+ in AA2P grafts were comparable to control grafts ($p>0.05$, $n=3$; Figure-8F). Thus, my in vivo findings show increased ST to LT platelet levels among AA2P cohorts. However, no significant difference was observed in leukocyte engraftment. Together, these results demonstrate improved activity of AA2P on the engraftment kinetics of platelets by expanded CB HSPCs because of their ability to speed recovery of platelet levels.

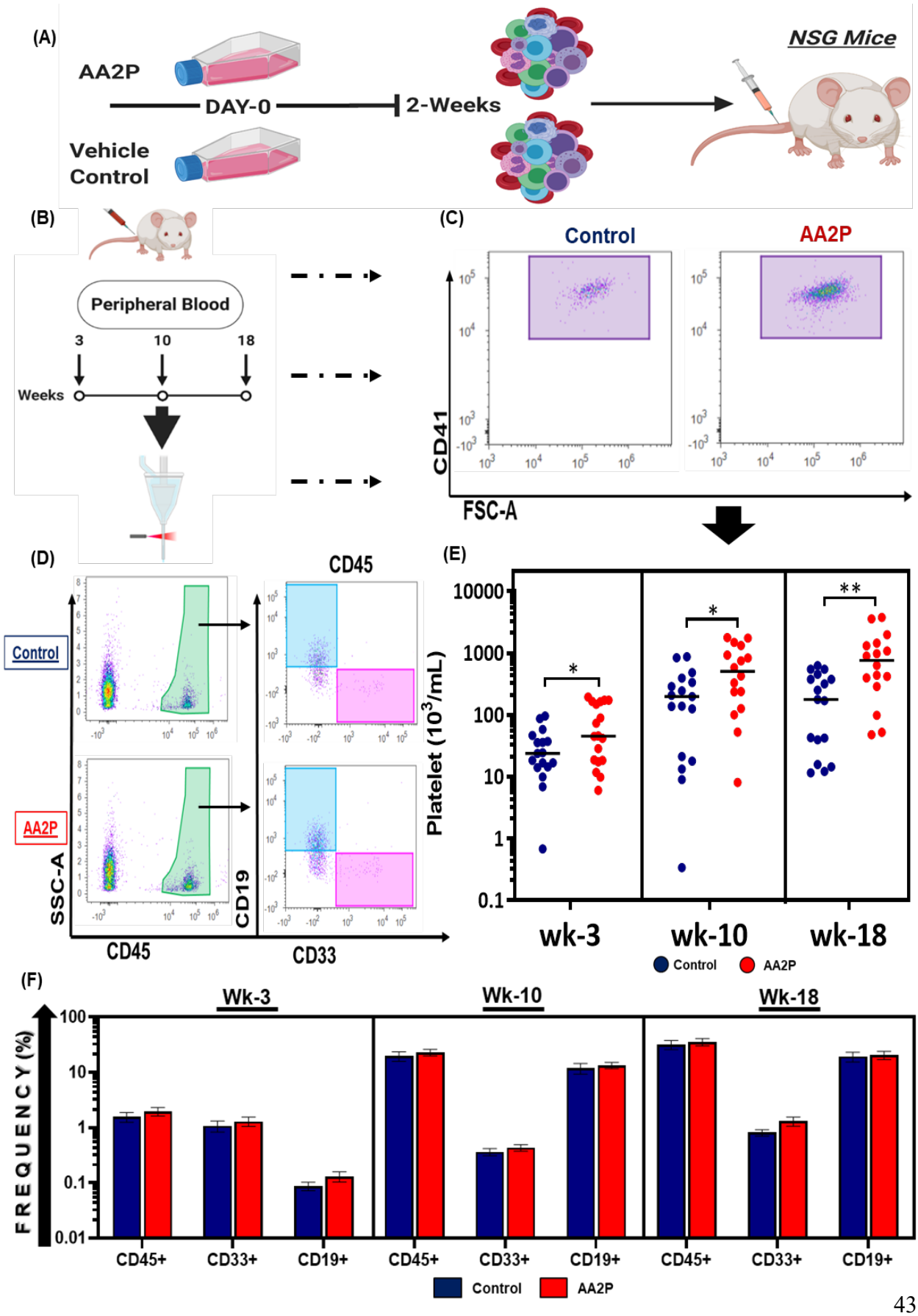
(A)
AA2P
DAY-0
2-Weeks
Vehicle Control
NSG Mice
(B)
Peripheral Blood
3
10
18
Weeks
(C)
Control
AA2P
CD41
FSC-A
(D)
CD45
Control
AA2P
SSC-A
CD19
CD45
CD33
(E)
Platelet (10^3/mL)
wk-3
wk-10
wk-18
Control
AA2P
(F)
Wk-3
Wk-10
Wk-18
FREQUENCY (%)
CD45+
CD33+
CD19+
Control
AA2P

Figure 8. AA2P grafts increased circulating platelets in humanized mice. (A) Mice were injected with a fraction of the final culture equivalent to the progeny of $5x10^3$ CB CD34+ cells cultured for 14 days with or without AA2P (5-7 mice per group; each symbol represents an individual mouse). **(B)** Circulating human CD41+ platelets were monitored on weeks 3, 10, and 18 via peripheral blood to account for early to late engraftment. **(C)** Representative FACS profile for human platelets using the CD41 marker in control and AA2P grafts. **(D)** Representative FACS profile for human leukocyte pan-hematopoietic CD45 with or without lineage specific CD19 and CD33 markers. **(E)** Significant early to late human platelets in mice transplanted with AA2P expanded CB cells compared to SFM control; lines show the median of indicated graft. Data presents the median of 3 independent experiments. Significant differences were determined by unpaired t-test (*$p<0.05$ and **$p<0.01$) from SFM control. **(F)** Frequency of human CD45+ cells, CD45+ CD19+ B-cells and CD45+ CD33+ myeloid cells in the periphery of humanized mice. Background levels for human leukocyte engraftment measured in saline control mice were inferior to 0.03%. Data presents the mean ± SEM of 3 independent experiments. P values were determined by 2-way ANOVA ($p>0.05$) from SFM control.

3.5 Impact of AA2P on multilineage chimerism of CB expanded HSPCs

The BM is the best-known location for HSCs and contains a large pool of progenitors important for the release of mature cells from all lineages, including neutrophils and platelets[22]. LT-hematopoiesis was measured 22 weeks after transplant to reduce potential contribution of ST-HSCs[91,92]. To evaluate the activity of HSCs in transplanted AA2P or SFM control grafts, isolated BM cells were used to measure human lympho-myeloid LT BM engraftment (Figure-9A). Representative of myeloid and lymphoid flow cytometric analyses of human BM engraftment in mice are presented in Figure-9B. Human chimerism was measured with CD45, which is a marker equally distributed on cytoplasmic membranes of most hematopoietic cells[173]. A stronger contribution for CD45+ engraftment was observed in AA2P-humanized mice cohorts when compared to control, with a median of 48% and 75% of CD45+ cells for control and AA2P ($p<0.01$), respectively (Figure 9C).

Furthermore, multilineage chimerism in AA2P or SFM control groups was assessed by BM cells positive for CD45 with lympho-myeloid lineage specific markers (Figure-9C). All lineages were found present in AA2P cohorts, and for the most part, at similar frequency to control groups. This included myeloid restricted lineage progenitors (CD45+CD33+)[172] and downstream monocytes (CD14+)[174]. Lymphoid T-cell populations (CD3+)[174] were revealed to be low in both groups, while B-cells (CD19+)[175] showed the strongest difference with a median of 3.1% for control and 6.6% for AA2P cohorts (%CD45+CD19+, $p=0.8$). Another population that exhibited increased engraftment was the CD34+ HSPCs[51–53] with a median of 3.1% and 6.6% for control and AA2P cohort groups.

In summary, in vivo findings of AA2P graft cohorts showed a significant increase in human BM chimerism. Moreover, normal differentiation was observed in AA2P grafts that

produced all major lineages, which suggests that AA2P can support the maintenance and perhaps expansion of HSCs when used with early acting cytokines.

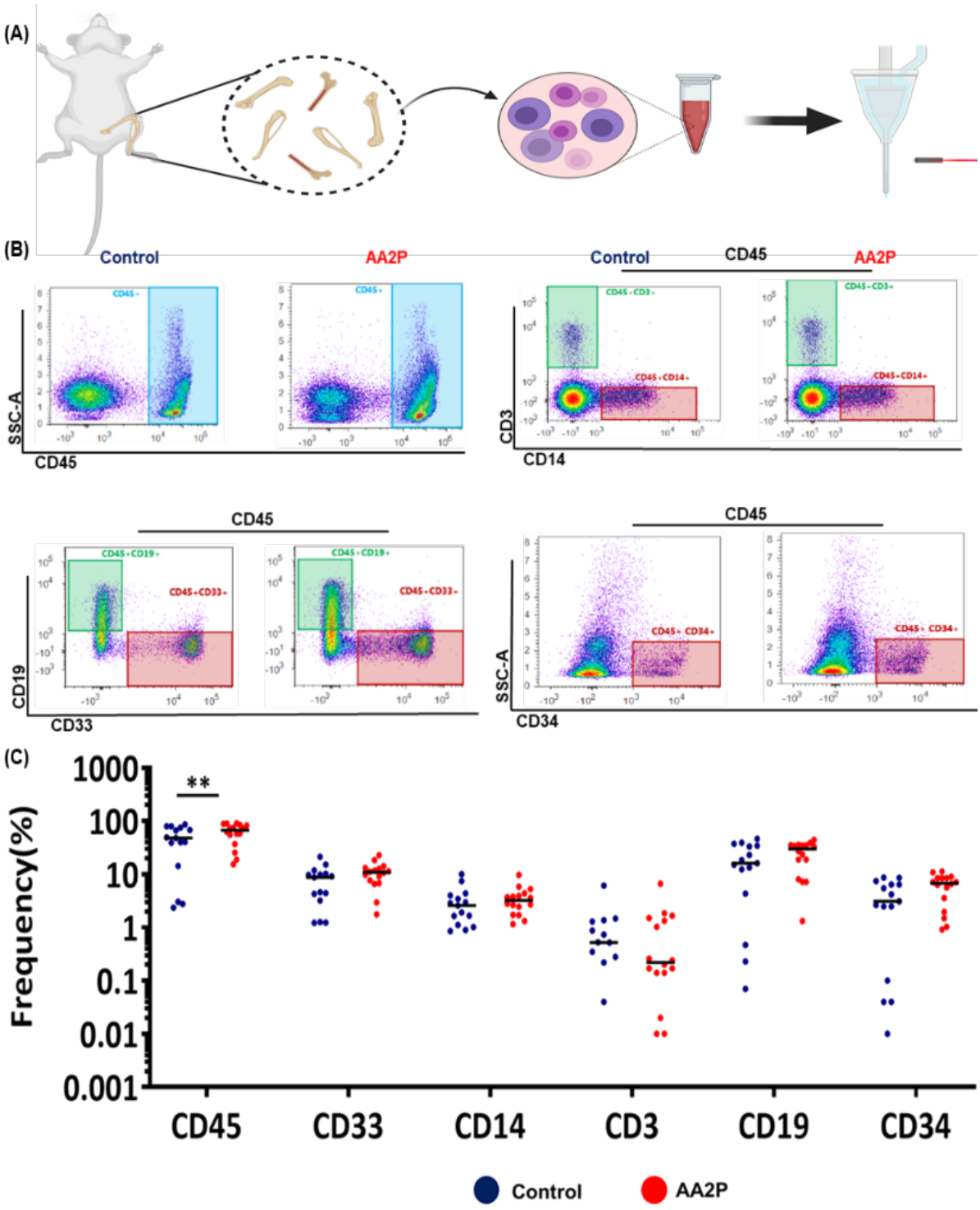
(A)
(B)
Control
AA2P
Control
CD45
AA2P
SSC-A
CD45
CD3
CD14
CD45
CD19
CD33
CD45
SSC-A
CD34
(C)
Frequency(%)
1000
100
10
1
0.1
0.01
0.001
**
CD45
CD33
CD14
CD3
CD19
CD34
Control
AA2P

Figure 9. AA2P grafts promote leukocyte engraftment in BM. (A) Mice hind limb long BM of SFM control- or AA2P- cells were isolated 22 weeks post transplantation **(B)** Gating and flow cytometry analyses of CD45+ with various hematopoietic lineages: myeloid cells (CD33+), monocyte (CD14+), T-cell (CD3+), B-cell (CD19+), and HSPC (CD34+). **(C)** Frequency of human CD45+ BM cells and other human hematopoietic lineages in mice transplanted with expanded CD34+ in AA2P or SFM control cultures. Each symbol represents an individual mouse; data derived from 5-7 mice per group. Significant differences is in 2-way ANOVA and is determined between independent variables, control and AA2P, on the cell population. Data presents the median of 3 independent experiments. Significantly different (**$p<0.01$) from SFM control.

3.6 Mechanism of Action of AA2P

My results thus far were attained using the synthetic Vit-C analogue, AA2P. I therefore wanted to investigate whether the natural molecule[176], Vit-C (Figure-10A), would elicit a similar response. As aforementioned, Vit-C can act as both an anti- or pro-oxidant, and results presented in literature show that hematopoietic cells are sensitive to Vit-C at high concentrations; thereby, narrowing my concentration range[139].

Preliminary experimental results, not presented herein, guided the 0.01μM to 500μM concentration range for Vit-C testing. Following the previously mentioned procedures, enriched CB CD34+ cells were cultured in the presence or absence of Vit-C and examined for TNC and HSPC fractions. Vit-C activity on cell growth and expansion of CD34+ populations are presented in Figure-10B-E. Vit-C cultured CD34+ cells showed a small but non-significant difference in TNC among all concentrations. An absent response is the case in Vit-C at low doses, 0.01μM and 2.5μM, while cytotoxic activity is evident beyond 250μM. A significant response in CD34+ expansion was attained from 5μM to 250μM. This was a 1.5-fold expansion in CD34+ when Vit-C was added at 5μM, 10μM, and 250μM, while the best working concentration of 100μM produced a 2-fold expansion within this population. Furthermore, only the 100μM and 250μM concentrations resulted in a significant 2-fold expansion in the CD34+CD45RA- and CD34+CD45RA-CD38- populations, with higher expansion in the 100μM. Unlike the increased TNC number achieved by AA2P, Vit-C seems to only effect the expansion of HSPCs. Finally, I further examined the functional properties of Vit-C treated CB CD34+ cells using the transplant model. Preliminary in vivo results of transplanted Vit-C grafts displayed engraftment properties similar to AA2P grafts with increased mid (week 10, median of 16 and 159 for control and Vit-C, platelet 10^3/mL) to late (week 18, median of 13 and 21 for

control and Vit-C, platelet 10^3/mL) platelet levels when compared to SFM control though differences were nor significant (n=1, Figure-10F). Likewise, peripheral myeloid (CD45+CD33+) and lymphoid (CD45+CD19+) engraftment in Vit-C groups remained similar to SFM control as previously observed with A2P cohorts (data not shown). Consistent with the increased LT engraftment observed in the BM of AA2P grafts, Vit-C cohorts exhibited a promising trend with a median of 3% for control and 36% for Vit-C groups (%CD45+; Figure-10G). Taken together, my results support that AA2P acts similarly to Vit-C as both molecules stimulate ex vivo expansion of HSPCs, and when transplanted, AA2P/Vit-C grafts are capable of increased platelet levels and LT BM engraftment.

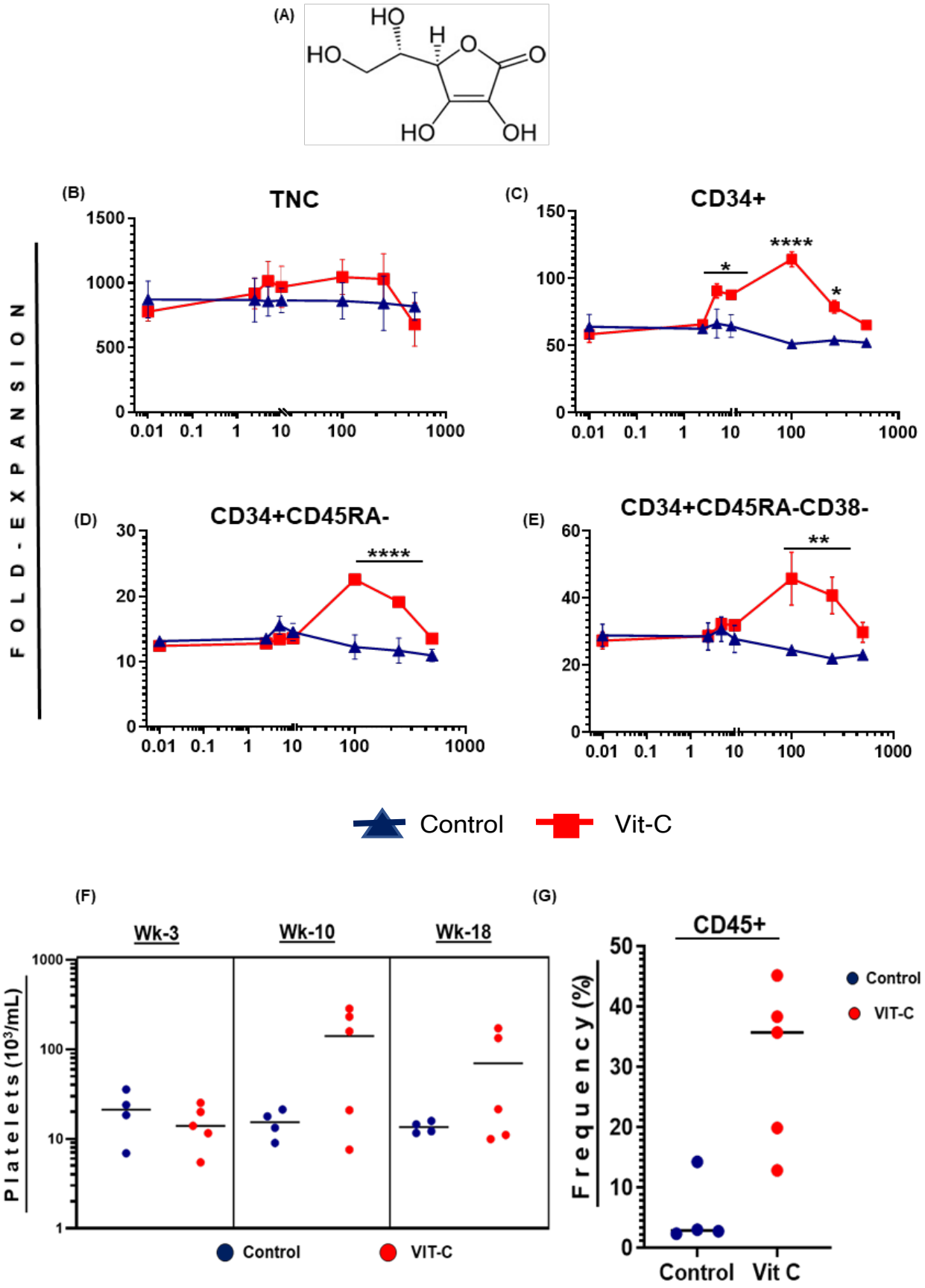
(A)
HO
H
O
HO
O
HO
OH
(B)
TNC
(C)
CD34+
(D)
CD34+CD45RA-
(E)
CD34+CD45RA-CD38-
FOLD-EXPANSION
Control
Vit-C
(F)
Wk-3
Wk-10
Wk-18
Platelets (10³/mL)
Control
VIT-C
(G)
CD45+
Frequency (%)
Control
VIT-C
Control
Vit C

Figure 10. Vit-C replicates some of the effects of AA2P on CB HSC and progenitors. Vit-C was added to CB CD34+ cells in SFM cultures. (**A)** Chemical structure of Vitamin-C (Vit-C). **(B-E)** Ex vivo fold expansion of phenotypically defined cell subsets after 14 days of culture supplemented with or without Vit-C at varying concentrations (0.01μM-500μM). SFM control (blue) and Vit-C (red) treated cultures. Significant difference when compared to SFM control in **(C-E)** HSPC enriched subpopulations (CD34+, CD34+CD45RA-, and CD34+CD45RA-CD38-) following treatment with Vit-C. Significant differences in 2-way ANOVA and is determined between independent variables, control and AA2P, on the cell population. Data presents the mean ± SEM of 3 independent experiments. **(F)** Human platelet levels in mice transplanted with SFM and Vit-C expanded CB cells. Data shows value in each mouse and the median of 1 independent experiment. **(G)** Frequency of human CD45+ BM cells in mice transplanted with expanded CD34+ in Vit-C or SFM control cultures 22-weeks post-transplantation. Each symbol represents an individual mouse and the median; data derived from 4-5 mice of 1 independent experiment. Significantly different (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$) from SFM control.

3.7 TET Inhibition and Mechanism of Action for AA2P

Gene expression can be epigenetically regulated by modulating the chromatin around target genes[74]. TET proteins aid in the demethylation of DNA by catalyzing oxidation of 5mc to 5hmc[149]. These enzymes are important epigenetic modifiers that govern HSC frequency, but deficiency or loss of their function causes DNA hypermethylation that is associated with various hematological diseases[140]. It is now clear that Vit-C modulates chromatin by augmenting TET-2[149]. I hypothesized that AA2P expands HSPCs by augmenting TET activity. With that being said, my final objective was to find a way to disrupt or inhibit TET-2 activity ex vivo, and whether this accompanies lower expansion of HSPCs in AA2P cultures. BOBCAT-339 (BOB; Figure-11A) is a mid-tier TET-1 and TET-2 inhibitor that prevents the removal of methyl groups on the genome[177]. Global 5hmc levels were significantly reduced in cells treated with BOB in comparison to vehicle control[177]. Overall, these results demonstrate a novel cytosine-based TET enzyme inhibitor that can used in culture to investigate AA2P and its mode of action. Preliminary effective concentration using BOB was tested on KG1 cell line to acquire faster results. KG1 cells were treated with BOB within a concentration range of 1μM to 500μM for 7 days. On day 7, cells were quantified by FACS for total nucleated cell count. Cytotoxic concentrations effecting the TNC population is achieved at 50μM and more when using BOB (figure-11B). Future dose-response assays using BOB on CD34+ CB enriched cells will range from 1μM to 50μM as cytotoxic levels are evident beyond the 50μM concentration.

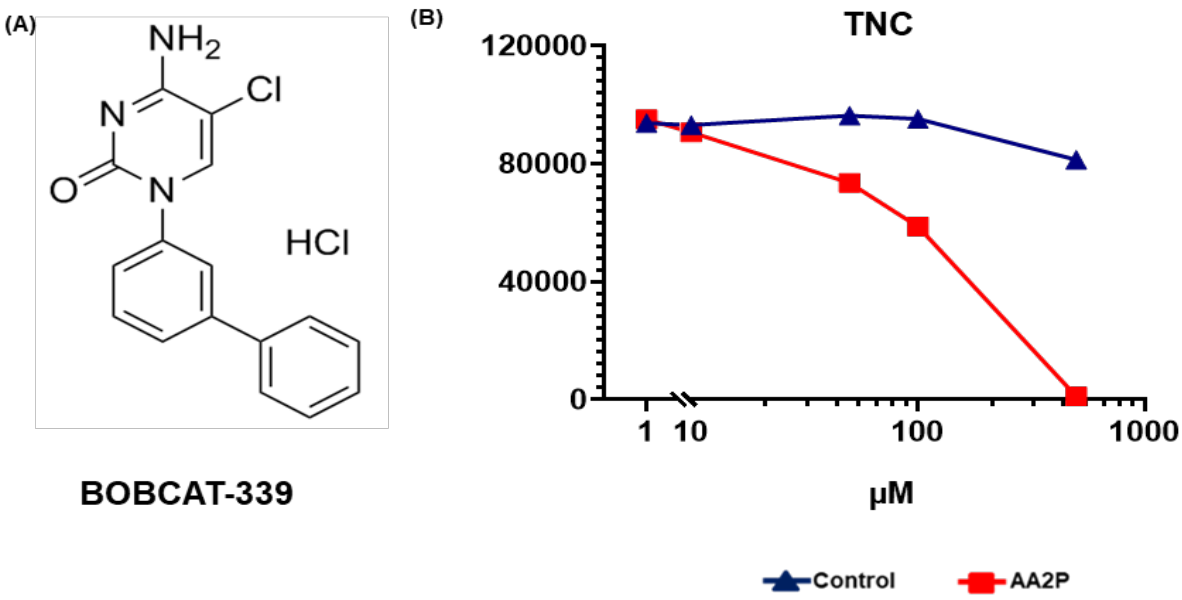

Figure 11. TET-2 inhibition with BOBCAT-339. BOB is a TET-2 inhibitor that will be used to hamper Vit-C induced expansion of CB CD34+ cells. **(A)** BOBCAT-339 chemical structure. **(B)** Optimal working concentration range using KG1 cell line; toxicity is the case beyond the 10µM concentration. Data presents 1 independent experiment.

Chapter 4: Discussion

HSC behavior is regulated by exogenous and intrinsic mechanisms that can be manipulated to alter differentiation and self-renewal programs[74]. Doing so can promote HSC expansion by functional maintenance of self-renewal and differentiation potential[178]. Many cytokines have been identified that regulate hematopoietic cells; however, cytokines alone cannot sustain self-renewal of HSCs and inevitably lead to differentiation[5]. Better results are achieved by adding small molecule to culture medium for ex vivo expansion of enriched CD34/CD133 HSPC grafts[179]. Examples of such molecules include 5azaD and TSA HDAC inhibitors that increase acetylation of histone H4[124]; VPA is another HDAC inhibitor that expands primitive HSCs by increased histone acetylation levels to promote expression of self-renewal genes[125]; SR1, that maintains less differentiated cells by inhibition of the AHR[127]; and the pyrimidoindole agonist UM171, which expands immature HSC by protecting these cells from inflammation[128,130]. All these compounds support expansion of CD34+ CB cells that have ST- and LT-repopulating potential.

Vit-C has recently been elucidated to modulate HSC frequency in vivo by augmenting TET protein activity—with ex vivo studies needed to further investigate this phenomenon[154]. Additionally, it is naturally the most enriched metabolite in primitive HSCs, underscoring its role as an important regulator for HSC frequency[140]. However, Vit-C is unstable in solution and prone to degradation; therefore, complicating its use in prolonged studies[155–157]. Herein, AA2P was adopted into this study as a more stable alternative because it has long acting activity and similar bioactivity to Vit-C[158–163]. Combining some of the above-mentioned molecules, our lab has maximized the expansion of HSCs through individual and synergistic activities of SCACs. Of importance, we have shown that AA2P can synergize with strong stem cell agonists like UM171 and SR1 to promote robust ST- and LT-engrafting HSPCs, but its individual activity is still

unknown (J. Manesia et al., manuscript in preparation). In my thesis, I was interested in studying the individual effect of AA2P on the ex vivo expansion of CB CD34+ cells, as this has yet to be explored. To the best of my knowledge, this is the first study to investigate AA2P activity on CB-derived CD34+ cells.

4.1 Impact of AA2P on the growth and expansion of CB HSPCs

I first investigated whether AA2P could increase the proliferation of CB cells including progenitor cells when used in combination with early acting cytokines. AA2P was screened for an optimal working concentration; a dose-dependent increase in TNC, CD34+CD45RA- and CD34+CD45RA-CD38- populations was observed with optimal expansion achieved at 500µM.

The growth of CB CD34+ cells were dependent on the concentration of AA2P. Such variations in cell growth are also seen in other culture systems[180], and under in vitro conditions is associated with the effects of excess O2[181]. Nonetheless, nascent growth stimulated by AA2P is evident in TNC; CD34+CD45RA-; and CD34+CD45RA-CD38- beyond 10µM (Figure-5C-D). Furthermore, similar results were reproduced in Vit-C cultures, which suggested that both natural and synthetic forms have analogous behavior and likely act through similar mechanisms (Figure-10B-E). The modification of DNA methyl groups is regulated by TET proteins whereby Vit-C acts as cofactor for such enzymes[182]. By catalyzing intermediate steps in DNA demethylation, the conversion of 5mc to 5hmc, TET-2 can transform chromatic regions; thereby, modifying gene expression[182,141]. The removal of TET-2 or Vit-C leads to increased HSC numbers, as well as a block in HSC differentiation[150]. Consistent with previous studies done using other cell lines[183184], AA2P treated CB CD34+ displayed increased TNC number likely due to enhanced TET-2 enzymes.

Further examination of the increased TNC population, HSPC markers were found to be significantly increased, here identified as CD34+CD45RA- and CD34+CD45RA-CD38-. In this specific study, I have shown that qualitative progenitor populations displayed elevated LMPP production (Figure-6B). Other progenitor population downstream of MPP such as CMP, GMP and MEP were not found to be differently expanded, at least not to a significant level. Genes involved in lymphoid and myeloid commitment are simultaneously expressed in HSCs, and down- or up-regulation of these genes prime HSCs for lineage restriction and differentiation[185]. The HSC compartment contains a large population of lineage specific HSCs that generate all hematopoietic lineages, but with skewed myeloid lineage differentiation[186]. Several studies have imitated TET-2 loss or deficiency in mice and the consequent preference for myeloid lineage expansion[151,152,186,187]. These processes accompany a loss of 5hmc in HSPCs, DNA hypermethylation, and gene expression tailored for myeloid lineage bias in self-renewing progenitors[153]. Such observations align with findings made by An et al. that showed TET-2 deficiency followed impaired LMPP production, while myeloid progenitor production remained normal[188]. MPPs exhibit much less lineage-affiliated genes, and commitment is a consequence of alterations in chromatin structure that requires transcription machinery[185]. As discussed earlier, AA2P induced differentiation via activated TET-2. This data strongly argues that AA2P-increased LMPP production is caused by stimulated TET-2 epigenetic remodeling of transcription factor-binding regions that induce leukocyte differentiation by promoting genes such as *CEBPβ*, *HIF1α*, *RUNX1*; and obstructing *PU.1*—described by a recent study done by Mingay et al.[189].

However, the results mentioned above on HPC subsets are based on phenotypic characterization and have their limitation, as a substantial number of CD34+CD38+ populations become CD34+CD38- during culture, which may present unreliable data[190]. Overcoming this

limitation requires functional assays that are necessary to clarify whether the expanded population is in fact enriched in HSPCs[190]. My qualitative findings of increased expansion of immature HPC subsets were successfully complemented with functional morphological readings using the CFU assay, where significant increases in the production of CFU and CFU-M were observed. This is interesting given the close relationship between engraftment potential and CFU content of graft[77]. Furthermore, HSCs treated with Vit-C have been shown to reduce monocytic differentiation and promote erythroid differentiation via FACS[140]. These findings are consistent with studies that show Vit-C/AA2P to increase TET-2 function; whereby, TET-2 deficiency encourages monocytic differentiation and reduces erythroid differentiation in vitro[191,192]. On the contrary, my findings and others have shown the addition of AA2P to improve plating efficiency of all colonies, BFU-E and CFU-M included, in both CB and BM (Figure-6D)[193]. Although FACS analyses are important for revealing differentiation capacity through phenotypic expression, probing functional properties by CFU is better suited for determining Vit-C effects on HSC activity[74].

I also used combinations of 4 and 5 markers to decipher the effects of AA2P on MPP and HSC-enriched cells. Two subsets enriched in HSC activity were found increased in AA2P cultures, CD34+CD38-CD45RA-CD90+ and CD34+CD38-CD45RA-CD90+CD49f+ but the difference failed to be significant likely due in part to the high variation between independent experiments which is compounded by the very low frequency of HSC subsets. It was nonetheless reassuring to obtain results that are consistent with previous evidence that HSC frequency remains stable in AA2P cultures[140,153]. Early events in leukemogenesis inactivate TET-2 that is associated with increased HSC frequency and self-renewal[149,150,152,191]. Likewise, the same effects are provoked in Vit-C depleted HSCs that had much less 5hmc levels[140]. Taken together, these results completed

my 1st objective demonstrating that AA2P can, on its own, promote the expansion of hematopoietic subsets enriched in progenitor activity, and likely support the maintenance of HSC.

4.2 Migration response in AA2P treated CB CD34+ cells

My second objective was to determine whether culturing CB progenitors in the presence of AA2P would increase their capacity to migrate towards CXCL12/SDF-1a—a crucial step necessary for successful homing of HSPCs and engraftment potential[113]. First, I investigated whether AA2P could increased expression of CXCR4 by indirectly evaluating its expression based on fluorescent intensity measured by cytometry. For the most part, my results revealed that CXCR4 expression remained unchanged in most populations, except however, for a significant increase in the MFI of CXCR4 within the CD34+ population, raising the possibility that those cells may migrate towards SDF-1a more efficiently; thereby, leading to increased migration activity.

Consistent with this, enhanced migration activity for the CD34+ population was observed with AA2P cultured progenitors. This is aligned with a large body of work that elucidated the method of action for CXCR4 to be the most important determinant for homing and mobilization of hematopoietic cells[34,35,113]. Also, these results displayed that HSPC-enriched CD34+ cells in the CD34+CD45RA-CD38- and CD34+CD45RA-CD38-CD90+ fraction had migration activity comparable to control. Evidently, HSPCs in CB units have been shown to have intrinsically impaired migration activity that is likely the case in these reduced migration observations[102].

4.3 AA2P graft to support NSG engraftment in a transplant model

Finally, I investigated whether ex vivo expanded HSC and progenitors in AA2P treated cultures could improve engraftment outcome. The use of a xenotransplant model like NSG mice provide direct functional proof of the engraftment activity of graft of interest. Human chimerism

observed in the PB and BM of AA2P/Vit-C and SFM control mice cohorts were used to examine engraftment activity of the transplanted graft.

This study provides the first evidence for elevated early to late platelet levels in the PB among mice cohorts transplanted with AA2P progenies, with similar observations made in in preliminary Vit-C results. Megakaryocytes that produce platelets can be derived from MPPs and downstream CMPs that are shown to be increased in AA2P cultured CD34+ cells (Figure-6B)[194,195]. It is possible that the increased production with AA2P in the MPP/CMP population translates into increased ST platelet levels in vivo[196]. On that note, the sheer number in TNC (2-fold) of the transplanted AA2P fraction is maybe enough to elevate short- to mid-term (weeks 3-10) platelet levels when compared to vehicle control, which is associated with improved CB transplantation outcome[197]. However, these elevated platelet levels cannot be sustained by ST-HSCs and MPPs alone after 16 weeks and require LT-HSC to continue this trend[90]. With that being said, the origin of megakaryocyte is one of the most debated subjects, with recent studies suggesting that megakaryocytes can be generated from various pathways or not required to undergo the stages of differentiation that is followed by other progenitors[219]. Some studies have presented that HSCs are capable of spontaneous differentiation into megakaryocytes upon bodily demand[2,198–203]. Both HSCs and MPPs contain lineage-biased subsets that have not been thoroughly investigated in my study[90,204,205]. Although my FACS-based findings did not show significant increase in the expansion of HSC-enriched populations, it is clearly possible that AA2P did increase expansion of primitive HSC missed by the current markers defined. This is supported by the increased engraftment seen in AA2P mice. Moreover, it is also possible that AA2P-HSCs are more primitive than control-HSCs due to increased epigenetic 5hmc levels induced by AA2P/TET2[153]. This suggests that the composition of the HSC compartment established in the

primary recipients of AA2P grafts contain more primitive HSCs, which accompanied increased ST and LT platelet levels.

Studies using embryonic stem cells have shown that Vit-C can modify differentiation programs and enhance somatic cell reprogramming—pushing embryonic stem cells further to the naïve pluripotent state[206–208]. By modifying the chromatin landscape through Vit-C induced demethylation, HSCs are maybe reprogrammed to become more primitive. Herein, a fraction of transplanted AA2P progenies could consist of distinct platelet-primed HSCs demonstrated to reside at the apex of the hierarchy[198,209,199]. In vivo outcomes using AA2P progenies show ST to LT megakaryocyte engraftment activity, here marked by early to late platelet levels (weeks 3-18; Figure-8F) in the periphery, which is evident in platelet-primed HSCs that also generated higher ST and LT platelet output[198]. Although further research is needed to validate this rare and functional population in human HSCs—it is of interest because HSCs share characteristics closely similar to megakaryocytes that AA2P could increase[210]. Moreover, leucocyte engraftment was also tracked and in contrast to platelets, their levels was not significantly different between both mice groups at all time points analyzed. Also monitored was the B-lymphocyte population that, although increased, were not significant possibly because these cells have a longer lifespan (52+ days)[211] than other leucocyte subpopulations (1-5 days)[212,213]. More importantly, HSCs in AA2P grafts represented a multipotent population as platelets and lympho-myeloid leucocytes were readily detectable as in control.

Onwards, I wanted to track hematopoietic cells within the BM to investigate whether AA2P grafts exhibit increased BM engraftment, while also evaluating LT hematopoiesis that is solely reconstituted by LT-HSCs after 16-weeks[90]. BM CD45+ engraftment evaluated 22-weeks posttransplant was found to be superior among the AA2P-graft cohorts when compared to control

(Figure-9C), with comparable data established in the BM of Vit-C groups (Figure-10G). It is plausible that a fraction of AA2P-HSCs are lymphoid biased. These lymphoid biased HSCs have been found to remain longer in quiescence and display a significantly lower demethylation profile—thereby contributing to the LT increased leukocyte engraftment in AA2P groups[214]. With that being said, superior LT contribution to CD45+ engraftment in AA2P cohorts can also be associated with an in vivo niche in NSG mice that has recently been confirmed to mainly support LMPP populations[215]. This LMPP fraction, here identified as CD34+CD38–CD45RA+CD90–, has been shown to be significantly increased in AA2P cultures; thereby, warranting AA2P-grafts for their superior leucocyte engraftment.

Furthermore, multilineage capacity of LT-HSCs was assessed by CD45+ with lineage specific markers. This encompassed cells positive for CD33, CD14, CD3, CD19 and CD34. All lineages were found to be present in AA2P cohorts demonstrating normal multi-differentiation capacity of AA2P-grafts. The increased human chimerism in AA2P cohorts was the combine result of small increases mostly not significant on their own of several lineages including CD19 and CD34. Aside from other studies that have found Vit-C to promote leukocyte differentiation[189], B-lymphoid cells that express CD19 have been shown to be completed silenced for myeloid genes[216]. It is therefore possible that some contributed CD19 engraftment in AA2P-grafts, largely made up of LMPPs, is by facilitated myeloid silenced genes. Likewise, AA2P cultures were able to significantly expand the CD34+ population when compared to control. This can be associated with an increased contribution to the CD34+ engraftment found in AA2P grafts.

4.5 Future Directions

Pending TET-2 inhibition will help determine whether CB CD34+ expansion elicited by AA2P is dependent on TET-2 activity as many Vit-C effects are. However, like TET-2, TET-3 is

abundantly expressed in HSPCs and deficiency in this protein causes severe depletion of 5hmc in HSPC DNA that follows decreased efficacy of Vit-C in suppressing abnormal self-renewal programs; thereby, suggesting complementary activity[153,188]. Herein, TET-3 inhibition could also determine whether these programs play an important in the expansion of CB CD34+ cells. It should be taken into account that some cultures could have hypermethylated DNA or imbalanced DNA methylation.

Interestingly, HSPCs expressed the highest levels of Vit-C transporters, SLc23a2, 14-fold higher than in restricted HPC populations[140]. Also, both Vit-C concentrations and Slc23a2 expression were decreased with differentiation[140]. It is therefore important to determine whether in vitro expansion of CB HSPCs convey similar results, and specifically, in the LMPP population that has been found to be significantly expanded in my culture results.

Next, ascorbate acts as a cofactor for prolyl hydroxylases that reduce hypoxia inducible factors (HIFs)[217]but are said to not be required for HSC function[218]. However, other studies have found that Vit-C induced HIF1α could play an important role in Vit-C induced differentiation programs[189]. Tracking these enzymes and HIF1α in HSCs would be worth looking into.

Finally, full understanding of AA2P's activity on HSC populations requires the completion of a serial transplantation and limited dilution assay. Serial transplantation will determine AA2P's activity on self-renewal capacity in expanded HSCs, while the limited dilution assay will aid in defining the frequency of HSCs in AA2P grafts to determine whether HSC were expanded or just better maintained in the presence of AA2P.

4.6 Conclusion

In conclusion, CB units accompany delayed engraftment in neutrophil and platelet levels that is associated with low HSC content. The rarity of human HSCs and the limited ability to study

these cells in the human niche hurdle our understanding of the regulatory processes and cues that control self-renewal and differentiation—thereby, limiting their therapeutic application. Nonetheless, natural and synthetic small molecules promoting HSC expansion have been identified, such as UM171 and the HDAC inhibitor VPA. Similarly, AA2P is a putative molecule capable of inducing ex vivo expansion in CB-derived CD34+ cells and progenitors, when combined with cytokines. AA2P also appeared to have either increased HSC expansion or improved HSC maintenance as suggested by the superior engraftment of platelet and human BM in AA2P-recipients over a 22-week course. Interestingly, AA2P may do so by modulation of the epigenetic landscape that, in combination with complementary molecules, has been shown in our lab to induce robust HSPC expansion with exceptional ST and LT reconstitution. Overall, my study shows the independent activity of AA2P as a moderate HSPC agonist, and its potential to advance clinical application of CB transplantations as a complementary molecule.

References

1. Keohane, E. M. Intrinsic sedects leading to increased erythrocyte. in *Rodak's hematology : clinical principles and applications* (2016).
2. Notta, F. *et al.* Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science (80-.).* **351**, (2016).
3. Spangrude, G. J. *et al.* Mouse hematopoietic stem cells. *Blood* (1991) doi:10.1182/blood.v78.6.1395.1395.
4. Till, J. E. & McCulloch, E. A. Hemopoietic stem cell differentiation. *Biochim. Biophys. Acta - Rev. Cancer* (1980) doi:10.1016/0304-419X(80)90009-8.
5. Seita, J. & Weissman, I. L. Hematopoietic stem cell: Self-renewal versus differentiation. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine* (2010) doi:10.1002/wsbm.86.
6. Porwit, A., McCullough, J. & Erber, W. N. *Blood And Bone Marrow Pathology*. *Blood and Bone Marrow Pathology* (2011). doi:10.1016/C2009-0-52942-X.
7. Ogawa, M. Differentiation and proliferation of hematopoietic stem cells. *Blood* (1993) doi:10.1182/blood.v81.11.2844.2844.
8. Weissman, I. L., Morrison, S., Cheshier, S. & Uchida, N. Hematopoietc stem cells: Biology and transplantation. *Exp. Hematol.* (1997).
9. Shahriyari, L. & Komarova, N. L. Symmetric vs. asymmetric stem cell divisions: an adaptation against cancer? *PLoS One* (2013) doi:10.1371/journal.pone.0076195.
10. Fuchs, E., Tumbar, T. & Guasch, G. Socializing with the neighbors: Stem cells and their niche. *Cell* (2004) doi:10.1016/S0092-8674(04)00255-7.
11. Zhong, W. & Chia, W. Neurogenesis and asymmetric cell division. *Current Opinion in Neurobiology* (2008) doi:10.1016/j.conb.2008.05.002.
12. Ho, A. D. Kinetics and symmetry of divisions of hematopoietic stem cells. *Experimental Hematology* (2005) doi:10.1016/j.exphem.2004.09.004.
13. Zhang, Y. V., Cheong, J., Ciapurin, N., McDermitt, D. J. & Tumbar, T. Distinct Self-Renewal and Differentiation Phases in the Niche of Infrequently Dividing Hair Follicle Stem Cells. *Cell Stem Cell* (2009) doi:10.1016/j.stem.2009.06.004.
14. Loeffler, M. & Roeder, I. Tissue stem cells: Definition, plasticity, heterogeneity, self-organization and models - A conceptual approach. *Cells Tissues Organs* (2002) doi:10.1159/000057688.
15. Marshman, E., Booth, C. & Potten, C. S. The intestinal epithelial stem cell. *BioEssays* (2002) doi:10.1002/bies.10028.
16. Clayton, E. *et al.* A single type of progenitor cell maintains normal epidermis. *Nature* (2007) doi:10.1038/nature05574.
17. Guerrouahen, B. S., Al-Hijji, I. & Tabrizi, A. R. Osteoblastic and vascular endothelial niches, their control on normal hematopoietic stem cells, and their consequences on the development of leukemia. *Stem Cells International* (2011) doi:10.4061/2011/375857.
18. Morrison, S. J. & Spradling, A. C. Stem Cells and Niches: Mechanisms That Promote Stem Cell Maintenance throughout Life. *Cell* (2008) doi:10.1016/j.cell.2008.01.038.
19. Boulais, P. E. & Frenette, P. S. Making sense of hematopoietic stem cell niches. *Blood* (2015) doi:10.1182/blood-2014-09-570192.
20. Orford, K. W. & Scadden, D. T. Deconstructing stem cell self-renewal: Genetic insights into cell-cycle regulation. *Nature Reviews Genetics* (2008) doi:10.1038/nrg2269.
21. T., L., A., D. & O., K. How do stem cells find their way home? *Blood* (2005)

doi:10.1182/blood-2005-04-1417 LK - http://bj7rx7bn7b.search.serialssolutions.com?sid=EMBASE&issn=00064971&id=doi:10.1182%2Fblood-2005-04-1417&atitle=How+do+stem+cells+find+their+way+home%3F&stitle=Blood&title=Blood&volume=106&issue=6&spage=1901&epage=1910&aulast=Lapidot&aufirst=Tsvee&auinit=T.&aufull=Lapidot+T.&coden=BLOOA&isbn=&pages=1901-1910&date=2005&auinit1=T&auinitm=.

22. Morrison, S. J. & Scadden, D. T. The bone marrow niche for haematopoietic stem cells. *Nature* (2014) doi:10.1038/nature12984.
23. Kusumbe, A. P., Ramasamy, S. K. & Adams, R. H. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. *Nature* (2014) doi:10.1038/nature13145.
24. Kiel, M. J. *et al.* SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* (2005) doi:10.1016/j.cell.2005.05.026.
25. Sipkins, D. A. *et al.* In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature* (2005) doi:10.1038/nature03703.
26. Colmone, A. *et al.* Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. *Science (80-.).* (2008) doi:10.1126/science.1164390.
27. Lo Celso, C. *et al.* Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature* (2009) doi:10.1038/nature07434.
28. Itkin, T. *et al.* Distinct bone marrow blood vessels differentially regulate haematopoiesis. *Nature* (2016) doi:10.1038/nature17624.
29. Cohen, S. G. *et al.* PAR1 signaling regulates the retention and recruitment of EPCR-expressing bone marrow hematopoietic stem cells. *Nat. Med.* (2015) doi:10.1038/nm.3960.
30. Ikuta, K. & Weissman, I. L. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc. Natl. Acad. Sci. U. S. A.* (1992) doi:10.1073/pnas.89.4.1502.
31. Flanagan, J. G. & Leder, P. The kit ligand: A cell surface molecule altered in steel mutant fibroblasts. *Cell* (1990) doi:10.1016/0092-8674(90)90299-T.
32. Barker, J. E. SI/SI(d) hematopoietic progenitors are deficient in situ. *Exp. Hematol.* (1994).
33. Zsebo, K. M. *et al.* Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell* (1990) doi:10.1016/0092-8674(90)90300-4.
34. Zou, Y. R., Kottman, A. H., Kuroda, M., Taniuchi, I. & Littman, D. R. Function of the chemokine receptor CXCR4 in heaematopolesis and in cerebellar development. *Nature* (1998) doi:10.1038/31269.
35. Sugiyama, T., Kohara, H., Noda, M. & Nagasawa, T. Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. *Immunity* (2006) doi:10.1016/j.immuni.2006.10.016.
36. Tzeng, Y. S. *et al.* Loss of Cxcl12/Sdf-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myelosuppression. *Blood* (2011) doi:10.1182/blood-2010-01-266833.

37. McDermott, D. H. *et al.* Chromothriptic cure of WHIM syndrome. *Cell* (2015) doi:10.1016/j.cell.2015.01.014.
38. Lai, C. Y. *et al.* Stage-specific roles for Cxcr4 signaling in murine hematopoietic stem/progenitor cells in the process of bone marrow repopulation. *Stem Cells* (2014) doi:10.1002/stem.1670.
39. Kimura, S., Roberts, A. W., Metcalf, D. & Alexander, W. S. Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proc. Natl. Acad. Sci. U. S. A.* (1998) doi:10.1073/pnas.95.3.1195.
40. Qian, H. *et al.* Critical Role of Thrombopoietin in Maintaining Adult Quiescent Hematopoietic Stem Cells. *Cell Stem Cell* (2007) doi:10.1016/j.stem.2007.10.008.
41. Yoshihara, H. *et al.* Thrombopoietin/MPL Signaling Regulates Hematopoietic Stem Cell Quiescence and Interaction with the Osteoblastic Niche. *Cell Stem Cell* (2007) doi:10.1016/j.stem.2007.10.020.
42. De Sauvage, F. J. *et al.* Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. *Nature* (1994) doi:10.1038/369533a0.
43. Sungaran, R., Markovic, B. & Chong, B. H. Localization and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow, and spleen using in situ hybridization. *Blood* (1997) doi:10.1182/blood.v89.1.101.101_101_107.
44. Purton, L. E. & Scadden, D. T. Limiting Factors in Murine Hematopoietic Stem Cell Assays. *Cell Stem Cell* (2007) doi:10.1016/j.stem.2007.08.016.
45. Yang, Q. & Bhandoola, A. Decoding HSC heterogeneity. *Blood* (2012) doi:10.1182/blood-2012-03-417238.
46. Morrison, S. J. & Weissman, I. L. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* (1994) doi:10.1016/1074-7613(94)90037-X.
47. Christensen, J. L. & Weissman, I. L. Flk-2 is a marker in hematopoietic stem cell differentiation: A simple method to isolate long-term stem cells. *Proc. Natl. Acad. Sci. U. S. A.* (2001) doi:10.1073/pnas.261562798.
48. Yang, L. *et al.* Identification of Lin-Sca1+kit+CD34 +Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood* (2005) doi:10.1182/blood-2004-06-2159.
49. Lai, A. Y. & Kondo, M. T and B lymphocyte differentiation from hematopoietic stem cell. *Seminars in Immunology* (2008) doi:10.1016/j.smim.2008.05.002.
50. Sidney, L. E., Branch, M. J., Dunphy, S. E., Dua, H. S. & Hopkinson, A. Concise review: Evidence for CD34 as a common marker for diverse progenitors. *Stem Cells* (2014) doi:10.1002/stem.1661.
51. Huang, S. & Terstappen, L. W. M. M. Lymphoid and myeloid differentiation of single human CD34+, HLA-DR+, CD38- hematopoietic stem cells. *Blood* (1994) doi:10.1182/blood.v83.6.1515.bloodjournal8361515.
52. Osawa, M., Hanada, K. I., Hamada, H. & Nakauchi, H. Long-term lymphohematopoietic reconstitution by a single CD34- low/negative hematopoietic stem cell. *Science (80-.).* (1996) doi:10.1126/science.273.5272.242.
53. Tavian, M. *et al.* Aorta-associated CD34+ hematopoietic cells in the early human embryo. *Blood* (1996) doi:10.1182/blood.v87.1.67.bloodjournal87167.
54. Aksoy, P., White, T. A., Thompson, M. & Chini, E. N. Regulation of intracellular levels of NAD: A novel role for CD38. *Biochem. Biophys. Res. Commun.* (2006)

doi:10.1016/j.bbrc.2006.05.042.
55. Shubinsky, G. & Schlesinger, M. The CD38 lymphocyte differentiation marker: New insight into its ectoenzymatic activity and its role as a signal transducer. *Immunity* (1997) doi:10.1016/S1074-7613(00)80353-2.
56. Malavasi, F. *et al.* Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiological Reviews* (2008) doi:10.1152/physrev.00035.2007.
57. Baum, C. M., Weissman, I. L., Tsukamoto, A. S., Buckle, A. M. & Peault, B. Isolation of a candidate human hematopoietic stem-cell population. *Proc. Natl. Acad. Sci. U. S. A.* (1992) doi:10.1073/pnas.89.7.2804.
58. Hao, Q. L., Thiemann, F. T., Petersen, D., Smogorzewska, E. M. & Crooks, G. M. Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. *Blood* (1996) doi:10.1182/blood.v88.9.3306.bloodjournal8893306.
59. Miller, J. S., McCullar, V., Punzel, M., Lemischka, I. R. & Moore, K. A. Single adult human CD34+/Lin-/CD38- progenitors give rise to natural killer cells, B-lineage cells, dendritic cells, and myeloid cells. *Blood* (1999) doi:10.1182/blood.V93.1.96.
60. Uchida, N. *et al.* HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* (1998) doi:10.1073/pnas.95.20.11939.
61. Petzer, A. L., Hogge, D. E., Lansdorp, P. M., Reid, D. S. & Eaves, C. J. Self-renewal of primitive human hematopoietic cells (long-term-culture-initiating cells) in vitro and their expansion in defined medium. *Proc. Natl. Acad. Sci. U. S. A.* (1996) doi:10.1073/pnas.93.4.1470.
62. Manz, M. G., Miyamoto, T., Akashi, K. & Weissman, I. L. Prospective isolation of human clonogenic common myeloid progenitors. *Proc. Natl. Acad. Sci. U. S. A.* (2002) doi:10.1073/pnas.172384399.
63. Majeti, R., Park, C. Y. & Weissman, I. L. Identification of a Hierarchy of Multipotent Hematopoietic Progenitors in Human Cord Blood. *Cell Stem Cell* (2007) doi:10.1016/j.stem.2007.10.001.
64. Sharma, Y., Astle, C. M. & Harrison, D. E. Heterozygous Kit Mutants with Little or No Apparent Anemia Exhibit Large Defects in Overall Hematopoietic Stem Cell Function. *Exp. Hematol.* (2007) doi:10.1016/j.exphem.2006.10.001.
65. Larochelle, A. *et al.* Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: Implications for gene therapy. *Nat. Med.* (1996) doi:10.1038/nm1296-1329.
66. Sutherland, H. J., Eaves, C. J., Eaves, A. C., Dragowska, W. & Lansdorp, P. M. Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. *Blood* (1989) doi:10.1182/blood.v74.5.1563.bloodjournal7451563.
67. Notta, F. *et al.* Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science (80-.).* (2011) doi:10.1126/science.1201219.
68. Tarunina, M. *et al.* A Novel High-Throughput Screening Platform Reveals an Optimized Cytokine Formulation for Human Hematopoietic Progenitor Cell Expansion. *Stem Cells Dev.* (2016) doi:10.1089/scd.2016.0216.
69. Kondo, M. Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *Immunol. Rev.* (2010) doi:10.1111/j.1600-065X.2010.00963.x.

70. Terstappen, L. W. M. M., Huang, S., Safford, M., Lansdorp, P. M. & Loken, M. R. Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. *Blood* (1991) doi:10.1182/blood.v77.6.1218.bloodjournal7761218.
71. Lebien, T. W. B-cell lymphopoiesis in mouse and man. *Curr. Opin. Immunol.* (1998) doi:10.1016/S0952-7915(98)80248-3.
72. Karamitros, D. *et al.* Single-cell analysis reveals the continuum of human lympho-myeloid progenitor cells article. *Nat. Immunol.* (2018) doi:10.1038/s41590-017-0001-2.
73. Laurenti, E. & Göttgens, B. From haematopoietic stem cells to complex differentiation landscapes. *Nature* (2018) doi:10.1038/nature25022.
74. Buckley, S. M. & Verfaillie, C. Regulation of hematopoiesis. in *Blood and Bone Marrow Pathology* (2011). doi:10.1016/B978-0-7020-3147-2.00004-3.
75. Sarma, N. J., Takeda, A. & Yaseen, N. R. Colony forming cell (CFC) assay for human hematopoietic cells. *J. Vis. Exp.* (2010) doi:10.3791/2195.
76. Pamphilon, D. *et al.* Current practices and prospects for standardization of the hematopoietic colony-forming unit assay: A report by the cellular therapy team of the Biomedical Excellence for Safer Transfusion (BEST) Collaborative. *Cytotherapy* (2013) doi:10.1016/j.jcyt.2012.11.013.
77. Page, K. M. *et al.* Total colony-forming units are a strong, independent predictor of neutrophil and platelet engraftment after unrelated umbilical cord blood transplantation: A single-center analysis of 435 cord blood transplants. *Biol. Blood Marrow Transplant.* (2011) doi:10.1016/j.bbmt.2011.01.011.
78. Liu, M., Miller, C. L. & Eaves, C. J. Human long-term culture initiating cell assay. *Methods Mol. Biol.* (2013) doi:10.1007/978-1-62703-128-8-15.
79. Cheng, H., Liang, P. H. & Cheng, T. Mouse hematopoietic stem cell transplantation. *Methods Mol. Biol.* (2013) doi:10.1007/978-1-62703-317-6_3.
80. Ito, M. *et al.* NOD/SCID/γcnull mouse: An excellent recipient mouse model for engraftment of human cells. *Blood* (2002) doi:10.1182/blood-2001-12-0207.
81. Doulatov, S., Notta, F., Laurenti, E. & Dick, J. E. Hematopoiesis: A human perspective. *Cell Stem Cell* (2012) doi:10.1016/j.stem.2012.01.006.
82. Manz, M. G. Human-Hemato-Lymphoid-System Mice: Opportunities and Challenges. *Immunity* (2007) doi:10.1016/j.immuni.2007.05.001.
83. Rongvaux, A. *et al.* Human Hemato-Lymphoid System Mice: Current Use and Future Potential for Medicine. *Annu. Rev. Immunol.* (2013) doi:10.1146/annurev-immunol-032712-095921.
84. Gille, C. *et al.* Monocytes derived from humanized neonatal NOD/SCID/IL2Rγ null mice are phenotypically immature and exhibit functional impairments. *Hum. Immunol.* (2012) doi:10.1016/j.humimm.2012.01.006.
85. Tanaka, S. *et al.* Development of Mature and Functional Human Myeloid Subsets in Hematopoietic Stem Cell-Engrafted NOD/SCID/IL2rγKO Mice. *J. Immunol.* (2012) doi:10.4049/jimmunol.1103660.
86. Lang, J. *et al.* Studies of Lymphocyte Reconstitution in a Humanized Mouse Model Reveal a Requirement of T Cells for Human B Cell Maturation. *J. Immunol.* (2013) doi:10.4049/jimmunol.1202810.
87. Martinez-Torres, F., Nochi, T., Wahl, A., Garcia, J. V. & Denton, P. W. Hypogammaglobulinemia in BLT humanized mice - An animal model of primary

antibody deficiency. *PLoS One* (2014) doi:10.1371/journal.pone.0108663.
88. Brehm, M. A. *et al.* Parameters for establishing humanized mouse models to study human immunity: Analysis of human hematopoietic stem cell engraftment in three immunodeficient strains of mice bearing the IL2rγnull mutation. *Clin. Immunol.* (2010) doi:10.1016/j.clim.2009.12.008.
89. Covassin, L. *et al.* Human immune system development and survival of non-obese diabetic (NOD)-scid IL2rγnull (NSG) mice engrafted with human thymus and autologous haematopoietic stem cells. *Clin. Exp. Immunol.* (2013) doi:10.1111/cei.12180.
90. Ema, H., Morita, Y. & Suda, T. Heterogeneity and hierarchy of hematopoietic stem cells. *Experimental Hematology* (2014) doi:10.1016/j.exphem.2013.11.004.
91. Iscove, N. N. & Nawa, K. Hematopoietic stem cells expand during serial transplantation in vive without apparent exhaustion. *Curr. Biol.* (1997) doi:10.1016/s0960-9822(06)00341-1.
92. Jordan, C. T. & Lemischka, I. R. Clonal and systemic analysis of long-term hematopoiesis in the mouse. *Genes Dev.* (1990) doi:10.1101/gad.4.2.220.
93. Lin, K. K. & Goodell, M. A. *Detection of Hematopoietic Stem Cells by Flow Cytometry*. *Methods in Cell Biology* (2011). doi:10.1016/B978-0-12-385493-3.00002-4.
94. Rossi, D. J., Jamieson, C. H. M. & Weissman, I. L. Stems Cells and the Pathways to Aging and Cancer. *Cell* (2008) doi:10.1016/j.cell.2008.01.036.
95. Broxmeyer, H. E. & Farag, S. Background and future considerations for human cord blood hematopoietic cell transplantation, including economic concerns. *Stem Cells Dev.* (2013) doi:10.1089/scd.2013.0382.
96. Gluckman, E. *et al.* Hematopoietic Reconstitution in a Patient with Fanconi's Anemia by Means of Umbilical-Cord Blood from an HLA-Identical Sibling. *N. Engl. J. Med.* (1989) doi:10.1056/NEJM198910263211707.
97. Ballen, K. K., Gluckman, E. & Broxmeyer, H. E. Umbilical cord blood transplantation: the first 25 years and beyond. *Blood* (2013) doi:10.1182/blood-2013-02-453175.
98. Broxmeyer, H. E., Farag, S. S. & Rocha, V. Cord Blood Hematopoietic Cell Transplantation. in *Thomas' Hematopoietic Cell Transplantation: Fifth Edition* (2016). doi:10.1002/9781118416426.ch39.
99. Hidalgo, A. & Frenette, P. S. Enforced fucosylation of neonatal CD34+ cells generates selectin ligands that enhance the initial interactions with microvessels but not homing to bone marrow. *Blood* (2005) doi:10.1182/blood-2004-03-1026.
100. Kahn, J. *et al.* Overexpression of CXCR4 on human CD34+ progenitors increases their proliferation, migration, and NOD/SCID repopulation. *Blood* (2004) doi:10.1182/blood-2003-07-2607.
101. Ohno, N., Kajiume, T., Sera, Y., Sato, T. & Kobayashi, M. Short-term culture of umbilical cord blood-derived CD34 cells enhances engraftment into NOD/SCID mice through increased CXCR4 expression. *Stem Cells Dev.* (2009) doi:10.1089/scd.2008.0298.
102. Xia, L., McDaniel, J. M., Yago, T., Doeden, A. & McEver, R. P. Surface fucosylation of human cord blood cells augments binding to P-selectin and E-selectin and enhances engraftment in bone marrow. *Blood* (2004) doi:10.1182/blood-2004-02-0650.
103. Michel, G. *et al.* Single- vs double-unit cord blood transplantation for children and young adults with acute leukemia or myelodysplastic syndrome. *Blood* (2016) doi:10.1182/blood-2016-01-694349.
104. Pineault, N. & Abu-Khader, A. Advances in umbilical cord blood stem cell expansion and

clinical translation. *Experimental Hematology* (2015) doi:10.1016/j.exphem.2015.04.011.
105. Genovese, P. *et al.* Targeted genome editing in human repopulating haematopoietic stem cells. *Nature* (2014) doi:10.1038/nature13420.
106. Baron, F., Ruggeri, A. & Nagler, A. Methods of ex vivo expansion of human cord blood cells: Challenges, successes and clinical implications. *Expert Rev. Hematol.* (2016) doi:10.1586/17474086.2016.1128321.
107. Darghouth, D. *et al.* Bio-engineered and native red blood cells from cord blood exhibit the same metabolomic profile. *Haematologica* (2016) doi:10.3324/haematol.2015.141465.
108. Henschler, R. & Richter, R. Transmigration Assays for the Determination of Molecular Interactions Between Hematopoietic Stem Cells and Niche Cells. in *Methods in Molecular Biology* (2019). doi:10.1007/978-1-4939-9574-5_5.
109. He, X. C. *et al.* Homing and migration assays of hematopoietic stem/progenitor cells. *Methods Mol. Biol.* (2014) doi:10.1007/978-1-4939-1133-2_19.
110. Naiyer, A. J. *et al.* Stromal derived factor-1-induced chemokinesis of cord blood CD34+ cells (long-term culture-initiating cells) through endothelial cells is mediated by E-selectin. *Blood* (1999) doi:10.1182/blood.V94.12.4011.
111. Peled, A. *et al.* The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34+ cells: Role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood* (2000) doi:10.1182/blood.v95.11.3289.011k33_3289_3296.
112. Heesen, M., Berman, M. A., Höpken, U. E., Gerard, N. P. & Dorf, M. E. Alternate splicing of mouse fusin/CXC chemokine receptor-4: stromal cell-derived factor-1alpha is a ligand for both CXC chemokine receptor-4 isoforms. *J. Immunol.* (1997).
113. Cottler-Fox, M. H. *et al.* Stem cell mobilization. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program* (2003) doi:10.1182/asheducation-2003.1.419.
114. Hoggatt, J., Singh, P., Sampath, J. & Pelus, L. M. Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. *Blood* (2009) doi:10.1182/blood-2009-01-201335.
115. Sahin, A. O. & Buitenhuis, M. Molecular mechanisms underlying adhesion and migration of hematopoietic stem cells. *Cell Adhesion and Migration* (2012) doi:10.4161/cam.18975.
116. Popat, U. *et al.* Enforced fucosylation of cord blood hematopoietic cells accelerates neutrophil and platelet engraftment after transplantation. *Blood* (2015) doi:10.1182/blood-2015-01-607366.
117. Wang, L. *et al.* A small-molecule/cytokine combination enhances hematopoietic stem cell proliferation via inhibition of cell differentiation. *Stem Cell Res. Ther.* (2017) doi:10.1186/s13287-017-0625-z.
118. Flores-Guzmán, P., Fernández-Sánchez, V. & Mayani, H. Concise Review: Ex Vivo Expansion of Cord Blood-Derived Hematopoietic Stem and Progenitor Cells: Basic Principles, Experimental Approaches, and Impact in Regenerative Medicine. *Stem Cells Transl. Med.* (2013) doi:10.5966/sctm.2013-0071.
119. Aggarwal, R., Lu, J., J. Pompili, V. & Das, H. Hematopoietic Stem Cells: Transcriptional Regulation, Ex Vivo Expansion and Clinical Application. *Curr. Mol. Med.* (2011) doi:10.2174/156652412798376125.
120. De Wynter, E. A. *et al.* CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell

progenitors. *Stem Cells* (1998) doi:10.1002/stem.160387.
121. Jung, M. Inhibitors of Histone Deacetylase as New Anticancer Agents. *Curr. Med. Chem.* (2012) doi:10.2174/0929867013372058.
122. Issa, J. P. Decitabine. *Current Opinion in Oncology* (2003) doi:10.1097/00001622-200311000-00007.
123. Pietrobono, R. *et al.* Quantitative analysis of DNA demethylation and transcriptional reactivation of the FMR1 gene in fragile X cells treated with 5-azadeoxycytidine. *Nucleic Acids Res.* (2002) doi:10.1093/nar/gkf434.
124. Araki, H. *et al.* Expansion of human umbilical cord blood SCID-repopulating cells using chromatin-modifying agents. *Exp. Hematol.* (2006) doi:10.1016/j.exphem.2005.10.002.
125. De Felice, L. *et al.* Histone deacetylase inhibitor valproic acid enhances the cytokine-induced expansion of human hematopoietic stem cells. *Cancer Res.* (2005) doi:10.1158/0008-5472.CAN-04-3063.
126. Chaurasia, P., Gajzer, D. C., Schaniel, C., D'Souza, S. & Hoffman, R. Epigenetic reprogramming induces the expansion of cord blood stem cells. *J. Clin. Invest.* (2014) doi:10.1172/JCI70313.
127. Boitano, A. E. *et al.* Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science (80-.).* (2010) doi:10.1126/science.1191536.
128. Fares, I. *et al.* Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. *Science (80-.).* (2014) doi:10.1126/science.1256337.
129. Cheung, A. M. S. *et al.* Analysis of the clonal growth and differentiation dynamics of primitive barcoded human cord blood cells in NSG mice. *Blood* (2013) doi:10.1182/blood-2013-06-508432.
130. Chagraoui, J. *et al.* UM171 induces a homeostatic inflammatory-detoxification response supporting human HSC self-renewal. *PLoS One* (2019) doi:10.1371/journal.pone.0224900.
131. Dickinson, B. C. & Chang, C. J. Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nature Chemical Biology* (2011) doi:10.1038/nchembio.607.
132. Duarte, T. L. & Lunec, J. Review: When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. *Free Radical Research* (2005) doi:10.1080/10715760500104025.
133. Finkel, T. Oxygen radicals and signaling. *Curr. Opin. Cell Biol.* (1998) doi:10.1016/S0955-0674(98)80147-6.
134. Carr, A. & Frei, B. Does vitamin C act as a pro-oxidant under physiological conditions? [In Process Citation]. *FASEB J* (1999).
135. Bevan, R. J. *et al.* Can vitamin C induce nucleotide excision repair? Support from in vitro evidence. *Br. J. Nutr.* (2010) doi:10.1017/S0007114509992285.
136. Lenton, K. J. *et al.* Vitamin C augments lymphocyte glutathione in subjects with ascorbate deficiency. *Am. J. Clin. Nutr.* (2003) doi:10.1093/ajcn/77.1.189.
137. Johnston, C. S., Meyer, C. G. & Srilakshmi, J. C. Vitamin C elevates red blood cell glutathione in healthy adults. *Am. J. Clin. Nutr.* (1993) doi:10.1093/ajcn/58.1.103.
138. Ohwada, R., Ozeki, Y. & Saitoh, Y. High-dose ascorbic acid induces carcinostatic effects through hydrogen peroxide and superoxide anion radical generation-induced cell death and growth arrest in human tongue carcinoma cells. *Free Radic. Res.* (2017) doi:10.1080/10715762.2017.1361533.
139. Chen, Q. *et al.* Pharamacologic ascorbic acid concentrations selectively kill cancer cells:

Action as a pro-drug to deliver hydrogen peroxide to tissuse. *Proc. Natl. Acad. Sci. U. S. A.* (2005) doi:10.1073/pnas.0506390102.
140. Agathocleous, M. *et al.* Ascorbate regulates haematopoietic stem cell function and leukaemogenesis. *Nature* (2017) doi:10.1038/nature23876.
141. Tahiliani, M. *et al.* Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science (80-.).* (2009) doi:10.1126/science.1170116.
142. Williams, K., Christensen, J. & Helin, K. DNA methylation: TET proteins-guardians of CpG islands? *EMBO Reports* (2012) doi:10.1038/embor.2011.233.
143. Blackledge, N. P. & Klose, R. J. CpG island chromatin: A platform for gene regulation. *Epigenetics* (2011) doi:10.4161/epi.6.2.13640.
144. Bogdanović, O. & Veenstra, G. J. C. DNA methylation and methyl-CpG binding proteins: Developmental requirements and function. *Chromosoma* (2009) doi:10.1007/s00412-009-0221-9.
145. Garaycoechea, J. I. *et al.* Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. *Nature* (2012) doi:10.1038/nature11368.
146. Yan, H. *et al.* IDH1 and IDH2 mutations in gliomas. *N. Engl. J. Med.* (2009) doi:10.1056/NEJMoa0808710.
147. Mihaylova, M. M., Sabatini, D. M. & Yilmaz, Ö. H. Dietary and metabolic control of stem cell function in physiology and cancer. *Cell Stem Cell* (2014) doi:10.1016/j.stem.2014.02.008.
148. Ito, S. *et al.* Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science (80-.).* (2011) doi:10.1126/science.1210597.
149. Jan, M. *et al.* Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. *Sci. Transl. Med.* (2012) doi:10.1126/scitranslmed.3004315.
150. Moran-Crusio, K. *et al.* Tet2 Loss Leads to Increased Hematopoietic Stem Cell Self-Renewal and Myeloid Transformation. *Cancer Cell* (2011) doi:10.1016/j.ccr.2011.06.001.
151. Ko, M. *et al.* Ten-eleven-translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. *Proc. Natl. Acad. Sci. U. S. A.* (2011) doi:10.1073/pnas.1112317108.
152. Quivoron, C. *et al.* TET2 Inactivation Results in Pleiotropic Hematopoietic Abnormalities in Mouse and Is a Recurrent Event during Human Lymphomagenesis. *Cancer Cell* (2011) doi:10.1016/j.ccr.2011.06.003.
153. Cimmino, L. *et al.* Restoration of TET2 Function Blocks Aberrant Self-Renewal and Leukemia Progression. *Cell* (2017) doi:10.1016/j.cell.2017.07.032.
154. Yin, R. *et al.* Ascorbic acid enhances tet-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals. *J. Am. Chem. Soc.* (2013) doi:10.1021/ja4028346.
155. Yuan, J. P. & Chen, F. Degradation of Ascorbic Acid in Aqueous Solution. *J. Agric. Food Chem.* (1998) doi:10.1021/jf9805404.
156. ROBERTSON, G. L. & SAMANIEGO, C. M. L. Effect of Initial Dissolved Oxygen Levels on the Degradation of Ascorbic Acid and the Browning of Lemon Juice during Storage. *J. Food Sci.* (1986) doi:10.1111/j.1365-2621.1986.tb10866.x.
157. Van Den Broeck, I., Ludikhuyze, L., Weemaes, C., Van Loey, A. & Hendrickx, M. Kinetics for Isobaric-Isothermal Degradation of L-Ascorbic Acid. *J. Agric. Food Chem.* (1998) doi:10.1021/jf9708251.

158. Zheng, K. *et al.* Enzymatic Production of Ascorbic Acid-2-phosphate by Recombinant Acid Phosphatase. *J. Agric. Food Chem.* (2017) doi:10.1021/acs.jafc.7b00612.
159. Kuwahara, T., Homma, T., Kondo, M. & Shimomura, M. A novel system combining biocatalytic dephosphorylation of l-ascorbic acid 2-phosphate and electrochemical oxidation of resulting ascorbic acid. *Biosens. Bioelectron.* (2011) doi:10.1016/j.bios.2010.12.041.
160. Choi, K. M. *et al.* Effect of ascorbic acid on bone marrow-derived mesenchymal stem cell proliferation and differentiation. *J. Biosci. Bioeng.* (2008) doi:10.1263/jbb.105.586.
161. Shima, N., Kimoto, M., Yamaguchi, M. & Yamagami, S. Increased proliferation and replicative lifespan of isolated human corneal endothelial cells with L-ascorbic acid 2-phosphate. *Investig. Ophthalmol. Vis. Sci.* (2011) doi:10.1167/iovs.11-7592.
162. Takamizawa, S. *et al.* Effects of ascorbic acid and ascorbic acid 2-phosphate, a long-acting vitamin C derivative, on the proliferation and differentiation of human osteoblast-like cells. *Cell Biol. Int.* (2004) doi:10.1016/j.cellbi.2004.01.010.
163. Tsutsumi, K. *et al.* Effects of L-ascorbic acid 2-phosphate magnesium salt on the properties of human gingival fibroblasts. *J. Periodontal Res.* (2012) doi:10.1111/j.1600-0765.2011.01430.x.
164. Mitsumoto, Y., Liu, Z. & Klip, A. A long-lasting vitamin C derivative, ascorbic acid 2-phosphate, increases myogenin gene expression and promotes differentiation in L6 muscle cells. *Biochem. Biophys. Res. Commun.* (1994) doi:10.1006/bbrc.1994.1242.
165. Senoo, H. & Hata, R. Extracellular matrix regulates cell morphology, proliferation, and tissue formation. *Kaibogaku zasshi. Journal of anatomy* (1994).
166. Hata, R. -I & Senoo, H. L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. *J. Cell. Physiol.* (1989) doi:10.1002/jcp.1041380103.
167. Cashman, J. D. *et al.* Kinetic evidence of the regeneration of multilineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. *Blood* (1997) doi:10.1182/blood.v89.12.4307.
168. Hogan, C. J. *et al.* Engraftment and development of human CD34+-enriched cells from umbilical cord blood in NOD/LtSz-scid/scid mice. *Blood* (1997) doi:10.1182/blood.v90.1.85.85_85_96.
169. Pineault, N. & Boyer, L. Cellular-based therapies to prevent or reduce thrombocytopenia. *Transfusion* (2011) doi:10.1111/j.1537-2995.2011.03369.x.
170. Brunstein, C. G., Setubal, D. C. & Wagner, J. E. Expanding the role of umbilical cord blood transplantation. *British Journal of Haematology* (2007) doi:10.1111/j.1365-2141.2007.06521.x.
171. Oran, B. & Shpall, E. Umbilical cord blood transplantation: a maturing technology. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program* (2012) doi:10.1182/asheducation.v2012.1.215.3798291.
172. Wagner, J. E. *et al.* One-unit versus two-unit cord-blood transplantation for hematologic cancers. *N. Engl. J. Med.* (2014) doi:10.1056/NEJMoa1405584.
173. Ngo, N., Patel, K., Isaacson, P. G. & Naresh, K. N. Leucocyte common antigen (CD45) and CD5 positivity in an 'undifferentiated' carcinoma: A potential diagnostic pitfall. *J. Clin. Pathol.* (2007) doi:10.1136/jcp.2006.044750.
174. Naeim, F., Nagesh Rao, P., Song, S. X. & Grody, W. W. Principles of

Immunophenotyping. in *Atlas of Hematopathology* (2013). doi:10.1016/b978-0-12-385183-3.00002-4.
175. Gustafson, M. P. *et al.* A method for identification and analysis of non-overlapping myeloid immunophenotypes in humans. *PLoS One* (2015) doi:10.1371/journal.pone.0121546.
176. Figueroa-Méndez, R. & Rivas-Arancibia, S. Vitamin C in health and disease: Its role in the metabolism of cells and redox state in the brain. *Frontiers in Physiology* (2015) doi:10.3389/fphys.2015.00397.
177. Chua, G. N. L. *et al.* Cytosine-Based TET Enzyme Inhibitors. *ACS Med. Chem. Lett.* (2019) doi:10.1021/acsmedchemlett.8b00474.
178. Dahlberg, A., Delaney, C. & Bernstein, I. D. Ex vivo expansion of human hematopoietic stem and progenitor cells. *Blood* (2011) doi:10.1182/blood-2011-01-283606.
179. Lang, P. *et al.* Transplantation of a combination of CD133+ and CD34+ selected progenitor cells from alternative donors. *Br. J. Haematol.* (2004) doi:10.1046/j.1365-2141.2003.04747.x.
180. Clément, M. V., Ramalingam, J., Long, L. H. & Halliwell, B. The in vitro cytotoxicity of ascorbate depends on the culture medium used to perform the assay and involves hydrogen peroxide. *Antioxidants Redox Signal.* (2001) doi:10.1089/152308601750100687.
181. Makino, Y., Sakagami, H. & Takeda, M. Induction of cell death by ascorbic acid derivatives in human renal carcinoma and glioblastoma cell lines. *Anticancer Res.* (1999).
182. Miller, P. G. & Ebert, B. L. Vitamin C regulates stem cells and cancer. *Nature* (2017) doi:10.1038/nature23548.
183. Kim, J. H. *et al.* Vitamin C Promotes Astrocyte Differentiation Through DNA Hydroxymethylation. *Stem Cells* (2018) doi:10.1002/stem.2886.
184. D'Aniello, C., Cermola, F., Patriarca, E. J. & Minchiotti, G. Vitamin C in Stem Cell Biology: Impact on Extracellular Matrix Homeostasis and Epigenetics. *Stem Cells International* (2017) doi:10.1155/2017/8936156.
185. Hu, M. *et al.* Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev.* (1997) doi:10.1101/gad.11.6.774.
186. Muller-Sieburg, C. E., Cho, R. H., Karlsson, L., Huang, J. F. & Sieburg, H. B. Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. *Blood* (2004) doi:10.1182/blood-2003-10-3448.
187. Li, Z. *et al.* Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. in *Blood* (2011). doi:10.1182/blood-2010-12-325241.
188. An, J. *et al.* Acute loss of TET function results in aggressive myeloid cancer in mice. *Nat. Commun.* (2015) doi:10.1038/ncomms10071.
189. Mingay, M. *et al.* Vitamin C-induced epigenomic remodelling in IDH1 mutant acute myeloid leukaemia. *Leukemia* (2018) doi:10.1038/leu.2017.171.
190. Von Laer, D. *et al.* Loss of CD38 antigen on CD34+ CD38+ cells during short-term culture [5]. *Leukemia* (2000) doi:10.1038/sj.leu.2401740.
191. Ko, M. *et al.* Tet2 Negatively Regulates Homeostasis and Differentiation of Hematopoietic Stem Cells in Mice. *Blood* (2011) doi:10.1182/blood.v118.21.2354.2354.
192. Pronier, E. *et al.* Inhibition of TET2-mediated conversion of 5-methylcytosine to 5-

hydroxymethylcytosine disturbs erythroid and granulomonocytic differentiation of human hematopoietic progenitors. *Blood* (2011) doi:10.1182/blood-2010-12-324707.
193. Li, Y. *et al.* Antioxidant Small Molecule Compound Chrysin Promotes the Self-Renewal of Hematopoietic Stem Cells. *Front. Pharmacol.* (2020) doi:10.3389/fphar.2020.00399.
194. Akashi, K., Traver, D., Miyamoto, T. & Weissman, I. L. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* (2000) doi:10.1038/35004599.
195. Debili, N. *et al.* Characterization of a bipotent erythro-megakaryocytic progenitor in human bone marrow. *Blood* (1996) doi:10.1182/blood.v88.4.1284.bloodjournal8841284.
196. Imond, H., Boyer, L., Roy, D. C. & Pineault, N. Cotransplantation of ex vivo expanded progenitors with nonexpanded cord blood cells improves platelet recovery. *Stem Cells Dev.* (2012) doi:10.1089/scd.2012.0142.
197. Barker, J. N., Scaradavou, A. & Stevens, C. E. Combined effect of total nucleated cell dose and HLA match on transplantation outcome in 1061 cord blood recipients with hematologic malignancies. *Blood* (2010) doi:10.1182/blood-2009-07-231068.
198. Sanjuan-Pla, A. *et al.* Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature* (2013) doi:10.1038/nature12495.
199. Yamamoto, R. *et al.* Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell* (2013) doi:10.1016/j.cell.2013.08.007.
200. Shin, J. Y., Hu, W., Naramura, M. & Park, C. Y. High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. *J. Exp. Med.* (2014) doi:10.1084/jem.20131128.
201. Grinenko, T. *et al.* Clonal expansion capacity defines two consecutive developmental stages of long-term hematopoietic stem cells. *J. Exp. Med.* (2014) doi:10.1084/jem.20131115.
202. Carrelha, J. *et al.* Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. *Nature* (2018) doi:10.1038/nature25455.
203. Rodriguez-Fraticelli, A. E. *et al.* Clonal analysis of lineage fate in native haematopoiesis. *Nature* (2018) doi:10.1038/nature25168.
204. Cabezas-Wallscheid, N. *et al.* Identification of regulatory networks in HSCs and their immediate progeny via integrated proteome, transcriptome, and DNA methylome analysis. *Cell Stem Cell* (2014) doi:10.1016/j.stem.2014.07.005.
205. Pietras, E. M. *et al.* Functionally Distinct Subsets of Lineage-Biased Multipotent Progenitors Control Blood Production in Normal and Regenerative Conditions. *Cell Stem Cell* (2015) doi:10.1016/j.stem.2015.05.003.
206. Blaschke, K. *et al.* Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. *Nature* (2013) doi:10.1038/nature12362.
207. Hore, T. A. *et al.* Retinol and ascorbate drive erasure of Epigenetic memory and enhance reprogramming to naïve pluripotency by complementary mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* (2016) doi:10.1073/pnas.1608679113.
208. Takahashi, T. *et al.* Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. *Circulation* (2003) doi:10.1161/01.CIR.0000064899.53876.A3.
209. Wilson, N. K. *et al.* Combined Single-Cell Functional and Gene Expression Analysis Resolves Heterogeneity within Stem Cell Populations. *Cell Stem Cell* (2015) doi:10.1016/j.stem.2015.04.004.
210. Woolthuis, C. M. & Park, C. Y. Hematopoietic stem/progenitor cell commitment to the

megakaryocyte lineage. *Blood* (2016) doi:10.1182/blood-2015-07-607945.
211. Macallan, D. C. *et al.* B-cell kinetics in humans: Rapid turnover of peripheral blood memory cells. *Blood* (2005) doi:10.1182/blood-2004-09-3740.
212. Lahoz-Beneytez, J. *et al.* Human neutrophil kinetics: Modeling of stable isotope labeling data supports short blood neutrophil half-lives. *Blood* (2016) doi:10.1182/blood-2016-03-700336.
213. Tak, T. *et al.* Circulatory and maturation kinetics of human monocyte subsets in vivo. *Blood* (2017) doi:10.1182/blood-2017-03-771261.
214. Yu, V. W. C. *et al.* Epigenetic Memory Underlies Cell-Autonomous Heterogeneous Behavior of Hematopoietic Stem Cells. *Cell* (2017) doi:10.1016/j.cell.2017.02.010.
215. Radtke, S. *et al.* Human multipotent hematopoietic progenitor cell expansion is neither supported in endothelial and endothelial/mesenchymal co-cultures nor in NSG mice. *Sci. Rep.* (2019) doi:10.1038/s41598-019-49221-x.
216. Wouters, B. J. *et al.* Distinct gene expression profiles of acute myeloid/T-lymphoid leukemia with silenced CEBPA and mutations in NOTCH1. *Blood* (2007) doi:10.1182/blood-2007-02-073486.
217. Knowles, H. J., Raval, R. R., Harris, A. L. & Ratcliffe, P. J. Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. *Cancer Res.* (2003).
218. Vukovic, M. *et al.* Adult hematopoietic stem cells lacking Hif-1α self-renew normally. *Blood* (2016) doi:10.1182/blood-2015-10-677138.
220. Ugarte, Fernando, and E Camilla Forsberg. "Haematopoietic Stem Cell Niches: New Insights Inspire New Questions." *EMBO Journal* 32.19 (2013): 2535–2547. Web.
221. Elisa Laurenti, and Berthold Göttgens. "From Haematopoietic Stem Cells to Complex Differentiation Landscapes." Nature 553.7689 (2018): 418–426. Web.
222. Görgens, A. et al. Revision of the human hematopoietic tree: granulocyte subtypes derive from distinct hematopoietic lineages. Cell Reports 3, 1539–1552 (2013).