ENABLING THE NEXT GENERATION OF HUMAN INDUCED PLURIPOTENT STEM CELL DERIVED HEMATOPOIETIC STEM CELL-BASED THERAPIES

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Abstract

Human induced pluripotent stem cells (iPSCs) represent a scalable cell source for the generation of hematopoietic progenitor cells (iHPCs); however, a lack of efficient iHPC expansion *in vitro* currently limits translational applications. To address this translational bottleneck, we assessed a panel of stem cell agonist cocktails (SCACs), originally developed to enhance cord-blood derived HSPC (CB-HSPC) expansion, on iHPC expansion. Three SCACs and GAS6 (X2A, X2A+GAS6, SM6, or SMA) were supplemented during iHPC differentiation and subsequent expansion using the STEMdiff™ Hematopoietic Kit. This monolayer differentiation strategy yielded a population of $CD34^+CD43^+$ and $CD45^+CD34^+$ iHPC. SCAC supplementation during iHPC differentiation yielded up to 2.5-fold higher frequency of $CD34^+CD43^+$ hematopoietic progenitors and up to 2.9-fold higher frequency of $CD45^+CD34^+CD45RA^-CD90^+$ HSC-like cells compared to non-treated controls. Subsequent SCAC supplementation during 2 weeks of expansion culture also significantly increased iHPC expansion (X2A+GAS6: 3.8-fold, X2A: 3.5-fold, SM6: 2.8-fold, SMA: 2.0-fold). The expanded iHPCs retained high levels of $CD34^+CD43^+$ expression but we observed an increase in the expansion of HSC-like cell fraction. The collective expansion observed with the SCACs was 1.5- to 2.8-fold higher than UM171 treatment alone. Furthermore, all SCAC-supplemented iHPCs retained multilineage potency, producing erythroid and granulocyte-macrophage progenitors in CFU assays. However, prolonged expansion, beyond 7 days, reduced multilineage potential, indicating a limited expansion window. Although optimal timing and composition of SCAC supplementation remains to be refined, these results highlight that exploiting the additive and synergistic effects of multiple small molecules represents a promising approach for enhancing iHPC expansion yields and biomanufacturing.

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List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AA2P	L-Ascorbic acid 2-phosphate
AGM	Aorta-gonad-mesonephros
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BME	β-mercaptoethanol
BMP4	Bone morphogenic protein 4
C/BFU-E	Erythroid colony-forming unit / burst-forming unit
CAR	Chimeric antigen receptor
CB	Cord blood
CFC	Colony forming cells
CFU	Colony-forming unit
CFU-G/M/GM	Granulocyte colony-forming unit, macrophage colony-forming unit, granulocyte-macrophage colony-forming unit
CFU-GEMM	Granulocyte-erythrocyte-monocyte-macrophage colony-forming unit
DNAm	DNA methylation
EB	Embryoid body
EP	Erythroid progenitors
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FGFs	Fibroblast growth factors
GAS6	Growth arrest-specific 6
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host disease
HAF	Human amniotic fluid
HDAC	Histone deacetylases
HE	Hemogenic endothelium
HLA	Human leukocyte antigen
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem progenitor cell
IGFBPs	IGF binding protein
IGFs	Insulin-like growth factors
iHPC	Induced hematopoietic progenitor cell
IL-3	Interleukin-3
IL-6	Interleukin-6

IMDM	Iscove's Modified Dulbecco's Medium
iMEP	Induced myeolo-erythroid progenitor cell
iMPP	Induced multipotent progenitor cell
iPSC	Human induced pluripotent stem cell
KO SR	Knockout serum replacement
LiCl	Lithium chloride
LSD1	Lysine-specific demethylase 1A
LT-HSC	Long-term HSC
MEP	Megakaryocytic-erythroid progenitor
MGG	May-Grunwald Giemsa
Mk	Megakaryocyte
mPB	Mobilized peripheral blood
MPP	Multipotent progenitor
MSC	Mesenchymal stromal cell
My	Myeloid
NK	Natural killer
NSG	NOD SCID gamma
PBS	Phosphate-buffered saline
PBSC	Peripheral blood stem cell
pen-strep	Penicillin-streptomycin
ROS	Reactive oxygen species
SCA	Stem cell agonist
SCAC	Stem cell agonist cocktail
SCAC Diff+Exp	SCAC-supplemented iHPCs during differentiation and expansion
SCAC Exp	SCAC-supplemented iHPCs during expansion only
SCF	Stem cell factor
SEM	Standard error of the mean
SR1	Stemregenin1
ST-HSC	Short-term HSC
TET	Ten-eleven translocation
TNC	Total nucleated cells
TPO	Thrombopoietin
VEGF	Vascular endothelial growth factor
Vit-C	Vitamin C
VPA	Valproic acid

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Chapter 1 Introduction

1. 1 HSC development

Hematopoiesis refers to the process of blood cell formation during both embryonic and adult stages of an organism. It involves the development, self-renewal, and differentiation of hematopoietic stem cells (HSCs), which serve as the origin for all blood cell lineages [1–4].

During mammalian embryogenesis, there are three defined waves of hematopoiesis, each with distinct timing, anatomical origins and lineage potentials [5–7]. The first wave, known as the "primitive" hematopoiesis, takes place in the yolk sac blood islands and mainly gives rise to large nucleated CD235a^{+} erythroid progenitor cells expressing embryonic globins, as well as some macrophage and megakaryocyte progenitors [8–12].The second wave, termed “definitive” hematopoiesis, is characterized by the emergence of HSCs within the aorta-gonad-mesonephros (AGM) [13,14]. These HSCs undergo specification and budding from the generation of lymphoid progenitor cells and erythro-myeloid progenitors that produce predominantly adult-type γ- and β-globin expressing red cells. The third wave, also hemogenic endothelium (HE), a specialized subset of vascular endothelium, through a process called endothelial-to-hematopoietic transition [15–17]. The formation of HE with definitive hematopoietic potential is crucial to produce engraftable HSCs from human induced pluripotent stem cells (iPSCs) in *ex vivo* settings.

The hierarchical organization of hematopoiesis has been extensively studied, leading to various models that describe the process. According to the classical and balanced hematopoietic hierarchical model (as illustrated in Figure 1.1), HSCs occupy the top position and give rise to all

immune cell types. HSCs can be categorized into self-renewing cell types, including long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs), which have an extended lifespan and are rare within the hierarchy[18]. LT-HSCs differentiate into ST-HSCs, and subsequently, ST-HSCs differentiate into multipotent progenitors (MPPs), which lack self-renewal capacity [19]. Further down the hierarchy, oligopotent and unipotent progenitors exist with shorter lifespans but higher expansion potential [20].These progenitors can differentiate into over 10 functional blood cell types [20].Oligopotent progenitors consist of common myeloid progenitors with myeloid, erythroid, and megakaryocytic potential, as well as common lymphoid progenitors with lymphoid potential only [21,22]. Common myeloid progenitors further differentiate into bipotent granulocyte-macrophage progenitors and megakaryocytic-erythroid progenitors (MEPs). Common lymphoid progenitors give rise to T cells, B cells, natural killer (NK) cells, and dendritic cells, while granulocyte-macrophage progenitors differentiate into granulocytes/monocytes and MEPs generate megakaryocytes/erythrocytes. This hierarchical model is maintained through the precise regulation of key transcription factors and cytokines that orchestrate the stepwise differentiation of HSCs into mature blood cells [23–27].

Previous studies have proposed models suggesting that individual HSCs gradually acquire lineage bias as they progress through hierarchically and distinct organized progenitor populations [18]. However, recent advancements in single-cell RNA sequencing have challenged the traditional hierarchical models of hematopoietic differentiation [28]. Studies on human bone marrow (BM) hematopoietic stem progenitor cells (HSPCs) and cord blood (CB) lympho-myeloid progenitor cells suggest that lineage-specific fate acquisition is a continuous process. Uni-lineage restricted cells emerge directly from a continuum of undifferentiated HSPCs, without a major transition

through multi- and bipotent stages [28–30]. This new perspective emphasizes the continuous nature of hematopoietic differentiation.

To ensure hematopoietic homeostasis throughout the lifespan, the balance between self-renewal and differentiation of HSPCs needs to be tightly regulated. HSPC activity is regulated by both cell-intrinsic factors, including transcriptional, epigenetic, and metabolic regulators, as well as cell-extrinsic factors, which include long-range humoral and neural signals and local cues from the stem cell niche. The stem cell niche, or the BM microenvironment, is a complex network of cells that provides molecular cues and physical interactions to regulate HSPC activity[3,31]. Understanding methods to harness HSPC self-renewal and differentiation can be useful for many downstream research and clinical applications.

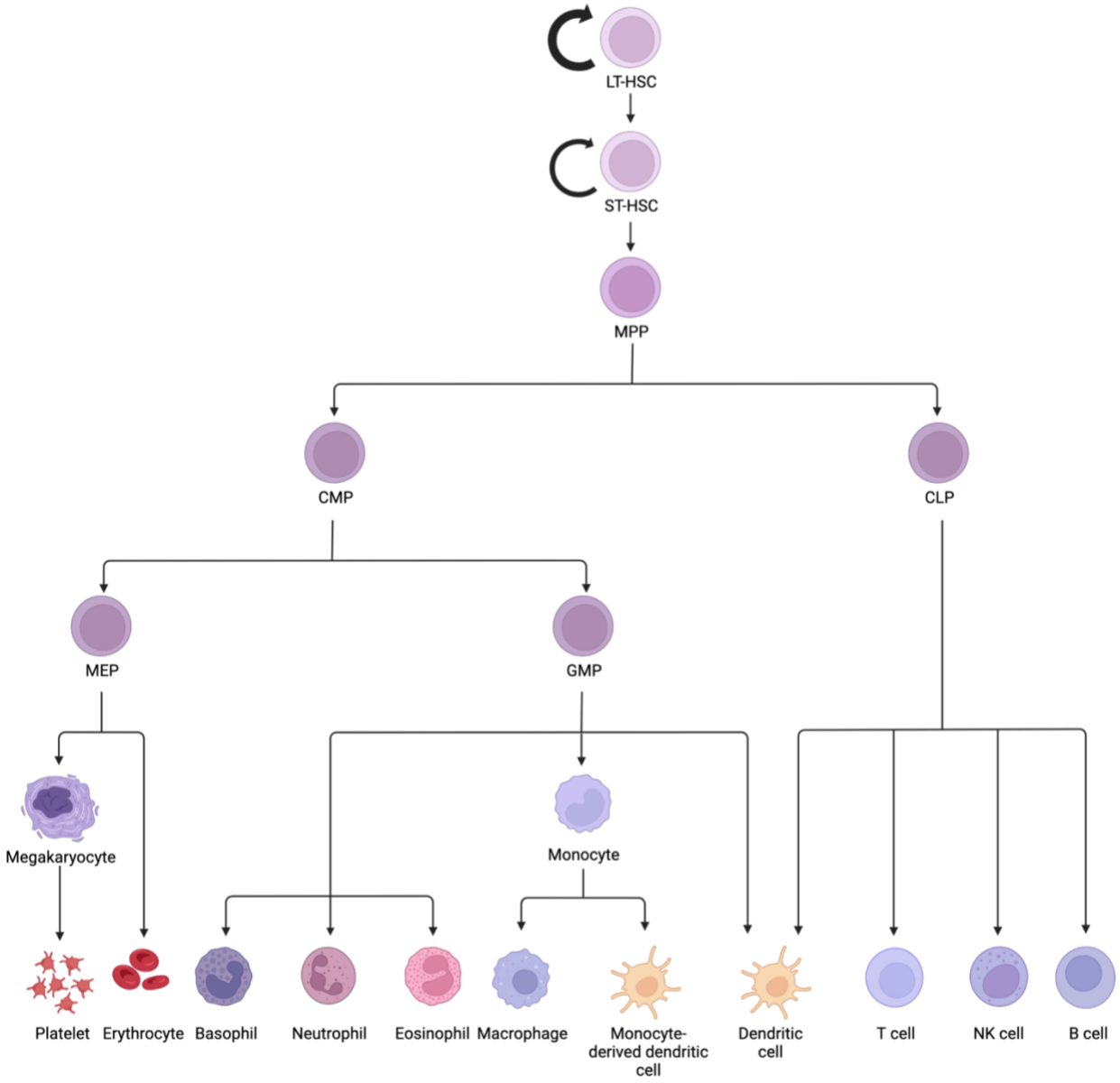


Figure 1.1. Classical hematopoietic hierarchical model.
Image generated by Biorender and adapted from schematic by Cheng *et al.*, 2020 [18].

1. 2 Sources and clinical applications of HSPCs

Hereditary blood disorders, such as sickle cell disease and thalassemia [32], and hematological malignancies [33], present challenges in terms of treatment and patient management [3,34–37]. Patients

often rely on frequent blood transfusions or BM transplants, which require compatible donors [32,38]. Allogeneic HSC transplantation is the only curative treatment for most hereditary blood disorders, but the limited availability of human leukocyte antigen (HLA)-matched donors and associated complications restrict its use [39,40]. In gene therapy applications, obtaining sufficient autologous HSPCs for genetic modification is often not feasible, and safe methods for expanding these rare cells are lacking [41].

Different sources of HSPCs for allogeneic transplantation have been explored, including BM, adult mobilized peripheral blood (mPB), and CB, each with its own advantages and disadvantages. BM is a traditional source of HSPCs, being the first to offer a curative option to certain hematological diseases [42]. However, obtaining BM requires invasive procedures and contains a relatively low number of HSPCs compared to mPB, which may limit the availability of donor cells for transplantation [43–45]. Similar to mPB, the incidence of acute graft-versus-host disease (GVHD) has been reported with BM transplantation [43,46–53]. mPB is an attractive source due to its relative ease of collection through mobilization of HSPCs from the BM into the peripheral blood using growth factors. mPB, compared to BM results in more rapid engraftment and may confer a survival benefit in advanced disease [43,46–48,50,52–56]. However, the limited number of HLA-matched donors and the risk of GVHD are significant drawbacks associated with mPB transplantation [47,48,53,55]. CB offers advantages such as non-invasive collection, potent anti-tumour activity [57], rapid availability, low risk of virus transmission, lower incidence of GVHD, and greater flexibility in HLA matching [58]. However, limitations include limited cell dose, associated with delayed neutrophil engraftment, graft failure, high incidence of infections, transplant-related mortality, and high costs [58–64]. Overall, traditional sources of HSPCs from

adult mPB, BM and CB have limitations in accessibility, scalability and donor-to-donor variability[65].

1. 3 Induced pluripotent stem cells (iPSC) and induced hematopoietic progenitor cells (iHPC)

More recently, iPSCs are being explored as a novel renewable and scalable cell source to generate patient-specific HSPCs and blood cell products[66–68]. iPSCs are a type of pluripotent stem cell derived from adult somatic cells that have been genetically reprogrammed into an embryonic stem cell (ESC)-like state through the ectopic expression of key pluripotency transcription factors, termed Yamanaka factors (i.e. Oct3/4, Sox2, Klf4 and c-Myc) that are important for maintaining the defining properties of ESCs [69]. Established iPSC lines have the same properties as ESCs, such as unlimited self-renewal and the capacity to differentiate into all cell types of the body (except for cells in extra-embryonic tissues such as the placenta)[70]. iPSCs also represent a renewable and scalable cell source to generate patient-specific induced hematopoietic progenitor cells (iHPC) and supply blood products, circumventing poor accessibility of CB- and BM-derived HSPC. Furthermore, iPSCs can be genetically modified to correct disease-causing mutations or enhance therapeutic properties, making them highly amenable to genome engineering to enable downstream applications such as autologous, patient-specific transplantation, gene therapies, and cell-based immunotherapies [71,72]. These unique features position iPSCs as a versatile tool for regenerative medicine and hold great promise for the treatment of blood malignancies and other hematological disorders.

Protocols aiming to generate iHPCs from iPSCs were developed based on knowledge of embryonic development [73]. Hematopoiesis takes place in distinct waves, known as primitive and definitive hematopoiesis. Primitive hematopoiesis occurs in the yolk sac and placenta, while definitive hematopoiesis originates in the AGM region [12]. In both cases, the formation of mesoderm and the induction of HE play critical roles in subsequent hematopoietic specification [15–17]. The differentiation of iPSCs to iHPCs is guided by the goal of replicating this complex process *in vitro.*

iHPCs differentiated from iPSCs have been developed using two well-established protocols: directed monolayer and spontaneous embryoid body (EB) based differentiation strategies [74,75]. The monolayer differentiation process yields two distinct cell populations: suspension and adherent cells. The suspension cells consist mainly of iHPCs and the adherent cells are mainly composed of endothelial and mesenchymal stromal cell (MSC) populations, as previously described [76]. In brief, the differentiation strategies aim to recapitulate HSPC development *in vivo* and are divided into key developmental stages in which specific signals are provided for mesodermal, hemangioblast/hemogenic-endothelium and hematopoietic stem/progenitor cell specification and maturation (Figure 1.2)[77]. It has been found that iHPCs demonstrate similar but distinct epigenetic profiles compared to CB-derived $CD34^+$ cells[78]. However, studies comparing iHPCs to CB-HSPCs have shown that both HSPC sources share similar immunophenotypes [76,79]. A summary of differentiation markers used to delineate lineages in iHPC differentiation protocols (e.g. HE, induced multipotent progenitor cell [iMPP], iHPC) can be discriminated using published markers summarized in
Table 1.1 [76,80–84].

Robust differentiation protocols have also been developed for iHPC to generate almost all the various lineage specific blood cells such as red blood cells, megakaryocytes, myeloid and lymphoid cells[77]. However, it has become evident that human iPSC-derived cells do not completely recapitulate their *in vivo* counterparts [67,85]. In contrast to CB- and BM-derived cells, challenges remain in generating long-term repopulating HSCs [83,86], as well as mature red blood cells, mature monocyte/macrophage lineage cells, and functional T cells [87–91]. To enhance the self-renewal and multilineage potential of iHPCs, forced expression of hematopoietic transcription factors has been employed [92,93]. One study reported the production of iHSCs with long-term engraftment though expression of the onco-fusion gene MLLAF4 [79]. These findings represent significant advances over the initial finding of iHSCs based on the *in vivo* differentiation approach via iPSC-teratoma formation [94,95]. However, these protocols have major translational limitations as they exhibit low efficiency, poor scalability, and rely on integrating lentiviral vectors to maintain the expression of potentially oncogenic transcription factors. The production of functional platelets at a therapeutic scale also presents significant obstacles [96]. These observations support the notion that iPSC differentiation primarily recapitulates embryonic hematopoiesis rather than adult hematopoiesis [97]. Despite these challenges of incomplete assimilation, iPSCs-derived HSPCs still possess robust differentiation potential.

Similar, to CB and BM-derived HSPC, a major bottleneck hindering translation of this promising strategy is the efficient production yields of iHPC cell populations derived from iPSCs. Although optimization towards HPC differentiation have been well optimized for iPSCs [98], the biggest challenge remains the generation of functional HSPCs *in vitro* that are expandable [76,79,99,100]. This

is a critical step towards further optimizing the efficiency and yields of desired iHPCs and iMPPs for various downstream hematological applications, such as chimeric antigen receptor (CAR) based immunotherapies. Towards the latter, iPSC-CAR and HPC-CAR progenitors represent a novel, scalable and renewal source of iPSC-derived immunotherapy cell products such as CAR-T cells and CAR-NK cells [66,101,102] as well as a source of HPCs to supply growing demands for cell transplantations. As such, there is a great deal of interest in validating whether *ex vivo* expansion strategies developed for CB- and BM-derived HSPC, could also improve iHPC expansion.

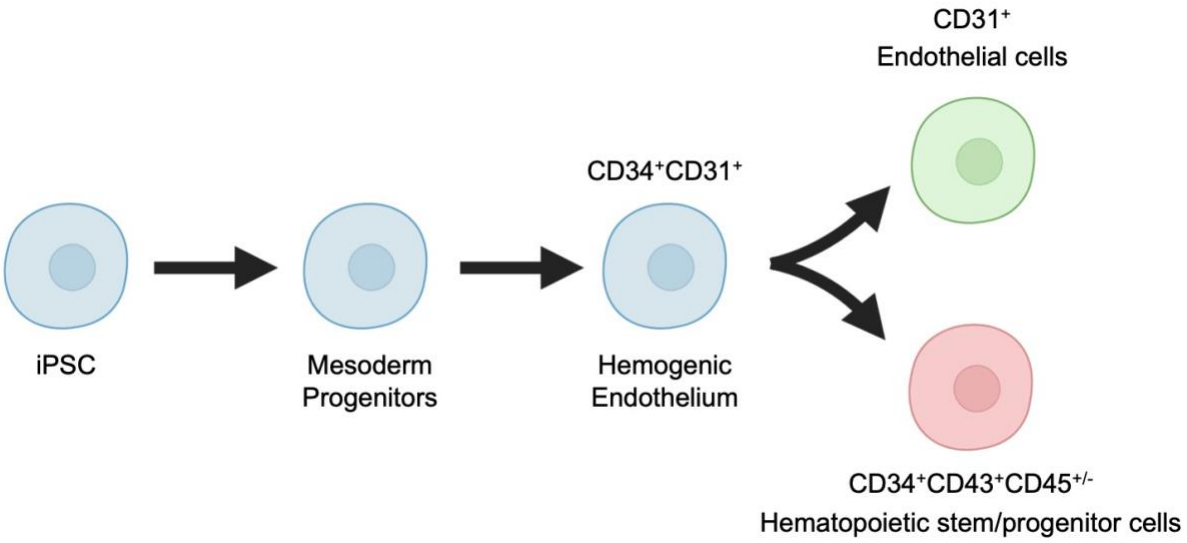


Figure 1.2. iHPC differentiation from iPSC progresses through distinct stages.

Table 1.1. Markers used to track iPSC-derived sub-populations by flow cytometry analysis. iHPC, induced hematopoietic progenitor cell; iMEP, induced myeolo-erythroid progenitor cell; iMPP, induced multipotent progenitor cell; MSC, mesenchymal stromal cell.

Cell population	Markers	References
Mesoderm	Brachyury $(T)^+$	Choi et al., 2012[103]
Hemangioblast	$APLNR^+PDGFR\alpha^+$	Choi et al., 2012[103]

Hemogenic Endothelium	VE-CAD^{+}, CD34^{+}, CD31^{+}, CD235a^{-}, CD43^{-},	Choi et al., 2012[103],
Endothelial	CD73^{-}	Uenishi et al., 2014[81],
	CD31, FLK1, CD34, CD201, ESAM, CD146	Ditadi et al., 2015[82]
iMPP	Lin^{-}CD43^{+}CD34^{+}CD45$^{+/-}$	Mesquitta et al., 2019[80],
iHPC	CD34^{+}CD43^{+}CD45^{+}	Uenishi et al., 2014[81],
iMEP	CD43^{+}CD45$^{+/-}$CD235^{+}CD41^{+}	Ruiz et al., 2019[76]
MSC	CD43^{-}CD45^{-}CD90^{+}CD73^{+}CD105^{+}	Ruiz et al., 2019[76]

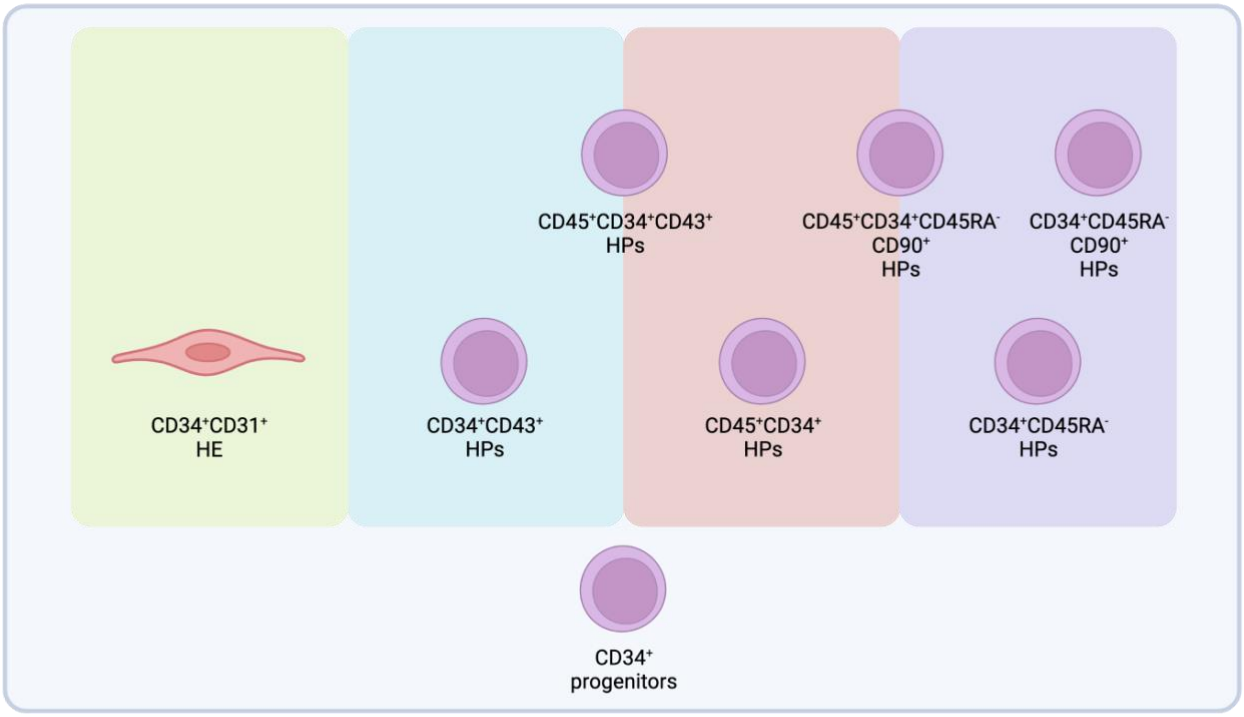


Figure 1.3. Venn diagram of hematopoietic stem cell-like, hematopoietic progenitor-like and hemogenic endothelial-like cell surface markers.
HE, hemogenic endothelium; HP, hematopoietic progenitors

1. 4 Strategies for HSPC expansion

Historically, CB-HSPC expansion has been bolstered by manipulating developmental pathways, coculture procedures, cytokine cocktails, and small molecules [104,105]. The major challenge in *ex vivo* expansion of HSPCs is achieving a balance between self-renewal and differentiation. *In vivo*, this balance is tightly regulated by extracellular molecules and complex intracellular signaling pathways, such as Wnt and Notch signaling [106–108]. Genetic manipulation of HSCs, such as the HOXB4 gene, has shown significant increases in self-renewal capacity [109]. Excessive self-renewal or insufficient differentiation increases the risk of leukemic transformation, while inadequate self-renewal or accelerated differentiation can exhaust the HSC population [41]. To address this, transient and removable activation using extrinsic agents is desired for *ex vivo* HSPC expansion.

Coculture systems utilizing stromal cells have also shown promise in expanding HSPCs. These systems enhance HSPC proliferation [110–112], maintain HSPC phenotype [111,113,114], and improve engraftment potential [110,111,115–120]. The interaction between HSPCs and stromal cells involves both contact-dependent and -independent activities, regulated by a diverse array of cytokines and chemokines secreted by stromal cells [113–115,121–126]. Coculture systems have demonstrated efficacy in promoting HSPC expansion from CB mononuclear cells, and engineering stromal cells to express specific factors can further enhance HSPC expansion and long-term repopulating capacity [127–129]. Further advancements are required to enhance the effectiveness and safety of co-culture conditions. This includes establishing well-defined protocols for expanding stromal cells,

such as determining the appropriate culture media, number of cell passages, and addressing the risk of pathogen transmission [130].

Attempts at HSPC expansion involved the use of cytokines or growth factors and their combinations. The combination of stem cell factor (SCF), thrombopoietin (TPO), FMS-like tyrosine kinase 3 ligand (FLT3L), and interleukin-6 (IL-6) is a widely used cytokine cocktail for HSC expansion cultures. Furthermore, additional developmental growth factors such as fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), IGF binding protein (IGFBPs), angiopoietin-like proteins, sonic hedgehog proteins, bone morphogenic protein 4 (BMP4), Wnt proteins, and Notch ligands have been identified for HSC expansion [41]. While these approaches supported considerable HSPC expansion while maintaining long-term repopulating capacity, challenges with cytokine-driven culture include optimizing conditions for stemness preservation, high cost and variable potency of cytokines, cytotoxicity from non-physiological concentrations, and inevitable differentiation occurred after transient expansion [41,130]. This highlights the need for additional agents.

Small molecules have emerged as promising candidates for HSC expansion due to their specific targets, ease of manipulation *in vitro*, and cost-effectiveness, rendering them suitable candidates for clinical use. The use of small molecule stem cell agonists (SCAs) has been particularly successful for the expansion of CB-derived, adult-derived HSPCs and embryonic/induced pluripotent stem cells. Key SCAs include StemRegenin1 (SR1), UM171, valproic acid (VPA), and L-Ascorbic acid 2-phosphate (AA2P), among others[104].

1. 5 Single stem cell agonists - StemRegenin 1 (SR1)

SR1 is an antagonist of the aryl hydrocarbon receptor, a ligand-activated transcription factor involved in pathways regulating hematopoiesis [131,132]. SR1 has been shown to increase expansion of CB-derived $CD34^+$ cells by 50-fold and to induce a 17-fold increase in cells able to engraft immunodeficient mice [131]. Notably, SR1 appears to have less durable self-renewal activity compared to UM171. SR1 may also induce lineage-bias of BM-derived and mPB-derived $CD34^+$ HSPCs towards conventional and plasmacytoid dendritic cell and NK cell differentiation [133].

1. 6 Single stem cell agonists - UM171

UM171 is a pyrimidoindole derivative that acts independent of the Ahr pathway and has been reported to target multiple cellular processes involved in HSPC expansion, including self-renewal, differentiation, cell metabolism, cell cycle regulation, and epigenetic regulation[134]. UM171 upregulates the expression of HSPC-specific genes and self-renewal pathways, such as the non-canonical Wnt signaling pathway, while simultaneously suppressing erythroid and megakaryocytic differentiation signals that would otherwise drive HSPC towards mature blood cell lineages[135,136]. The enhancement of HSPC expansion by UM171 may also be associated with its ability to regulate glucose metabolism and reactive oxygen species (ROS) levels[136]. Moreover, UM171 selectively increases the number of proliferating HSPC while decreasing apoptosis[137]. UM171 is also involved in epigenetic regulation as it facilitates the *ex vivo* expansion of HSPC by activating the CULLIN3KBTBD4 ubiquitin ligase, which selectively targets the lysine-specific demethylase 1A (LSD1)-CoREST complex responsible for chromatin

acetylation regulation. This leads to the poly-ubiquitination and subsequent degradation of the LSD1-CoREST complex [138–140]. Overall, UM171 exerts its effects on HSPC expansion through multiple mechanisms, including upregulation of non-canonical Wnt signaling, suppression of erythroid and megakaryocytic differentiation signaling, regulation of glucose metabolism and ROS levels, and the proteasomal degradation of the LSD1-CoREST complex.

In terms of cell expansion effects, UM171 has been shown to stimulate 10-fold *ex vivo* expansion of short- and long-term *in vivo* repopulating HSPCs [134]. Of interest, UM171 has demonstrated additive effects on HSPC expansion when used in combination with SR1 (unpublished data from StemCell Technologies). A clinical trial by Cohen *et al*. has shown that UM171-mediated CB-derived stem cell expansion is feasible, safe, and engraftable using small single cords. UM171-expanded CB may overcome severe acute GVHD and treatment-related mortality, while maintaining benefits of low incidence of chronic GVHD and relapse, making UM171 a highly valuable and clinically relevant candidate for HSPC expansion[141].

1. 7 Single stem cell agonists - Valproic acid (VPA)

VPA is an inhibitor of histone deacetylases (HDAC) and in the family of chromatin modifying agents[142]. HDAC are enzymes that regulate gene expression by removing acetyl groups from histone proteins, leading to a condensed chromatin structure that inhibits transcription. By inhibiting HDAC activity, VPA promotes an open chromatin structure that allows for enhanced HSPC expansion and self-renewal by forcing expression of pluripotency genes, such as Oct4, Nanog, and Sox2[142–144]. VPA has been found to induce HSPC proliferation and self-renewal

while delaying the cell cycle, without inducing apoptosis and maintaining short-term and long-term HSCs [145,146]. Studies have shown that VPA treatment not only expands HSCs but also leads to phenotypic transformation and cellular reprogramming of HSCs[142,147–149]. Furthermore, VPA promotes HSC multi-lineage reconstitution in serial transplantation in NOD scid gamma (NSG) mice and enhances the expression of HOXB4 and AC133, as well as histone acetylation on their regulatory sites[145,146,149–151].

In cells isolated from adult mPB, VPA has be found to induce an 807-fold increase in numbers of $CD34^+CD45RA^-CD90^+$ cells and a 3000-fold increase in numbers of $CD34^+CD45RA^-CD90^+CD49f^+$ cells. VPA-mediated expansion of adult BM-derived cells resulted in a 138-fold increase in numbers of $CD34^+CD45RA^-CD90^+$ cells and a 1507-fold increase in $CD34^+CD45RA^-CD90^+CD49f^+$ HSPC cells[142]. Of note, there has been conflicting data on whether VPA induces lineage bias in $CD34^+$ cells. A study by Zini *et al.* found that VPA has the capacity to enhance erythrocyte and megakaryocyte differentiation[152]. While another study by Chateauvieux *et al*., claimed that VPA interferes with hematopoietic lineage commitment by inhibiting erythroid differentiation and activating the myelo-monocytic pathway[153]. Finally, a study by Zimran *et al*., found that VPA-treated cultures produced cultures with the capacity to generate progenitors of each hematopoietic lineage in similar proportion to unmanipulated $CD34^+$ cells[142]. Although the expansive effects of VPA on HSPCs were well-established, the impact of VPA on HSPC differentiation remains inconclusive.

1. 8 Single stem cell agonists - L-Ascorbic acid 2-phosphate (AA2P)

AA2P is a vitamin C (Vit-C) analog used to bolster HSPC expansion. Previous studies have found that Vit-C is an epigenetic regulator that enhances the activity of epigenetic erasers such as Jumonji-C domain-containing histone demethylase and ten-eleven translocation (TET) proteins, which are also known as α-ketoglutarate dependent dioxygenases[154]. Vit-C was found to be essential for the generation of HSPCs during later stages of differentiation and the specification of functional hematopoietic endothelial cells [154,155]. Vit-C-dependent epigenetic regulation, particularly through lysine demethylase 6, played a critical role in establishing an epigenetically competent state in endothelial cells that supports endothelial-to-hematopoietic transition during early hematopoiesis[155]. In studies conducted by the Pineault lab (Canadian Blood Services), AA2P-supplemented CB-derived stem and progenitor subsets expanded by 2-fold and AA2P-expanded cells were capable of increased engraftment in immunodeficient mice compared to controls[156].

1. 9 Stem cell agonist cocktails (SCAC)

Historically, HSPC expansion was induced by addition of individual SCAs [131,134,142]. More recently, small molecules have been used in combination to optimize their capacity to induce HSPC expansion *ex vivo*. A cocktail consisting of three small molecules (SR1, VPA, and BML-210, an HDAC inhibitor) and four cytokines (TPO, SCF, FLT3L, and IL-6) was reported to induce the expansion of $CD34^+$ cells and $CD34^+CD38^-$ cells to 28.0-fold and 27.9-fold, respectively. Stem cell cocktail-expanded $CD34^+$ cells demonstrated sustained multilineage differentiation potential in primary and secondary murine *in vivo* murine xenotransplantation

experiments[157]. Similarly, culture with a cocktail consisting of CHIR-99021, forskolin and OAC1 enhanced the expansion of $CD34^+$ cells by 8.1-fold compared with controls, while maintaining multilineage differentiation potential *in vitro*[158]. In another study, CB-$CD34^+$ cells were expanded with a cocktail consisting of cytokines and seven small molecules (SB, PD, Chir, Bpv, Pur, Pμ, and NAM). Supplementation of CB-$CD34^+$ cell cultures with SB431542 (a TGF-β inhibitor), Chir9901 (a GSK3 inhibitor), and Bpv (a PTEN inhibitor) resulted in a 50-fold increase in $CD34^+CD38^-$ cells. Furthermore, these expanded cells exhibited a 1.5-fold higher engraftment potential in the peripheral blood of the NMRI murine model of *in utero* transplantation[159].

Recently, the Pineault lab has used statistical design to formulate stem cell agonist cocktails (SCAC, as detailed in

Table 1.2) which leverage synergistic and additive effects of a combination of SCAs as a novel strategy to increase expansion of HSPCs [156]. The cocktails X2A, SMA, and SM6 were formulated using various concentrations of the same molecules, SR1, UM171, VPA, and AA2P [156,160–162]. The variation in concentration of small molecules resulted in diverse outcomes; however, it was evident that certain effects were redundant, particularly in terms of expanding distinct HSPC subsets [156,160–162]. Two lead cocktails, X2A and SM6, demonstrated potent growth promoting activities and demonstrated high levels of engraftment in NSG immunodeficient mice [156,160–162]. Importantly, X2A enhanced expansion of long-term scid-repopulating cells (SRC) by 15-fold over input level, significantly surpassing that obtained by the C6 combination of SR1 and UM171 [161]. However, the effects of SCAC expansion on iHPCs and iMPPs is unknown. In this project, I aim to test SCAC and novel formulations to improve iHPC differentiation and

advance personalized iPSC-derived blood therapy products as novel treatment modalities for patients afflicted with hematological diseases.

Table 1.2. SCACs formulated using different concentrations of SCAs.
AA2P, L-Ascorbic acid 2-phosphate; SR1, StemRegenin 1; VPA, valproic acid.

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

1. 10 Growth arrest-specific 6 (GAS6)

Growth arrest-specific 6 (GAS6) promotes the expansion of HSPCs through the activation of the TAM receptor tyrosine kinases (TYRO3, AXL, and MERTK), which are expressed on the surface of HSPCs [163]. The TAM receptor signaling pathway plays a critical role in regulating HSPC survival, self-renewal, and differentiation. Jin *et al.* found that GAS6/AXL signaling was found to regulate the self-renewal of chronic myeloid leukemia stem cells [164]. Meanwhile, Dormady *et al.* found that overexpression of GAS6 in 3T3 fibroblast monolayer supports the generation of hematopoietic progenitor cell colonies[165].

Most recently, Maganti *et al*. demonstrated that AA2P-mediated upregulation of GAS6/AXL is accomplished by DNA demethylation in a TET-dependent manner, which leads to the increased expression of AXL and its ligand GAS6, with X2A showing the greatest upregulation of AXL

amongst all SCACs [160,161]. The supplementation of X2A with GAS6 increased the expansion of HSPCs without inducing differentiation (e.g., 640- vs. 1975-fold for $CD34^{+}CD45RA^{-}$ HSPC in X2A and X2A+GAS6 cultures, respectively)[160]. Transplant assays using day-14 expanded HSPCs demonstrated that supplementation of GAS6 in X2A cultures significantly increased engraftment by 10-fold in both primary and secondary NSG mice. GAS6 also resulted in a remarkable increase in the number of human colony-forming units and CD34+ BM cells, with a 7- to 15-fold increase in primary transplants and a 20- to 26-fold increase in secondary transplants[160]. This study highlights GAS6 as a potent growth factor, capable of promoting the robust expansion of human HSPCs, including the serial engraftment of HSPCs with high levels of engraftment. In this study, I wanted to assess whether the effects of X2A with the addition of GAS6 could be recapitulated in iHPCs.

1. 11 Stem cell agonist mediated expansion of iHPC

The capacity of SCAs UM171 and SR1 to improve the generation of iPSC-derived HPC is now under investigation with preliminary studies showing positive results (
Supplementary Table 1 and Supplementary Table 2). UM171 was also shown to promote the expansion of myeloid and NK cell progenitors from iPSCs [137]. In several studies, UM171-treated $CD45^{+}CD34^{+}$ iHPCs demonstrated 1.5- to 4-fold expansion[137,166,167]. Even fewer studies have been conducted on SR1-mediated expansion of iPSC-derived HPCs and progenitors. A study conducted by Angelos *et al.* found that SR-1 treated iHPCs did not demonstrate significantly different expansion of $CD34^{+}CD43^{+}$ and $CD45^{+}CD34^{+}$ subsets[168]. When used individually, UM171 and SR1 promoted expansion of myeloid progenitors; however, demonstrated weak

engraftment activity in NSG immunodeficient mice [156,160–162]. Taken together, preliminary data testing individual SCAs shows promise in increasing iHPC expansion. Despite these promising results to date, no work has been done on assessing the synergistic and additive effects of the SCAs on iHPC differentiation and expansion.

1. 12 Objectives and Hypothesis

The production of sufficient numbers of iHPCs and iMPPs from iPSCs has been a roadblock to downstream applications. I hypothesised that supplementing the current iHPC culture platform with SCACs previously shown to promote HSC and HSPC expansion should increase the yield of iHPCs produced from iPSCs. The following objectives were set in my work to test my hypothesis:

Objective 1. Optimization of iHPC differentiation from iPSC

Methods: Two distinct strategies for differentiating iPSCs to iHPCs, namely EB formation and directed monolayer, were employed in my experimental study. The resulting yield of cells was evaluated, and the hematopoietic cells generated were characterized using flow cytometry analysis.

Objective 2. Expansion of terminally differentiated iHPC

Methods: I assessed the impact of supplementing iHPCs with SCACs, as well as GAS6, during post-differentiation expansion. I analyzed the yield of cells, characterized their phenotype by

flow cytometry, and assessed the multilineage potential of the expanded iHPCs using the colony forming unit (CFU) assay.

Objective 3. Impact of SCACs on the differentiation and expansion of iHPCs

Methods: I aimed to determine if earlier SCAC supplementation during mesodermal induction and iHPC differentiation could increase iHPC yields by day 12. Cell yield, phenotype characterization via flow cytometry, and multilineage potential using the CFU assay were analyzed.

Chapter 2 Materials and Methods

2. 1 Generation and Culture of iPSCs

Human amniotic fluid (HAF) cells at 26 weeks of gestation were obtained from The Ottawa Hospital (Ottawa, Ontario, Canada) following routine amniocentesis. Informed consent was obtained from each woman to use the amniotic fluid for research purposes and all the methods were carried out in accordance with relevant guidelines and regulations as approved by the Ottawa Hospital Research Ethics Board. All experimental protocols using HAF cells were performed following the guidelines established and approved by the National Research Council Canada Research Ethics Board. In brief, HAF cells were reprogrammed into iPSCs using non-integrating episomal reprograming vectors expressing Yamanaka factors, as previously described [169]. The iPSCs were maintained on 6-well tissue culture plates (Corning, 353046) coated with Matrigel® (Corning, 354277) in mTeSR™1 medium (StemCell Technologies, 85850). Culture medium was changed daily and iPSCs were passaged every four to seven days at 70 to 80% confluency. In brief, mTeSR™1 medium was aspirated and ReLeSR™ (StemCell Technologies, 05872) was added to the wells and incubated at room temperature for thirty seconds before being aspirated. iPSCs were incubated in the residual ReLeSR for three minutes at room temperature. The iPSCs were subsequently flushed with mTeSR1. To create appropriately sized iPSC aggregates, iPSCs were detached from the wells using a P1000 pipette to gently flush with mTeSR™1 medium. The detached iPSC aggregates (approximately 40 and 100 µm) were plated onto new Matrigel®-coated 6-well plates containing mTeSR™1 medium and colonies were visually assessed to monitor growth until the subsequent passage.

2. 2 Embryoid body (3D) hematopoietic differentiation of human iPSC

Briefly, on D0 of differentiation, iPSCs were dissociated into single cells by adding ACCUTASE™ (StemCell Technologies, 07920) to the wells, incubating at room temperature for 5 minutes, aspirating ACCUTASE™, and incubating at 37°C for 5 minutes. Singularized iPSCs were dislodged from wells by using a P1000 pipette and DMEM/F12 with 15 mM HEPES (Gibco™, 11330032) to mix the single-cell suspension 3-5 times. Viable cells were counted via trypan blue staining, centrifuged at 300 x g for 5 minutes, and diluted to 3.6 x 10^5 cells/mL in EB Formation Medium, which consisted of STEMdiff™ Hematopoietic - EB Basal Medium (StemCell Technologies, 100-0171), STEMdiff™ Hematopoietic - EB Supplement A at 1X (StemCell Technologies, 100-0172), and Y-27632 at 10 µM (StemCell Technologies, 72302).

To pre-treat the AggreWell™400 24-well plates (StemCell Technologies, 34411), 500 µL of Anti-Adherence Rinsing Solution (StemCell Technologies, 07010) was added to each well and the plates were centrifuged at 1300 x g for 5 minutes. After centrifugation, the plates were inspected using a Leica DMi1 inverted microscope (Leica Microsystems) to ensure bubbles were removed from the wells. If bubbles remained, the plates were centrifuged again at 1300 x g for 5 minutes. Anti-Adherence Rinsing Solution was aspirated from the wells and the plates were washed using DMEM/F12 with 15 mM HEPES. 1 mL of EB Formation Medium was added to each well of the AggreWell™400 plate. The singularized iPSCs were seeded into the plate and dispersed evenly into the microwells by gently pipetting several times. To force aggregation of cells into the microwells, the plate was centrifuged at 100 x g for 3 minutes. The cells were incubated at 37˚C and 5% CO_2 overnight.

On D2 of differentiation, a half medium change was performed with EB Medium A, which consisted of STEMdiff™ Hematopoietic - EB Basal Medium and STEMdiff™ Hematopoietic - EB Supplement A. The cells were incubated at 37˚C and 5% CO_2 overnight. On D3 of differentiation, a half medium change was performed with EB Medium B, which consisted of STEMdiff™ Hematopoietic - EB Basal Medium and STEMdiff™ Hematopoietic - EB Supplement B (StemCell Technologies, 100-0173) at 1X. The cells were incubated at 37˚C and 5% CO_2 for 2 days. On D5 of differentiation, the EBs were harvested, filtered, and eluted with EB Medium B using a 40 µm reversible strainer. The eluted EBs were transferred to a 6-well non-tissue culture-treated plate (Corning, 353046) and incubated at 37˚C and 5% CO_2 for 2 days. On D7 of differentiation, EB Medium B was added to each well and the cells were incubated at 37˚C and 5% CO_2 for 3 days. On D10 of differentiation, a half medium change was performed with EB Medium B and the cells were incubated at 37˚C and 5% CO_2 for 2 days. On D12 of differentiation, the EBs were harvested and dissociated into single-cell suspension by incubating in 1 mL of Collagenase Type II at 2500 U/mL (StemCell Technologies, 07418) at 37˚C for 20 minutes, followed by addition of 3 mL of TrypLE™ Express (Thermo Fisher, 12604-013) and incubation at 37˚C for 20 minutes. The cell suspension was gently pipetted to break up clumps, suspended in 6 mL of DMEM/F-12 with 15 mM HEPES, and centrifuged 300 x g for 5 minutes. The cell pellet was resuspended in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) and 1 mM EDTA. The dissociated cells were counted and prepared for further analysis by flow cytometry.

2. 3 Monolayer (2D) hematopoietic differentiation of human iPSC

The monolayer differentiation strategy was very similar to the EB differentiation strategy, following a similar timeline and cytokine treatment regime, to induce iHPC differentiation from iPSCs. iPSCs were differentiated using a defined, xeno- and feeder-free monolayer STEMdiff™ Hematopoietic Kit (StemCell Technologies, 05310) according to manufacturer's instructions. In brief, one day prior to differentiation (D-1), iPSC aggregates were detached from cultures as described above. To collect iPSC aggregates between 40 and 100 µm, detached aggregates were filtered using 100 µm (Corning, 352360) and 40 µm (Corning, 352340) cell strainers. The appropriately-sized iPSC aggregates were plated onto Matrigel®-coated plates (24-well plates (Corning, 353047) for differentiation experiments or 12-well plates (Corning, 353503) for expansion experiments), in mTeSR™1 medium and at a density of 19-22 aggregates / cm^2. The iPSCs were incubated at 37˚C and 5% CO_2 overnight. On day 0 (D0) of differentiation, wells containing the appropriate densities of iPSC aggregates were selected for differentiation. To induce iPSC differentiation towards mesodermal progenitor cells, a full medium change to medium A was performed followed by a half medium A change on D2. On D3 of differentiation, mesodermal cells were subsequently differentiated towards hemangioblasts, multipotent precursor cells with the capacity to differentiate into hematopoietic and endothelial cells (hemogenic endothelium, HE), by performing a full medium change to medium B, followed by half medium B changes on D5, D7 and D10. The protocol yielded two distinct cell populations: suspension cells that are iHPCs and adherent cells that are mainly composed of endothelial and mesenchymal stem cell (MSC) populations, as previously described [76]. The suspension cells were harvested by gently flushing with a P1000 pipette, counted, and prepared for analysis by flow cytometry, seeded for expansion and/or CFU assays using methylcellulose-based medium.

2. 4 Expansion of iPSC-derived hematopoietic progenitor cells

On D12 of monolayer differentiation, the floating supernatant cells containing iHPCs were harvested, re-suspended in expansion media, and transferred to non-coated 24-well plates (Corning, 353047) containing 300 µL of cell suspension at a density of $5x10^4$ cells/mL. The cells were then expanded for either 7 or 14 days in Expansion media contained StemSpan™ SFEM II (StemCell Technologies, 09605) and StemSpan™ CD34+ Expansion Supplement (StemCell Technologies, 02691), in the presence or absence of a panel of SCACs: X2A (1X), SM6 (1X) or SMA (0.25X, 0.5X or 1X), X2A+ GAS6 (10 ng/mL) or UM171 (35 nM) (all kindly gifted by Nicolas Pineault) were added during medium changes from D0-D12, D5-D12 or D7-D12 of differentiation. To maintain the cell density below $1x10^6$ cells/mL, at D4 of expansion, 400 µL of expansion media and the corresponding amount of SCAC, UM171 or GAS6 was added. After 7 or 14 days of expansion, the cells were counted and prepared for further analysis by flow cytometry and CFU assays using methylcellulose-based medium.

2. 5 Flow Cytometry

On D12 differentiation and days 7 and 14 of expansion, flow cytometry was used to analyze a portion of cultured cells for iHPC progenitors. The specific antibodies and combinations used for different screens are listed in the table below (Table 2.1). Compensation beads were employed to set voltages and gating parameters, ensuring accurate fluorescence signals for all fluorophore-conjugated antibodies. Cells were stained with fluorescence-conjugated antibodies in 100 µl of FACS buffer (PBS+2% FBS) for 30 minutes at 6°C, then washed and resuspended in FACS

buffer. Flow cytometry acquisitions were performed on a Attune NxT (Thermo Fisher), BD AccuriTM C6 Plus (BD Biosciences) or, BD LSR Fortessa (BD Biosciences). Forward- and side-scatter on unstained controls were used to gate on cells, respectively. Forward-scatter height vs. forward-scatter area was used to gate on single cells. Analysis was performed using FlowJo software. Data analysis was performed using FlowJo 10.8.1 software (Becton Dickinson & Company).

Table 2.1. Antibodies used to examine the differentiation and expansion of iPSC-derived hematopoietic cells via flow cytometry analysis.

Antigen	Fluorophore	Catalog #	Company
CD14	BV421	565283	BD Biosciences
CD14	APC	555399	BD Biosciences
CD31	PE	566125	BD Biosciences
CD34	PE	555822	BD Biosciences
CD34	PE-Cy7	560710	BD Biosciences
CD34	APC	60013AZ	StemCell Technologies
CD41a	FITC	555466	BD Biosciences
CD43	APC	60085AZ	StemCell Technologies
CD43	PE	60085PE	StemCell Technologies
CD45	FITC	555482	BD Biosciences
CD45	PE-Cy7	557748	BD Biosciences
CD45RA	BV421	562885	BD Biosciences
CD45	APC	60018AZ	StemCell Technologies
CD45	FITC	60018FI	StemCell Technologies
CD45RA	APC	550855	BD Biosciences
CD90	PE-Cy7	561558	BD Biosciences
CD144	Alexa Fluor 488	53-1449-42	eBiosciences
CD235a	PE	555570	BD Biosciences

2. 6 May-Grunwald Giemsa Stain

A minimum of 50,000 cells were washed in PBS at pH 6.8, fixed in absolute methanol, and stained with May-Grunwald Giemsa (MGG). Slides were examined using an inverted microscope (Olympus IX81), and images were acquired with a Photometrics Evolve® 512 Delta camera.

2. 7 Colony Forming Unit Assay

On D12 of monolayer differentiation, the floating cells were harvested, counted, and the cells were suspended in the Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2% FBS (StemCell Technologies, 07700). For each dish, 100 µL of cell suspension was mixed with 1 mL MethoCult™ SF H4636 **(**StemCell Technologies, 04636). Viable cells were seeded in each 35mm dish containing 1.1 mL of methylcellulose medium. The cells were plated in duplicates in non-treated 35mm culture dishes (StemCell Technologies, 27371) using a 3 mL syringe. The cells were then incubated for 14 days at 37°C with 5% CO_2. After 14 days of incubation, the number of colonies formed was counted using a Leica DMi1 inverted microscope (Leica Microsystems) with a magnification of 50x-100x. Colonies with 50-200 cells were considered for analysis. The colonies were identified and classified based on their morphology and size. Colonies were scored based on the following criteria: multipotent CFU–granulocyte-erythrocyte-monocyte-macrophage (CFU-GEMM), CFU–erythroid (C/BFU-E), and CFU–myeloid (CFU-G/CFU-M/GM) progenitors. Representative images of each colony were taken at day 14.

2. 8 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.1.2 for Windows (GraphPad Software). The data were presented as mean ± standard error of the mean (SEM), and one-way and two-way ANOVA was used to compare the means between groups. $P < 0.05$ was considered statistically significant.

Chapter 3 Results

3. 1 Optimization of iHPC differentiation from iPSCs

There are multiple differentiation methods of generating iHPCs from human pluripotent stem cells such as co-cultures with BM-derived stromal cell feeder layers[170], serum-free or feeder-free differentiation methods[171–173], and commercially available kits such as STEMCELL Technologies STEMdiff™ Hematopoietic Kits. In this first aim, I wanted to determine the optimal iHPC culture system for assessing the effects of SCACs on iHPC generation. Ideally, the iHPC culture system would yield an iHPC population similar to CB-HSPCs, the cell source for which the SCACs have been initially formulated and validated. For these studies, I used a HAF-derived iPSC line that has been generated and extensively validated in the Jezierski lab [169] to assess and optimize iHPC differentiation capabilities using two well-established STEMDiff™ Hematopoietic differentiation strategies that have been reported to recapitulate early embryonic hematopoiesis events [171,173–178]: spontaneous EB-based differentiation [175–179] and directed monolayer differentiation [174,180,181].

3.1.1 Embryoid Body (EB) iHPC Differentiation Strategy

First, I tested a commercially available animal component-, serum- and feeder-free STEMdiff™ Hematopoietic - EB kit and reagents for the generation of iHPCs through EBs (Stem Cell Technologies, depicted in Figure 3.1A). In brief, the iPSCs were dissociated into a single cell suspension and seeded in AggreWell plates in EB formation Medium A to generate EBs and induce mesoderm differentiation. After 3 days of mesoderm differentiation, the medium was changed to EB medium B to induce hematopoietic lineage differentiation. On day 5, the EBs

were transferred into non-tissue culture treated plates and on day 12, the EBs were harvested and dissociated. The harvested iHPCs were analyzed by flow cytometry for the expression of a panel of iHPC cell surface markers (summarized in Table 1.1) such as hematopoietic progenitor marker CD34, pan-embryonic hematopoietic progenitor marker CD43, pan-hematopoietic leukocyte marker CD45, and endothelial marker CD31[166,182,183] (Supplementary Table 1).

The EB differentiation yielded iHPCs primarily composed of a population of $CD45^-$ $CD34^+CD43^-$ population of definitive hemogenic endothelial-like cells [82,103] with an average yield of 1×10^6 cells/cm^2 (Figure 3.1B). While this protocol yielded $CD34^+$ cells, the lack of definitive hematopoietic progenitor CD43 and CD45 markers expression highlighted a lack of hematopoietic commitment in the $CD34^+$ population. This represented an early iHPC or non-hematopoietic phenotype [100,184,185].

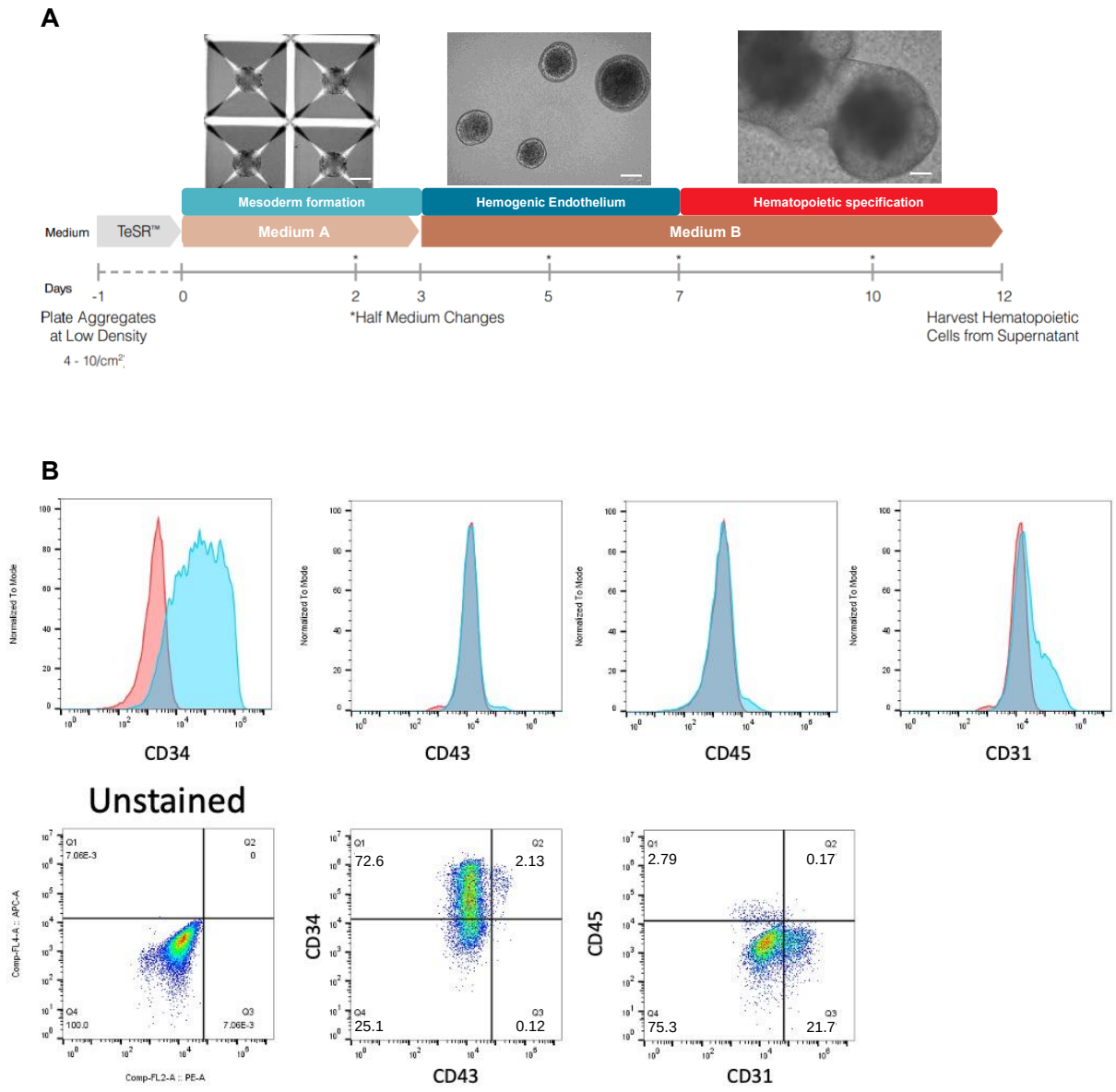


Figure 3.1. Hematopoietic embryoid body differentiation yields populations of hemogenic endothelial-like cells and hematopoietic progenitors.
(A) Representative images of cell morphology changes at different stages of human iPSC to iHPC differentiation using three-dimensional, animal component-, serum- and feeder-free conditions. Scale bars, 100 μm. (B) Representative flow cytometry profiles of a primarily $CD45^-CD34^+CD43^-$ cell population. After EB differentiation, cells dissociated from EBs were either unstained or stained with anti-CD34, anti-CD43, anti-CD45, and anti-CD31 (n=1 independent differentiation). Unstained, red; stained, blue. EB, embryoid body.

3.1.2 Directed Monolayer iHPC Differentiation Strategy

Second, I tested a directed monolayer differentiation strategy to generate iHPCs. I used a commercially available chemically-defined, serum- and feeder-free STEMdiff™ Hematopoietic Kit (Stem Cell Technologies) that requires no replating, no co-culture feeders, and no EB formation. Furthermore, this kit has been extensively validated for reproducibility across multiple human iPSC lines [76,180,181,186–190]. In brief, iPSCs were seeded on Matrigel coated plates, at a defined aggregate size and number (described in Materials and Methods) and iHPC differentiation was initiated via mesoderm induction (Medium A, day 0 – 3) resulting in the formation of an adherent monolayer. With the subsequent addition of hematopoietic cytokines (Medium B, day 3 – 12), hematopoietic clusters emerged from the monolayer, shedding iHPCs into the media (suspension cells; Figure 3.2A; phase contrast images). This monolayer differentiation process yielded two distinct cell populations (Figure 3.2A): suspension and adherent cells. The suspension cells consisted mainly of iHPCs and the adherent cells have been characterized as being mainly composed of endothelial and MSC populations, as previously described [76].

At the end of the differentiation (D12), the monolayer differentiation protocol produced an average yield of 2.19 x 10^5 cells/cm^2 total nucleated cells (TNCs). The harvested cells were subsequently characterized by flow cytometry using the hematopoietic markers previously described for the EB formation strategy (Figure 3.2B). Overall, the flow cytometry analysis of single marker expression shows that iHPCs expressed CD34 (95.60%), CD43 (95.49%), CD45 (58.30%), and CD31 (90.50%) (Figure 3.2B) reflecting a heterogenous iHPC population. Given that early definitive HSPCs are generated through endothelial-to-hematopoietic transition [191,192],

I found that a subset of the iHPC population were double positive for $CD34^+CD31^+$ (56.80%), delineating a hemogenic endothelial progenitor cell population [183,184] (Table 3.1). As hemogenic endothelial progenitor cells differentiate into hematopoietic cell types, they can gradually lose expression of CD31, while simultaneously acquiring the phenotype of hematopoietic progenitor cells (HPCs), as assessed by the expression of CD43, CD45, and CD45RA markers [76,192,193]. To further analyze these HPCs, I used previously defined dual markers to characterize the HPCs ($CD34^+CD43^+$ and $CD45^+CD34^+$) as described in iPSC-derived HSPCs [80,103,166,182] and HSC-enriched cells ($CD34^+CD45RA^-$) as described in CB-derived HSPCs [134,194–196]. Flow cytometry analysis showed distinct populations of $CD34^+CD43^+$ (91.40%), $CD45^+CD34^+$ (10.21%), and $CD34^+CD45RA^-$ (26.18%) cells (Table 3.1 and Figure 3.2). The use of CD34, CD43 and CD45 markers have been used in combination to further assess multipotent hematopoietic progenitor cells ($CD45^+CD34^+CD43^+$) in iPSC-derived HSPCs [76,103,166,184]. The iHPCs consisted of a subset of $CD45^+CD34^+CD43^+$ cells (8.98%) (Table 3.1). Finally, I assessed the expression of CD90, another marker used to enrich for HSC activity, to identify two additional HSC-like subsets: $CD34^+CD45RA^-CD90^+$ (2.55%) and $CD45^+CD34^+CD45RA^-CD90^+$ (0.62%) (Table 3.1) HSC-like cells shown with CB and peripheral blood stem cells (PBSC) to enrich in repopulating capacity [195,197,198]. This observed low frequency of iHSC-like cells is consistent with previous literature for iPSC-derived bona fide HPCs and HSC-like cells coincident with the lack of long-term engraftment potential of iPSC-derived HPC *in vivo* [76,166]. In summary, the directed monolayer differentiation strategy yielded a hierarchy of iHSPC cell populations, reminiscent of CB-derived HSPCs [134,194–196,198,199] and definitive hematopoiesis differentiation.

The SCACs have been formulated for the expansion of CB-derived HSPCs with $CD34^+$, $CD34^+CD45RA^-$, $CD34^+CD45RA^-CD38^-$, and $CD34^+CD45RA^-Epcr^{High}$ phenotypes [156,160,162,200]. Importantly, the monolayer differentiation strategy generated a more phenotypically CB-HSPC-like iHPC phenotype compared to the early iHPC or non-hematopoietic phenotype generated by EB differentiation. Additionally, the monolayer culture system offers greater control over the homogeneous exposure of small molecules in a two-dimensional (2D) environment as opposed to the three-dimensional (3D) culture system. For these reasons, I used the directed monolayer iHPC differentiation strategy for all subsequent experiments aimed at testing the different SCAC formulations.

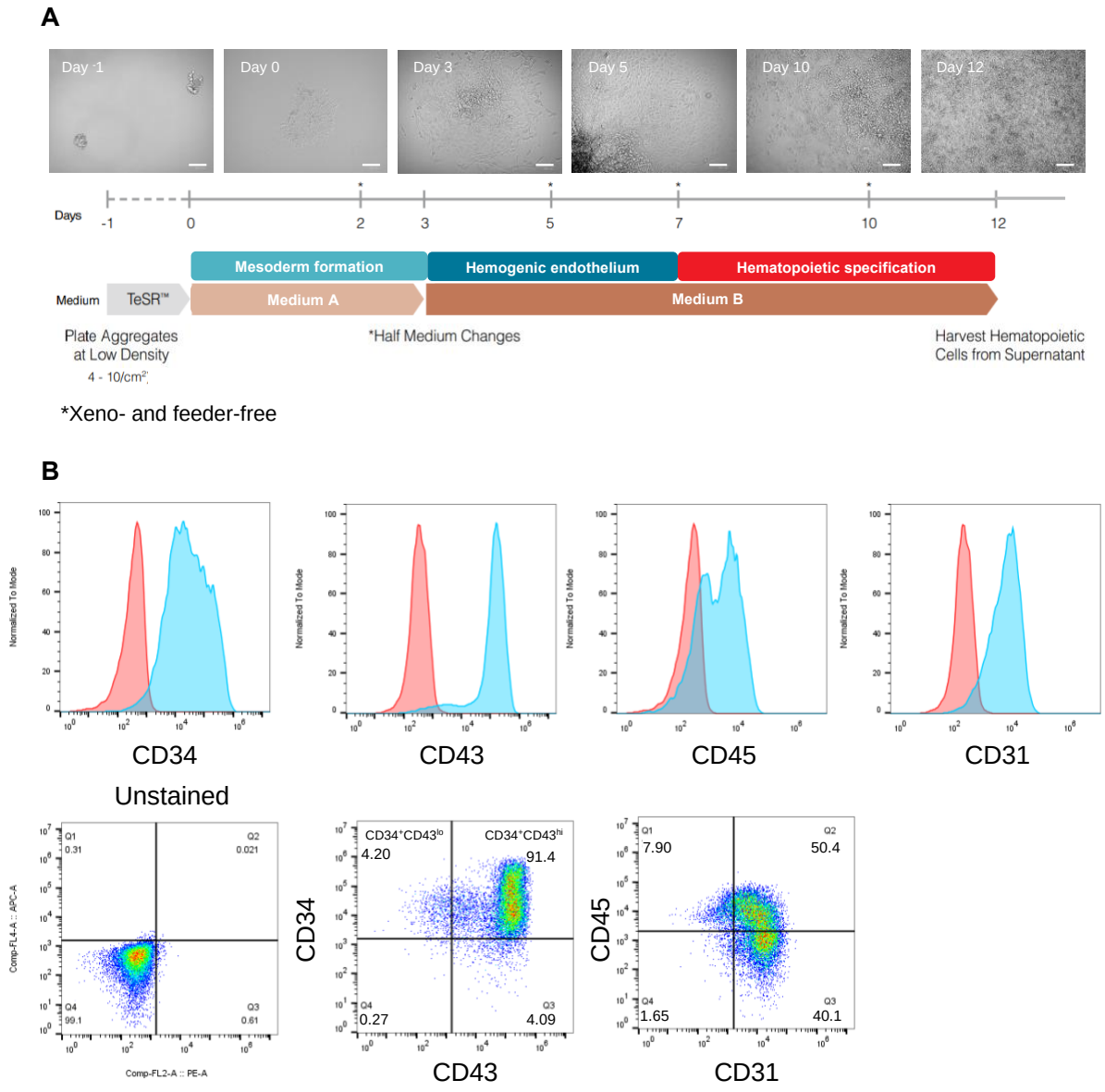


Figure 3.2. Hematopoietic monolayer differentiation yields a heterogenous hematopoietic progenitor population with a phenotype similar to CB-derived HPCs.
(A) Experimental overview of the monolayer differentiation strategy and representative images of cell morphology changes during iPSC to iHPC differentiation. Scale bars, 100 μm. (B) Representative flow cytometry profiles of cells generated after 12 days of differentiation. After monolayer differentiation, isolated suspension cells were either unstained or stained with anti-CD34, anti-CD43, anti-CD45, and anti-CD31 (n=3 independent differentiations for CD34, CD43 and CD45 staining; n=1 independent differentiation for CD31 staining). Unstained, red; stained, blue.

Table 3.1. Hematopoietic monolayer differentiation yields cell population with HSPC phenotypes.

Cell population	HPC markers	Ref	Mean ± SEM (%)
Definitive hematopoietic stem cell-like	$CD45^+CD34^+CD45RA^-CD90^+$	[76]	0.62
Hematopoietic stem cell-like	$CD34^+CD45RA^-CD90^+$	[76]	2.55 ± 1.10
Definitive hematopoietic progenitors	$CD34^+CD43^+CD45^+$	[76,103,166,184,201]	8.98 ± 4.35
Hematopoietic progenitors	$CD34^+CD45^+$	[103,166,182,202,203]	10.21 ± 4.76
Hematopoietic progenitors	$CD34^+CD45RA^-$	[204]	26.18 ± 2.06
Hemogenic endothelial cells	$CD34^+CD31^+$	[168,205]	56.8

Data is presented as mean ± SEM % of harvested suspension cells (n=3, except $CD34^+CD31^+$ and $CD45^+CD34^+CD45RA^-CD90^+$ which are n=1).

3. 2 Expansion of terminally differentiated iHPC

In the second aim, I hypothesized that supplementation of iHPCs during post-differentiation expansion culture with a panel of SCACs (Table 1.2) would increase the yields of iHPCs. I also wanted to determine whether GAS6, a growth factor recently shown to promote expansion of CB-HSPC cultures [160], could further increase expansion of iHPCs when supplemented with X2A. For this, at D12 of iHPC differentiation, the iHPCs were harvested and transitioned into expansion medium for two weeks, as described in Materials and Methods. Cultures were supplemented with a panel of SCACs: X2A (1X), SM6 (1X), SMA (1X), or X2A+GAS6. iHPCs with no SCAC supplementation (non-treated) were used as baseline controls. UM171 (35 nM) previously shown to increase iHPC expansion by 1- to 4-fold relative to non-treated controls

[80,166,167,206] (Supplementary Table 1) was used herein as a positive benchmark control as detailed in Figure 3.3A.

The iHPCs were harvested after 7 or 14 days of expansion, counted, and analyzed by flow cytometry for the emergence of iHPCs. Representative phase contrast images illustrating expansion of the iHPC are shown in Figure 3.4A. During the entire 14-day expansion period, all SCACs increased iHPC proliferation and expansion compared to non-treated controls (Figure 3.4B). In addition, the gain in expansion achieved with most SCACs was also superior to that provided by UM171 alone (Figure 3.4B). After 7 days of expansion, iHPCs supplemented with X2A resulted in a 2-fold increase when normalized to non-treated controls (Figure 3.4B). However, the addition of GAS6 to X2A cultures (X2A+GAS6) was not significantly different from X2A-treated cultures when normalized to non-treated controls. UM171, SM6, and SMA showed no significant increase over non-treated controls (Figure 3.4B). After 14 days of expansion, iHPCs yields increased by 4- (X2A+GAS6), 3- (X2A and SM6), and 2-fold (SMA) when normalized to non-treated controls (Figure 3.4B). While X2A+GAS6-treated iHPCs achieved superior expansion compared to X2A, there was no significant difference in fold expansion between the treatments. Interestingly, iHPCs supplemented with UM171 did not show a statistically significant fold increase over non-treated iHPCs beyond the first 4 days (Figure 3.4B). Importantly, no cytotoxicity was observed for any of the SCACs during the iHPC expansion phase.

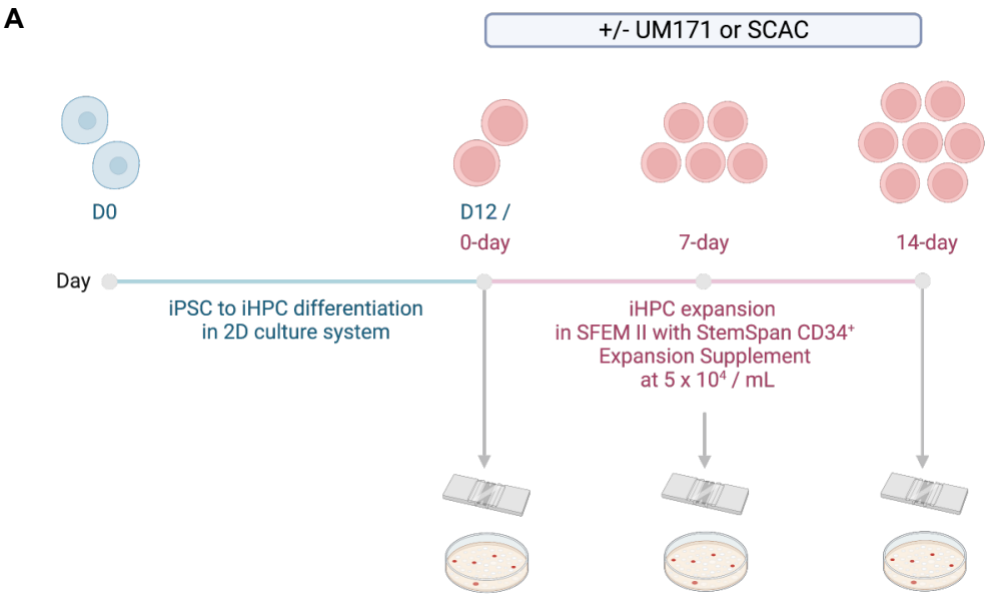


Figure 3.3. Schematic of experimental timeline of hematopoietic differentiation followed by expansion and colony forming assay.
After 12 days of differentiation, 50,000 cells/mL of iHPCs were expanded in expansion media (StemSpan SFEM II and StemSpan $CD34^+$ expansion supplement) and supplemented with UM171 (35 nM) or SCACs (X2A, SM6 or SMA all at 1X), and X2A+GAS6 (GAS6 at 10 ng/mL).

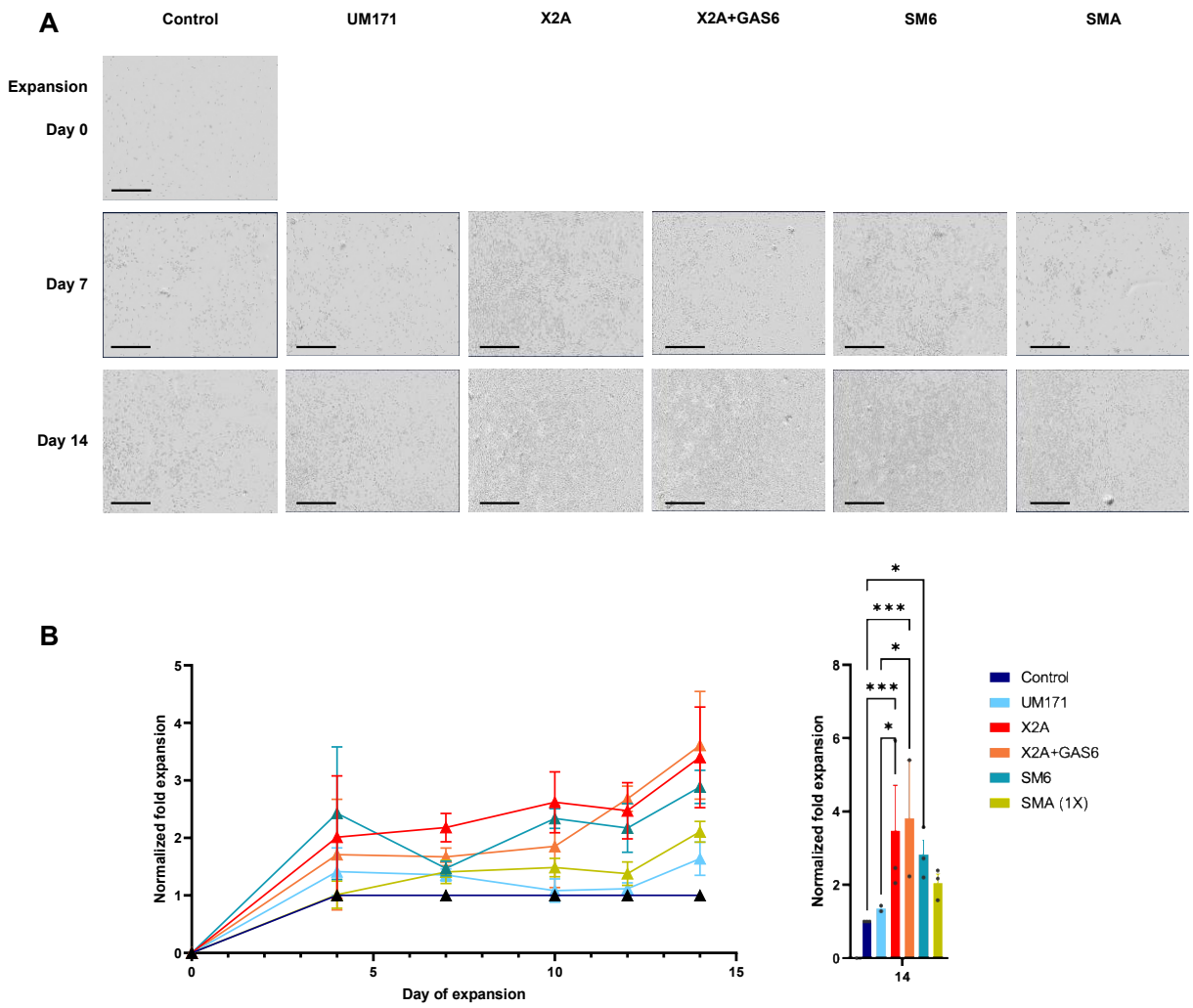


Figure 3.4. SCAC-supplemented iHPC demonstrate greater expansion compared to non-supplemented iHPC.
(A) Representative phase contrast images of iHPC expansion supplemented with UM171, X2A, X2A+GAS6, SM6 or SMA based on confluency assessments at day 0, 7 and 14. Scale bars, 100 µm. (B) Fold expansion (calculated based on TNC yields) of SCAC expanded iHPCs, normalized to control (untreated) over the 14-day expansion period. Cell counts were performed at day 4, 7, 10, 12 and 14 (n=2 independent differentiations for UM171 and X2A+GAS6 conditions, n=3 independent differentiations for other conditions). Data presented as both line (left) and bar (right) graphs for ease of interpretation. Data presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$ as assessed with two-way ANOVA.

3.2.1 Impact of SCAC supplementation on the emergence of lineage differentiated hematopoietic cells

Previous studies investigating SCA-mediated expansion of iHPCs have shown that some SCAs can preferentially affect the expansion of different hematopoietic progenitor/cell lineage subtypes [80,166,168,206]. As such, I assessed the expanded iHPCs using a panel of lineage-specific markers include pan-hematopoietic leukocyte marker CD45[182], megakaryocytic lineage marker CD41a [98,207], erythroid lineage marker CD235a (glycophorin A) [98,207], and myeloid lineage marker CD14 [208,209] (Supplementary Figure 1).

Initially, I examined changes in the expression of pan-hematopoietic marker CD45 during expansion. CD45 expression in non-treated controls increased with expansion over 0 (14.8%), 7 (76.6%), and 14 days (91.0%) (Supplementary Figure 4 and Supplementary Figure 5). Both UM171 and SCAC supplementation during 7 (UM171: 78.5%, X2A+GAS6: 82.0%, X2A: 80.6%, SM6: 80.3%, SMA: 71.4%) and 14 (UM171: 93.6%, X2A: 98.8%, X2A+GAS6: 97.9%, SM6: 96.0%, SMA: 95.1%) days of expansion increased the frequencies of $CD45^+$ hematopoietic cells compared to non-treated controls (Supplementary Figure 4 and Supplementary Figure 5). The expansion of iHPCs and SCAC supplementation are factors that resulted in a trending increased expression of hematopoietic CD45 cells, which suggests increased hematopoietic commitment during the expansion process. To determine the types of lineage-specific progenitors affected by SCACs, I assessed the frequencies of $CD235a^+CD41a^+$ MEP-like cells, $CD45^+CD235a^+$ erythroid progenitor-like cells, $CD45^+CD41a^+$ megakaryocyte-like cells, and $CD45^+CD14^+$ myeloid-like cells after expansion (Supplementary Figure 1). I found that SCAC supplementation during iHPC expansion did not significantly affect the

frequency or introduce lineage bias during expansion (Supplementary Figure 3 to Supplementary Figure 5, Supplemental Results).

3.2.2 SCAC-expanded iHPCs possess multilineage differentiation potential

After phenotypic characterization of the SCAC-expanded iHPCs, I used the functional CFU assay to assess the proliferative and multilineage differentiation potential (potency) of the expanded iHPCs. The CFU assay can assess the formation of multilineage and lineage-specific progenitors including multipotent CFU–GEMM, CFU–erythroid, and CFU–myeloid progenitors. Prior to expansion (day 0 of expansion), MGG staining demonstrated that control cultures contained monoblast-, myelocyte- and erythroblast-like cells (Figure 3.5A) and representative CFU colonies are shown in Figure 3.5B.

Non-treated control iHPCs showed formation of CFU-erythroid and CFU-myeloid progenitors with potent colony formation activity, giving rise to 0.04 colony forming cells (CFC) / seeded cell by day 7 of expansion and 0.04 CFC / seeded cell by day 14 of expansion when including all types of CFU colonies (Figure 3.5C). By comparison, all SCAC-expanded iHPCs gave rise to higher numbers of CFU colonies compared to non-treated controls and UM171-expanded cultures. SCAC-expanded iHPCs giving rise to both CFU-erythroid and CFU-myeloid colonies (Figure 3.5C, left and right); however, iHPCs expanded for 7 days with SCACs produced 0.17 CFC / seeded cell (X2A+GAS6), 0.15 CFC / seeded cell (X2A), 0.15 CFC / seeded cell (SM6), 0.12 CFC / seeded cell (SMA), compared to UM171 which produced 0.08 CFC / seeded cell (UM171) (Figure 3.5C, left). Meanwhile, iHPCs expanded for 14 days with SCACs produced

0.49 CFC / seeded cell (X2A+GAS6), 0.25 CFC / seeded cell (X2A), 0.19 CFC / seeded cell, (SM6), and 0.04 CFC / seeded cell (SMA), compared to UM171 which produced 0.03 CFC / seeded cell (UM171) (Figure 3.5C, right). All conditions gave rise to both CFU-erythroid and CFU-myeloid progenitors (Figure 3.5C, left and right), which is consistent with the presence of $CD45^{+}CD235a^{+}$ and $CD45^{-}CD235a^{+}$ erythroid progenitor-like cells and $CD45^{+}CD14^{+}$ myeloid committed-like cells in pre-expansion cultures (Supplementary Figure 4 and Supplementary Figure 5).

Furthermore, all treatment conditions showed a higher frequency of CFU-myeloid progenitors compared to CFU-erythroid progenitors, with little to no multipotent CFU-GEMM progenitors (Figure 3.5C, left and right). While SCAC supplementation did not change the proportion of CFU-erythroid and CFU-myeloid progenitors, I observed a bias towards CFU-myeloid progenitors to CFU-erythroid progenitor lineages by day 14 of expansion compared to day 7 of expansion in all conditions (Figure 3.5C, left and right). This observation is consistent with the increase in frequency of $CD45^{+}CD14^{+}$ myeloid committed-like cells and the decrease in frequency of $CD45^{-}CD235a^{+}$ erythroid progenitor-like cells (Supplementary Figure 4 and Supplementary Figure 5). Importantly, SCAC supplementation resulted in higher numbers of CFU colonies than non-treated controls and UM171-expanded cultures (Figure 3.5C, left and right). This recapitulates observations in CB-derived HSPCs made by the Pineault lab, where SCAC supplementation demonstrated greater expansion of MPPs compared to non-treated controls [156].

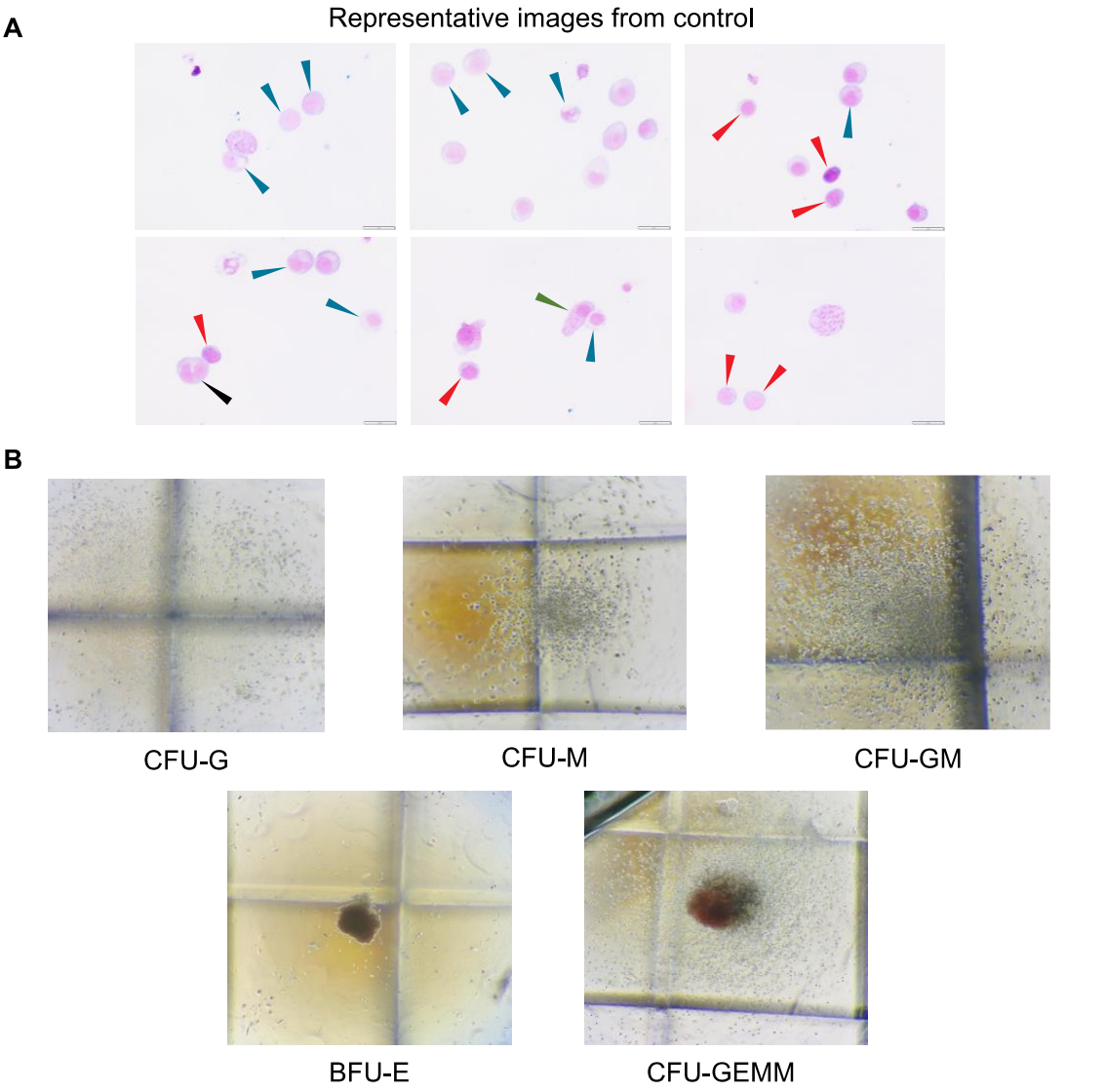

A
Representative images from control
B
CFU-G
CFU-M
CFU-GM
BFU-E
CFU-GEMM
C
SCAC Exp
7-day expansion
SCAC Exp
14-day expansion
CFC / seeded cell
Control
UM171
X2A
X2A+GAS6
SM6
SMA
Treatment
**
CFU-G/M/GM
C/BFU-E
CFU-GEMM

Figure 3.5. SCAC-supplemented iHPC demonstrate greater multilineage potency compared to non-supplemented iHPC.
(A) Representative MGG stain preparation of iHPCs at 12 days of iPSC to iHPC differentiation. Scale bar, 20 µm. Black arrowhead: band cell, blue arrowhead: monoblast, green arrowhead: myelocyte, red arrowhead: erythroblast. (B) Representative CFU colony morphologies. (C) CFC per seeded cell was assessed for iHPCs expanded for 7 and 14 days supplemented with UM171 (35 nM) or SCACs (X2A, SM6 or SMA), and/or GAS6 (10 ng/mL). CFU-G/M/GM, C/BFU-E and CFU-GEMM colonies were counted 14 days after initial plating (n=2 independent differentiations for UM171 and X2A+GAS6 conditions, n=3 independent differentiations for other conditions). Data presented as mean ± SEM. **$p < 0.01$ as assessed with two-way ANOVA. BFU, burst-forming unit; CFU, colony-forming unit; E, erythroid; G, granulocyte; GEMM, granulocyte, erythrocyte, monocyte, macrophage; GM, granulocyte/macrophage; M, macrophage.

3. 3 Impact of SCACs on the differentiation on iPSC towards the hematopoietic lineage

Given the positive effect of SCAC supplementation during the expansion of differentiated iHPCs (Aim 2), in the third aim, I sought out to determine whether earlier SCAC supplementation during mesodermal induction and iHPC differentiation would similarly increase iHPC yields by D12 of differentiation. Since iHPC differentiation is accompanied by epigenetic changes, I hypothesized that SCACs would be more effective at modulating proliferation early during the differentiation process.

To assess SCAC-mediated effects of iHPC proliferation during differentiation and subsequent yields, SCACs were supplemented at three time points during differentiation: D0-D12, D5-D12 or D7-D12 (treatment strategy is depicted in Figure 3.6A); cells with no SCAC supplementation were used as non-treated controls. The iHPCs were harvested on D12, counted, and phenotypically analyzed by flow cytometry. Overall, no SCAC-mediated cytotoxicity was observed during the differentiation process with the exception of SMA supplementation at 1X and 0.5X concentrations during D0-D12 (data not shown). As a result, SMA supplementation at

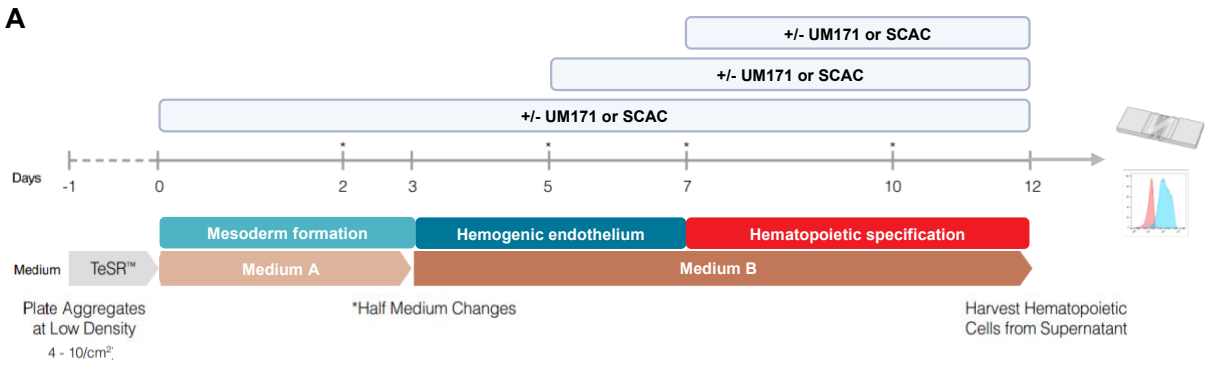
0.25X during differentiation was used for all the subsequent experiments in Aim 3. The TNCs harvested at the end of the D12 of iHPC differentiation did not show statistically significant differences in yield between non-treated controls and SCAC-treated iHPCs at all treatment time points (Figure 3.6B).

3.3.1 Effects of SCAC supplementation during different developmental steps of iPSC to iHPC differentiation

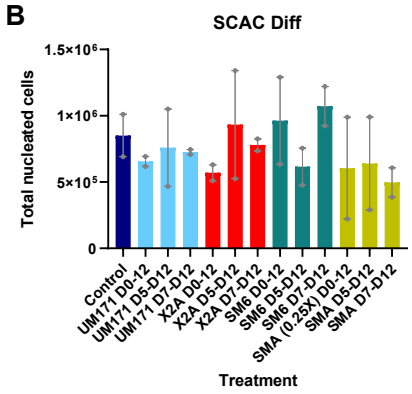
To assess whether SCAC supplementation during differentiation had an effect on the yield and phenotypic profile of iHPC frequencies, I performed flow cytometry analysis for the panel of iHPC and iHSC-like markers, as described in Aim 1 and 2. Overall, SCAC supplementation during D0-D12 produced the highest yield of HPCs and HSC-like cells compared to other supplementation periods (D5-D12 and D7-D12; Figure 3.6C and D).

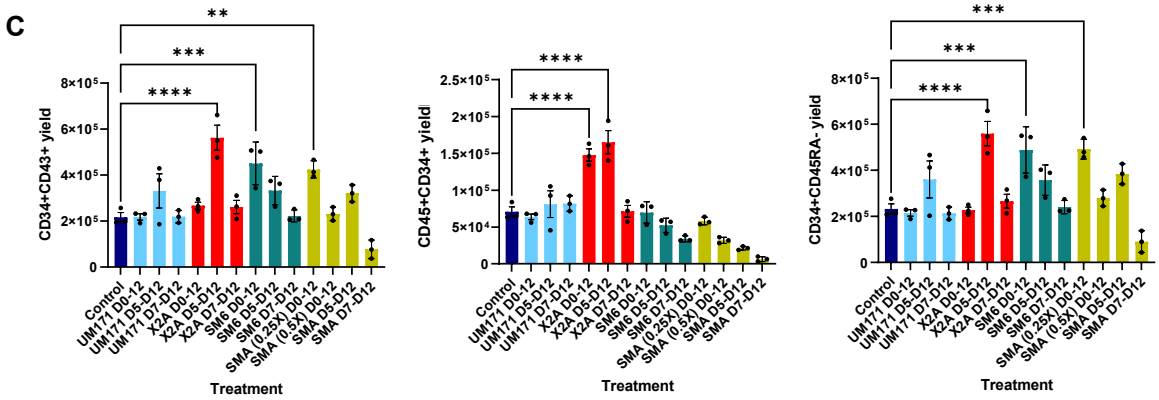
SCAC supplementation during D0-D12 consistently gave rise to the highest yield of $CD34^+CD43^+$ cells (SM6 D0-D12: 45.1 x 10^4 cells, ***$p < 0.001$; SMA (0.25X) D0-12: 42.4 x 10^4 cells, **$p < 0.01$) and $CD45^+CD34^+$ cells (X2A D0-12: 14.8 x 10^4 cells, ****$p < 0.0001$; SM6 D0-12: 7.0 x 10^4 cells, SMA (0.25X) D0-12: 5.8 x 10^4 cells), compared to other supplementation periods (D5-D12 and D7-D12; Figure 3.6C). Similarly, SCAC supplementation during the D0-D12 period also gave rise to the highest yield of $CD34^+CD45RA^-$ cells (SM6 D0-12: 48.9 x 10^4 cells, ***$p < 0.001$; SMA (0.25X) D0-12: 49.2 x 10^4 cells, ***$p < 0.001$) compared to the other supplementation periods (D5-D12 and D7-D12; Figure 3.6C).

Lastly, SCAC supplementation during D0-D12 also generally gave rise to the highest yield of $CD45^+CD34^+CD45RA^-CD90^+$ cells (SM6 D0-12: 3.5 x 10^4 cells, ****$p < 0.0001$; SMA (0.25X) D0-12: 3.3 x 10^4 cells, ***$p < 0.001$) compared to other supplementation periods (D5-D12 and D7-D12; Figure 3.6D). SCAC supplementation during D0-D12 also showed a trend towards giving rise to a higher yield of $CD45^+CD34^+CD43^+$ cells (X2A D0-12: 14.1 x 10^4 cells, ****$p < 0.0001$; SM6 D0-12: 6.3 x 10^4 cells; SMA (0.25X) D0-12: 5.3 x 10^4 cells) compared to other supplementation periods (D5-D12 and D7-D12; Figure 3.6D). As such, I decided to focus on the D0-D12 SCAC-treated iHPCs for all subsequent analyses.



*Xeno- and feeder-free





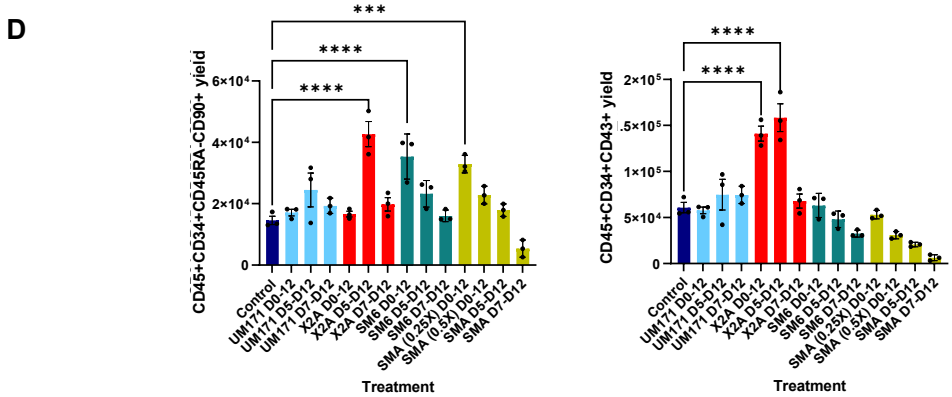


Figure 3.6. SCAC supplementation during different stages of hematopoietic differentiation modulates yield and phenotype of iHPCs.
(A) Experimental timeline of SCAC supplementation at different timepoints during iPSC to iHPC differentiation. (B) TNCs harvested at the end of iPSC to iHPC differentiation (12 days) in the presence or absence of UM171 (35 nM) and SCACs: X2A (1X), SM6 (1X) or SMA (0.25X, 0.5X or 1X) during different time periods. Cells were counted manually by hemacytometer at D12 (n=1 independent differentiation). (C) Effects of SCAC supplementation during different time points of iHPC differentiation on the production of $CD34^+CD43^+$, $CD45^+CD34^+$, and $CD34^+CD45RA^-$ HPCs. Flow cytometry analysis was performed at D12 (n=1 independent differentiation). (D) Yield of $CD45^+CD34^+CD43^+$ and $CD45^+CD34^+$ $CD45RA^-$ $CD90^+$ HSC-like cells after SCAC supplementation (n=1 independent differentiation). Mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 as assessed by one-way ANOVA.

3.3.2 Hematopoietic progenitors supplemented with SCAC during differentiation possess the potential to form multiple lineages *in vitro*

Although there were no statistically significant differences in TNC yields at D12 of differentiation between treatment conditions (Figure 3.6B and Figure 3.7B), I wanted to assess whether the increased frequencies of HSC-like cells and HPCs would have an impact on the multilineage potency. D0-D12 SCAC treated and non-treated control iHPCs were harvested on D12 of differentiation, counted and plated in semi-solid medium (CFU assay) to assess the potency of the harvested cells (as depicted in Figure 3.7A).

The CFU assay demonstrated that iHPCs supplemented with SCACs during D0-D12 retained the ability to give rise to both CFU–myeloid and CFU–erythroid progenitors, and to a lesser degree, CFU-GEMM progenitors (Figure 3.7C). The relative composition of CFU colonies was similar to that of non-treated controls; however, the SCAC-supplemented iHPCs (X2A D0-12: 0.06 CFC / seeded cell; SMA (0.25X) D0-12: 0.06 CFC / seeded cell; SM6 D0-12: 0.06 CFC / seeded cell) gave rise to a higher total number of CFCs compared to non-treated control (0.04 CFC / seeded

cell) and UM171 iHPCs (0.04 CFC / seeded cell) (Figure 3.7C). Interestingly, the SCAC-supplemented iHPCs also gave rise to a higher fraction of CFU–erythroid progenitors (SM6 D0-12: 0.031 CFC / seeded cell; SMA (0.25X) D0-12: 0.024 CFC / seeded cell; X2A D0-12: 0.021 CFC / seeded cell) compared to non-treated control (0.015 CFC / seeded cell) and UM171 iHPCs (0.014 CFC / seeded cell) (Figure 3.7C).

This data suggests that SCAC-supplementation during iHPCs differentiation did not affect the differentiation efficacy or multilineage potency despite showing a higher proportion of HSC-like phenotypic frequencies.

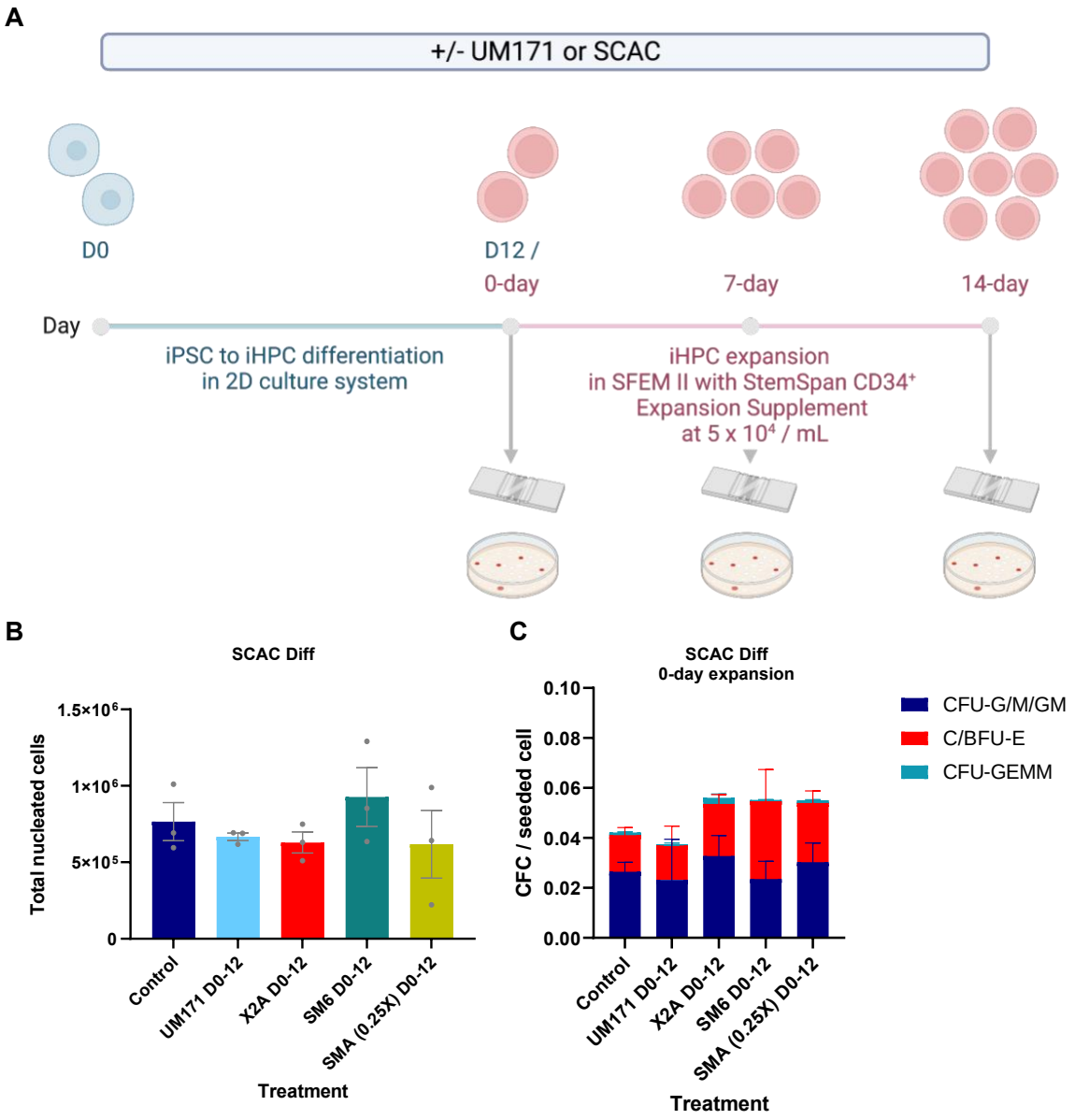


Figure 3.7. Schematic of experimental SCAC supplementation during D0-12 of iPSC to iHPC differentiation modulates potency of iHPC.
(A) Schematic diagram of supplementation of UM171 (35nM) or SCACs X2A (1X), SM6 (1X) or SMA (0.25X) during D0-12 of iPSC to iHPC differentiation and subsequent expansion. (B) TNCs harvested after 12 days of iPSC to iHPC differentiation (n=3 independent differentiations). (C) CFC per seeded cell of Control iHPC and D0-D12 SCAC iHPC at D12 of differentiation. iHPCs were plated into individual 35 mm dishes in MethoCult SF H4636 semi-solid medium for CFU assay. Colonies were scored 14 days after plating (n=3 independent differentiations for control and SMA 0.25X; n=2 independent differentiations for UM171, X2A, and SM6). Mean ± SEM, no significant differences compared to non-treated controls as assessed by one-way ANOVA for B and two-way ANOVA for C.

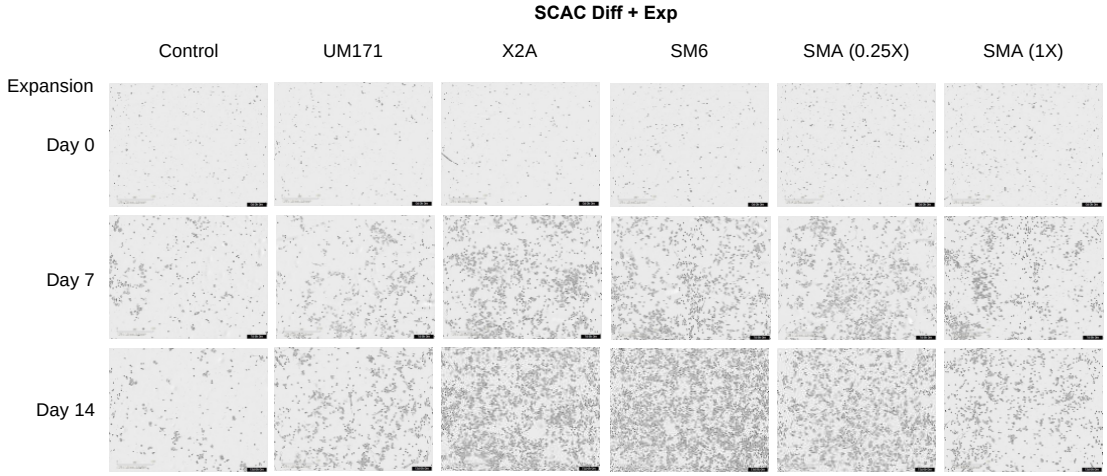
3.3.3 SCAC supplementation during differentiation and expansion enhances proliferation of iHPCs

Since D0-12 SCAC gave rise to a higher frequency of HSC-like cells, I wanted to assess whether subsequent SCAC-supplemented expansion would preferentially induce a higher expansion of these HSC-like cells and hence a higher overall yield. Hence, the D0-12 iHPCs were subsequently expanded for another 2 weeks in expansion media (SFEM II and StemSpan $CD34^+$ Expansion Supplement (StemCell Technologies)) supplemented with UM171 (35 nM) or X2A (1X), SM6 (1X), or SMA (0.25X or 1X). Cells with no SCAC supplementation were used as non-treated controls. Henceforth, SCAC-supplemented iHPCs during differentiation and expansion are referred to as "SCAC Diff+Exp" iHPC and SCAC-supplemented iHPCs during expansion only are referred to as "SCAC Exp".

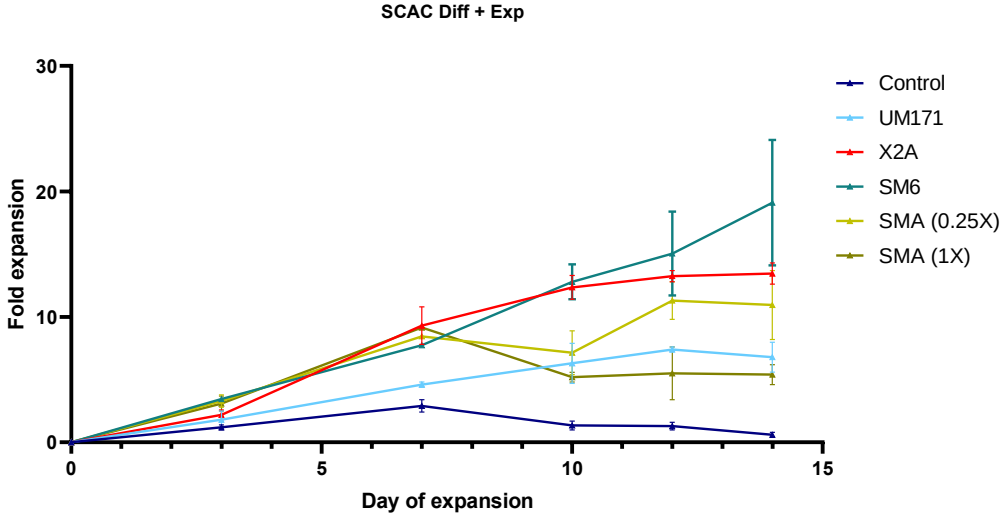
Fold expansion was assessed based on TNC counts at 3, 7, 10, 12 and 14 days of expansion and normalized to day 0 counts. Representative phase contrast images illustrating fold expansion of the iHPCs are shown in Figure 3.8A. All SCAC-supplemented iHPCs showed higher proliferation and expansion compared to non-treated controls and UM171 cultures (Figure 3.8A). SCAC Diff+Exp iHPCs demonstrated 9- (X2A and SMA, 1X) and 8-fold (SM6 and SMA, 0.25X) expansion by day 7 of expansion relative to the initial plating density (day 0; Figure 3.8B). By comparison, UM171-supplemented iHPCs gave rise to a 5-fold expansion relative to the initial plating density (day 0) (Figure 3.8B). After 14 days of expansion, SCAC Diff+Exp iHPCs demonstrated fold expansions of 19- (SM6), 14- (X2A), and 11-fold (SMA, 0.25X) relative to the initial plating density (day 0), whereas UM171 showed a 7-fold expansion relative to the initial plating density (day 0; Figure 3.8B).

Overall, I observed similar yields of iHPCs when comparing SCAC supplementation during iHPC expansion only (SCAC Exp; Figure 3.4B) and SCAC Diff+Exp (Figure 3.8B). These findings suggest that SCAC supplementation during differentiation does not have statistically significant additive and/or synergistic effects on downstream expansion capabilities. However, experiments from the same differentiation would need to be conducted in order to provide a rigorous comparison between SCAC Exp and SCAC Diff+Exp. Interestingly, in both conditions (SCAC Exp and SCAC Diff+Exp), X2A and SM6 had the highest increase in fold expansion, significantly higher than non-treated controls and UM171 iHPCs (Figure 3.8B).

A


SCAC Diff + Exp
Control
UM171
X2A
SM6
SMA (0.25X)
SMA (1X)
Expansion
Day 0
Day 7
Day 14

B


SCAC Diff + Exp
Fold expansion
Day of expansion
Control
UM171
X2A
SM6
SMA (0.25X)
SMA (1X)

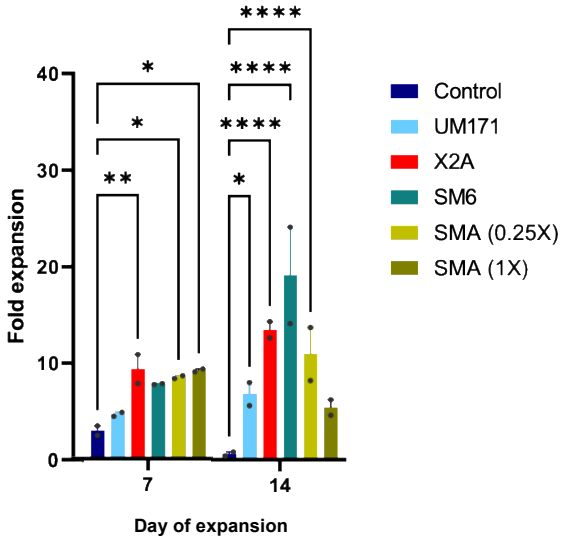

Fold expansion
Day of expansion
Control
UM171
X2A
SM6
SMA (0.25X)
SMA (1X)

Figure 3.8. SCAC supplementation during iPSC to iHPC differentiation and subsequent iHPC expansion.
(A) Representative phase contrast images (10X magnification) of iHPC expanded over 14 days supplemented with UM171 (35 nM) or SCACs: X2A (1X), SM6 (1X), or SMA (0.25X or 1X) or non-treated controls (control) (n=1 independent differentiation). Scale bars, 100 µm. (B) Fold expansion of iHPCs normalized to the initial plating density counts (day 0). Fold expansion represented as line (top row) and bar (bottom row) graphs for easier interpretation. Mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 as assessed by two-way ANOVA.

3.3.4 SCAC supplementation during differentiation and expansion enhances multilineage potential of iHPCs

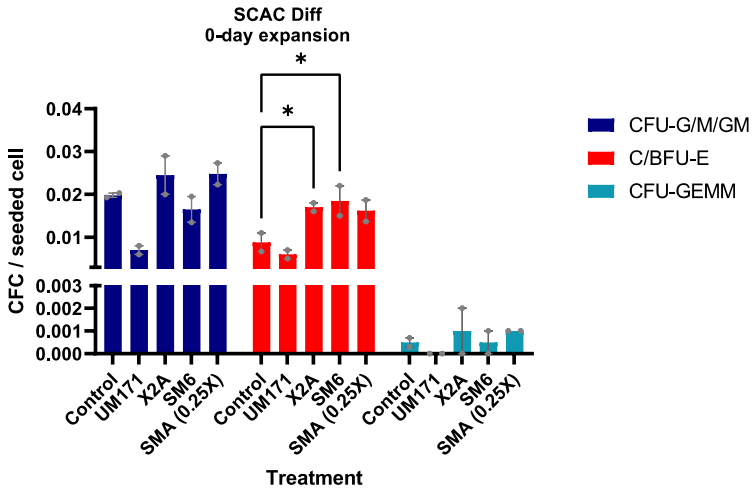
To confirm the retention of multilineage potency in SCAC Diff+Exp iHPCs, I subsequently performed CFU assays after 7 and 14 days of expansion. Non-treated control iHPC, gave rise to predominantly CFU-myeloid progenitors and a small fraction of CFU-erythroid progenitors; giving rise to a total of 0.0122 colony forming cells (CFC) / seeded cell by day 7 of expansion (Figure 3.9B). By 14 days of expansion, non-treated control iHPCs gave rise to only CFU-myeloid progenitors (0.04 CFC / seeded cell by day 14 of expansion, Figure 3.9C).

By comparison, almost all SCAC Diff+Exp iHPCs gave rise to higher numbers of CFU colonies than non-treated and UM171 cultures (Figure 3.9B and C). However, similar to non-treated controls, SCAC Diff+Exp iHPCs gave rise to predominantly CFU-myeloid progenitors with little to no CFU-erythroid progenitors (Figure 3.9B and C). More specifically, SCAC Diff+Exp iHPC expanded for 7 days gave rise to 0.0708 CFC / seeded cell (SM6, ****p < 0.0001), 0.0496 CFC / seeded cell (SMA 1X, ****p < 0.0001), 0.0437 CFC / seeded cell (SMA 0.25X, ****p < 0.0001), and 0.0403 CFC / seeded cell (X2A, ****p < 0.0001), compared to non-treated control

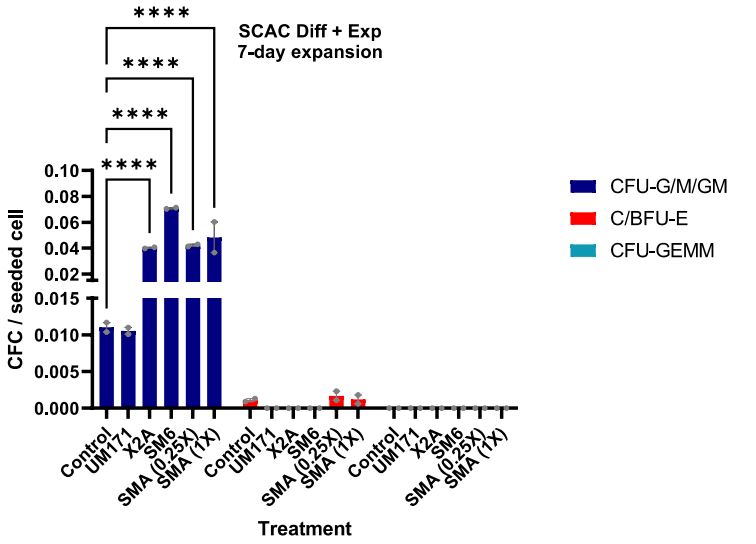
iHPC (0.0122 CFC / seeded cell) (Figure 3.9B). UM171 iHPCs gave rise to 0.0106 CFC / seeded cell (Figure 3.9B).

Meanwhile, SCAC Diff+Exp iHPC expanded for 14 days gave rise to 0.0922 CFC / seeded cell (SM6, ****$p < 0.0001$), 0.0215 CFC / seeded cell (X2A, ****$p < 0.0001$), 0.0199 CFC / seeded cell (SMA 1X, ****$p < 0.0001$), and 0.0110 CFC / seeded cell (SMA 0.25X, ***$p < 0.001$), compared to non-treated control iHPC (0.0002 CFC / seeded) and UM171 iHPCs 0.0098 CFC / seeded cell (Figure 3.9C). Compared to non-expanded control iHPCs (Figure 3.9A), higher numbers of CFU colonies at day 7 and 14 of expansion were observed for SCAC Diff+Exp iHPC (Figure 3.9B and C). These observations are consistent with CB-derived HSPCs, where SCAC supplementation demonstrated greater expansion of MPPs compared to controls [156]. However, the number of CFU colonies decreased substantially by day 14 of expansion suggesting potential iHPC exhaustion and loss of potency following prolonged SCAC supplementation and increased proliferation (Figure 3.9C). Similar to SCAC Exp, SCAC Diff+Exp iHPCs did not change the proportion of CFU-erythroid and CFU-myeloid progenitors compared to controls. Interestingly, I observed a progressive bias towards CFU-myeloid progenitors and a loss of CFU-erythroid progenitors during prolonged expansion (Figure 3.9B and C), which is consistent with trends of SCAC Exp iHPCs.

A


SCAC Diff
0-day expansion
*
*
CFC / seeded cell
0.04
0.03
0.02
0.01
0.003
0.002
0.001
0.000
Control
UM171
X2A
SM6
SMA (0.25X)
Treatment
CFU-G/M/GM
C/BFU-E
CFU-GEMM

B


****
****
****
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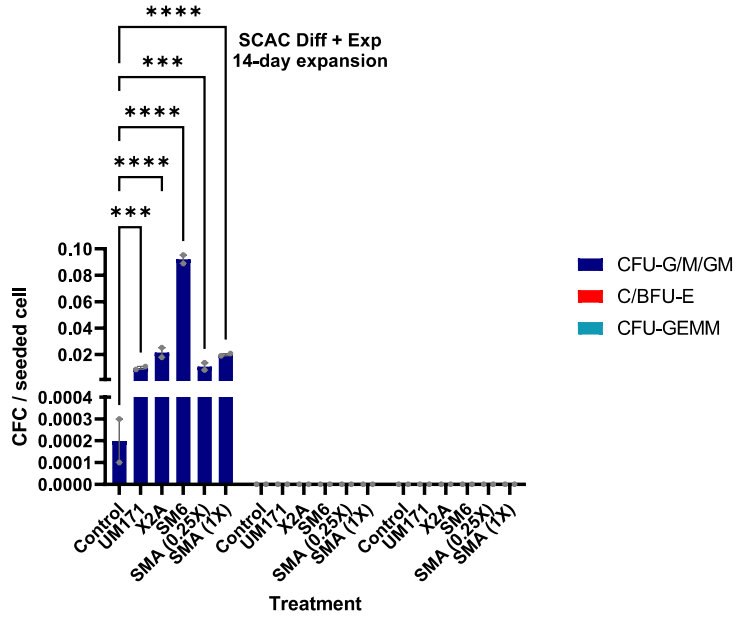
SCAC Diff + Exp
7-day expansion
CFC / seeded cell
0.10
0.08
0.06
0.04
0.02
0.015
0.010
0.005
0.000
Control
UM171
X2A
SM6
SMA (0.25X)
SMA (1X)
Treatment
CFU-G/M/GM
C/BFU-E
CFU-GEMM

C


****
***
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SCAC Diff + Exp
14-day expansion
CFC / seeded cell
0.10
0.08
0.06
0.04
0.02
0.0004
0.0003
0.0002
0.0001
0.0000
Control
UM171
X2A
SM6
SMA (0.25X)
SMA (1X)
Treatment
CFU-G/M/GM
C/BFU-E
CFU-GEMM

Figure 3.9. SCAC supplementation during iPSC to iHPC differentiation and iHPC expansion modulates potency of iHPC.
(A) CFC per seeded cell after iPSC to iHPC differentiation for 12 days with UM171 (35 nM) or SCACs (SCAC Diff): X2A (1X), SM6 (1X), or SMA (0.25X) (non-treated control and SMA 0.25X: n=3 independent differentiations; UM171, X2A and SM6: n=2 independent differentiations). (B) CFC per seeded cell after iPSC to iHPC differentiation and iHPC expansion over two weeks. Cells were supplemented with UM171 (35 nM) or SCACs (SCAC Diff+Exp): X2A (1X), SM6 (1X) or SMA (differentiation: 0.25X; expansion: 0.25X or 1X) (n=1 independent differentiation). Cells were plated into individual 35 mm dishes in iPSC-specific MethoCult SF H4636 semi-solid medium. Colonies were counted 14 days after plating. BFU, burst-forming unit; CFU, colony-forming unit; E, erythroid; G, granulocyte; GEMM, granulocyte, erythrocyte, monocyte, macrophage; GM, granulocyte/macrophage; M, macrophage. Mean ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ as assessed by two-way ANOVA.

Chapter 4 Discussion

The scaling up of iHPC production holds significant importance in various clinical applications, such as HSC transplantation and personalized cell therapies, which require a substantial number of functional cells [72,77,210,211]. More specifically, adult patients receiving autologous HSC transplantations often require a target dose of 5×10^6 $CD34^+$ cells/kg [212] and adults receiving *ex vivo* expanded CB cells require 1.8×10^7 TNC/kg and 1.5×10^5 $CD34^+$ cells/kg [213]. In fact, the success rate of engraftment and patient survival after CB-derived HSC transplantation is dependent upon the dose of $CD34^+$ cells and TNC [214,215]. Thus, there is a growing need to optimize a clinically relevant biomanufacturing process for generating an adequate number of functional iHSCs and iHPCs to support transplantation demands. Although iPSCs represent a promising alternative to CB- and PBMC-derived HSC sources, given their robust self-renewal and differentiation capabilities, current efforts of generating iHSCs and even clinically relevant doses of iHPCs remain a challenge. Currently, the differentiation of iPSCs into iHPCs yields only small quantities (average yield: 7.8×10^4 cells/cm^2), highlighting the need for iHPC expansion as a method to scale up their production [181,187]. However, current strategies utilizing small molecules like SR1 and UM171 have only achieved moderate expansion of iHPCs [137,166–168,206]. Thus, there is a pressing need to develop novel strategies that can effectively and robustly scale up the production of iHPCs while maintaining their functional properties.

Towards this goal, SCACs have demonstrated positive effects on the expansion of CB-HSPCs due to the unique additive and synergistic nature of the combined SCAs [156,160–162]. However, the impacts of SCACs in the expansion and differentiation of iHPCs has not been explored. My

study aimed to investigate the potential effects of SCACs on iHPC expansion. Additionally, as epigenetic modifications play a crucial role in the iHPC differentiation process, targeting epigenetic regulation represents an attractive approach for modulating iHPC differentiation and yields. Indeed, the Pineault lab has provided evidence that UM171 and AA2P promote expansion of CB-HSPCs through epigenetic reprogramming, exerting distinct effects on the epigenetic profile of HSPCs [156,160–162].

4. 1 Directed Monolayer Differentiation: A Reproducible and Adaptable Platform for iHPC Generation

The generation and expansion of iHPCs has been the focus of several studies, with each employing distinct strategies and culture conditions throughout various stages of iPSC differentiation. A notable finding in this field is the prevalent use of the monolayer hematopoietic differentiation strategy in the majority of HSC-focused studies [76,80,166,183,206,207,216,217], with only the study by Angelos *et al.* employing the EB formation approach [168]. Although I also assessed an EB strategy to generate iHPCs, I found that this yielded an iHPC cell population with an immature pro-definitive-like HPC phenotype that is more uniquely suited for further differentiation and specification into NK and T cells (unpublished data from Jezierski lab) and as described in previous studies [168,218,219].

In this study, I generated iHPCs using the commercial monolayer STEMdiff™ Hematopoietic Kit, a widely used and well-established strategy for the generation of hematopoietic cells from human iPSCs [76,180,181,186–188,190,220]. Although the specific composition of the STEMdiff™

Hematopoietic Kit is not disclosed due to proprietary reasons, the kit composition is based on previously reported studies [76]. These described methods include the induction of mesoderm formation with bFGF (basic fibroblast growth factor), BMP4, and VEGF (vascular endothelial growth factor), and HE formation and hematopoietic specification with bFGF, BMP4, FLT3L, SCF, TPO, and VEGF [76]. Similar cytokines and growth factors have been used in previous studies to differentiate iPSCs towards iHPCs [76,180,181,187,188,190,220].

This protocol generated 2.19 x 10^5 cells/cm^2 of iHPCs after D12 of differentiation, similar to previously reported yields of 2.50 x 10^4 cells/cm^2 and 1.31 x 10^5 cells/cm^2 using this kit [181,187]. Focusing on phenotypically defined iHPCs, I was able to generate 2.02 x 10^4 cells/cm^2 of $CD45^+CD34^+$ iHPCs which is similar to Ruiz *et al.* who generated 1 x 10^5 cells/cm^2 of $CD45^+CD34^+$ iHPCs and 4.86 x 10^4 cells/cm^2 of $CD45^{lo}CD34^{hi}$ iHPCs [76,186]. These yields are in line with what has been reported by Stem Cell Technologies which tested three iPSC and two ESC lines showing yields ranging from 1 x 10^5 to 2 x 10^5 $CD45^+CD34^+$ cells/cm^2 and 5 x 10^4 to 1 x 10^5 $CD45^+CD34^+$ cells/cm^2, respectively [221]. Collectively, these findings highlight that not all iPSC lines are created equal and that iHPC differentiation fidelity and yield are influenced by the iPSC line used. Differences between donor individuals, genetic stability and experimental variability contribute to iPSC model variation by impacting differentiation potency, cellular heterogeneity, morphology, and transcript and protein abundance [222]. Nevertheless, the protocol has been shown to consistently generate a population of $CD45^+CD34^+$ iHPCs across multiple iPSC and ESC lines.

Ruiz *et al.* further assessed the iHPCs derived through the monolayer STEMdiff™ kit demonstrating that it effectively reproduced the primitive (first) and definitive (second and third) waves of hematopoiesis observed in the yolk sac during embryonic development [76]. Consistent with the cell populations produced in primitive hematopoiesis, I observed $CD45^{+}CD235a^{+}$ and $CD45^{-}CD235a^{+}$ erythroid progenitor-like cells. Additionally, I also observed $CD235a^{lo}CD41a^{+}$ MEP-like cells, $CD34^{+}CD31^{+}$ hemogenic endothelial progenitor cells, and importantly, a yield of 4.16×10^{3} cells/cm^{2} of $CD45^{+}CD34^{+}CD45RA^{-}CD90^{+}$ immunophenotypically HSC-like cells, in line with cell populations present in definitive hematopoiesis. While Ruiz *et al.* focused on a more expanded marker panel, they also identified a low percentage of HSC-like immunophenotype (4×10^{3} cells/cm^{2} of $CD34^{+}CD38^{-}CD45RA^{-}CD90^{+}CD49f^{+}$ cells), albeit with no long-term engraftment potential *in vivo*, as previously described [76,166,186,220]. Nevertheless, this protocol has been shown to be reproducible and provides a clinically relevant and readily adaptable platform for the generation of erythroid and multilineage progenitors from iPSCs.

4. 2 UM171-mediated iHPC expansion

The expansion media that was used in this study was StemSpan™ SFEMII and $CD34^{+}$ Expansion Supplement. Although this media has been formulated for the expansion of TNCs and $CD34^{+}$ cells from CB, BM, or other cell sources [221], it has also been used for the expansion of iHPCs in other studies [80,223]. To date, the majority of SCA efforts focused on expanding human pluripotent stem cells has focused on using UM171 and SR1. In fact, the clinical use of UM171 has demonstrated its feasibility, safety, and ability to expand CB-derived HSPCs with successful

engraftment[141]. This underscores the significance of UM171 as a valuable and relevant candidate for expanding iHPCs.

In my study, the supplementation of iHPCs with UM171 did not significantly increase the fold expansion of iHPCs compared to non-treated iHPCs beyond the initial 4 days of culture. These observations are consistent with Brok-Volchanskaya *et al.* who assessed UM171 supplementation from day 3 to 29 of hematopoietic differentiation, but saw no significant effect of UM171 on the TNCs of iHPCs [206]. In another study, Mesquitta *et al.* supplemented UM171 during 5 or 7 days of iHPC expansion, following 9 days of differentiation for iPSC- and ESC-derived HPCs [137]. Using ESC-derived HPCs, Mesquitta *et al.* observed a significant UM171 induced increase in the frequency of $CD45^+CD34^+CD43^+$ cell populations (1.8×10^5 and 0.9×10^5 cells after 5 and 7 days of expansion, respectively) [137]. However, when Mesquitta *et al.* repeated the studies using iPSC-derived HPCs, they observed no significant change in the frequency of $CD45^+CD34^+CD43^+$ cell populations [137]. Furthermore, using the ESC-EB culture system, Angelos *et al.* supplemented SR1 to cultures during the hematoendothelial induction and differentiation phase (D6-15) [168]. SR1 treatment significantly increased $CD34^+CD43^+$ and $CD45^+CD34^+$ cell populations (1.4- and 1.6- fold increase of $CD34^+CD43^+$ and $CD45^+CD34^+$, respectively) from D6-12 [168]. However, SR1 treatment during D6-15 only increased yields of the $CD34^+CD43^+$ cell subset [168]. When Angelos *et al.* assessed SR1 treatment in two different iPSC lines, no significant changes were found in $CD34^+CD43^+$ and $CD45^+CD34^+$ cell populations [168]. Although these studies used different differentiation protocols and treatment regimens, and focused on different cell subsets for analysis, they highlight some key findings: ESC- and iPSC-derived HPCs respond differently to SCAs; the latter being less responsive to SCA-mediated

expansion, and that the timing of SCA supplementation is an important factor. This further underscores the need for optimal dosing and SCA formulations specifically tailored for iHPCs. Furthermore, it also highlights that the ideal window of SCA supplementation remains to be defined and is human pluripotent stem cell type dependent. The challenge of finding this ideal window is further compounded by the different differentiation protocols employed, which limits direct comparisons.

4. 3 SCAC-mediated iHPC expansion

Since the SCACs used in this study all contained UM171 and SR1, I sought to compare whether the additive and synergistic effects of a combination of SCAs would have an influence on iHPC expansion yields. In general, SCACs showed a trend towards a higher TNC fold-expansion after 7 (X2A: 2.1-fold, SM6: 1.5-fold, SMA (1X): 1.4-fold, UM171: 1.3) and 14 days (X2A: 3.5-fold, SM6: 2.8-fold, SMA (1X): 2.0-fold, UM171: 1.4-fold). Interestingly, instead of looking at TNCs, Mesquitta *et al.* looked at discrete cell populations of iHPCs following UM171 supplementation. UM171 treatment resulted in a 4.1-fold increase in $CD34^+CD43^+$ cell population and a 3.5-fold increase in $CD45^+CD34^+CD43^+$ cell population [80]. Additionally, they observed a frequency of 35% $CD45^+CD34^+CD43^+$ cells, which represents a 3.5-fold increase [80]. Overall, SCAC-mediated fold expansion of TNCs was lower than UM171-mediated fold change of $CD34^+CD43^+$ and $CD45^+CD34^+CD43^+$ cell populations observed by Mesquitta *et al* [80]. Collectively, these observations are consistent with previous findings that analyzing the fold expansion and frequency of discrete cell subsets may be a more sensitive method to detect SCAC-induced

changes and identifying target cells. Applying this approach in future studies will potentially enable identification of important alterations in population dynamics.

Nevertheless, all SCACs exhibited increased expansion of TNCs, with X2A and SM6 showing the highest levels of expansion. Interestingly, these findings are consistent with previous observations in the expansion of CB-derived TNC conducted by the Pineault lab [156,160–162]. The Pineault lab also showed that X2A supplementation resulted in a significantly higher expansion of the $CD34^{+}CD45RA^{-}$ HSPC subset compared to UM171 [156,160–162]. These findings suggests that X2A may be selectively targeting the expansion of this distinct stem cell population. We did not assess the presence of this subset in the iHPCs, but it is likely that this would represent a very small subset of iHPCs compared to CB-HSPCs. Interestingly, the fold expansion of TNCs in non-treated CB-HSPCs was superior to non-treated iHPCs, suggesting that CB-HSPCs have greater expansion capacities at baseline than iHPCs. However, when normalized to non-treated controls, CB-HSPCs (X2A: 2.8-fold, SM6: 2.8-fold, SMA: 1.4-fold) [156,160–162] and iPSC-derived iHPCs (X2A: 3.5-fold, X2A+GAS6: 3.8-fold, SM6: 2.8-fold, SMA: 2.0-fold) showed comparable fold expansion of the TNC. Overall, X2A and SM6 supported the highest expansion yields of iHPCs, in agreement with observations from the Pineault lab for CB-HSPCs. Furthermore, the expansion effects of SCACs on the TNCs of iHPC recapitulates the observations found in CB-HSPCs. However, it remains to be determined which specific hematopoietic cell subsets may be preferentially being expanded. Thus, it will be valuable to explore distinct HSPC subsets to determine whether the composition of the expanded TNC differ between iHPCs and CB-HSPCs.

Lastly, I also wanted to assess whether GAS6, shown to act as a growth factor in CB-HSPCs (personal communication) [161], would enhance the expansion of iHPCs. The Pineault lab demonstrated that GAS6 promoted expansion of CB-HSPCs through AXL signaling, with X2A showing the greatest upregulation of AXL amongst all SCACs [161]. To assess whether a similar effect could be seen in iHPCs, I expanded cells with X2A alone or X2A with the addition of GAS6 (X2A+GAS6). While there was no significant change in TNCs between X2A and X2A+GAS6, both X2A and X2A+GAS6 also showed significantly higher expansion of TNCs compared to non-treated controls and UM171 positive controls. Contrastingly, the Pineault lab demonstrated significantly higher expansion of $CD34^{+}CD45RA^{-}$ cells by X2A+GAS6 compared to X2A alone [156,160–162]. However, this observation does not rule out the possibility that there could be a difference between X2A- and X2A+GAS6-mediated expansion in the $CD34^{+}CD45RA^{-}$ iHSPC subsets. Thus, this will require further investigation.

Furthermore, to ensure the biological activity of the GAS6/AXL signaling pathway, two conditions must be met: (1) AXL, a receptor of GAS6, must be expressed by the iHPC and (2) GAS6 must be available in its carboxylated form. In my studies, I did not detect any significant differences in yield and phenotype when comparing iHPC cultures expanded with X2A alone and those with X2A+GAS6 supplementation. However, an important limitation of this study is that AXL expression in iHPCs was not assessed. Consequently, I cannot rule out that the observed minimal impact of X2A+GAS6 supplementation might stem from low AXL expression on iHPCs. Notably, no previous research has explored AXL expression in iHPCs, underscoring the need for further exploration in this area. Furthermore, the production of biologically active GAS6 involves a vitamin K-dependent posttranslational modification catalyzed by gamma-

glutamyl carboxylase [224]. This modification extensively γ-carboxylates the newly synthesized peptide within the endoplasmic reticulum, a process that is essential for GAS6 to interact with and activate its corresponding tyrosine kinase receptors [165]. Therefore, I cannot rule out that the limited impact of X2A+GAS6 supplementation could due to the absence of carboxylated GAS6. Thus, in future studies, it would be beneficial to validate that the exogenously supplemented GAS6 is carboxylated to ensure GAS6 is available in its biologically active form.

4. 4 Comparative analysis of SCACs and individual SCAs during iHPC differentiation and/or expansion

In addition to SCA/SCAC supplementation during terminally differentiated iHPC expansion, I also investigated SCA/SCAC supplementation during various differentiation phases of hematopoietic differentiation to assess their impact on differentiation fidelity and yields. In brief, SCAs/SCACs were supplemented during hematopoietic specification (D7-12), HE formation to hematopoietic specification (D5-12), or during the entire hematopoietic differentiation period (D0-12). We subsequently assessed both TNC yields and further examined the yields of a subset of hematopoietic cell subtypes. Although there was no significant difference in TNCs between SCAC and UM171 after 12 days of iHPC differentiation; there was a significantly higher proportion of HPCs and HSC-like cells in SCAC-treated cells compared to UM171 and non-treated controls. These observations highlight that the SCACs may be preferentially targeting these discrete cell population compared to UM171 alone, in alignment with previous reports [80,166]. Comparatively, Li *et al.* also assessed UM171 supplementation at multiple stages of hematopoietic differentiation, including from iPSCs to HE (D0–6), from mesoderm to HPCs

(D4–14), and from HE to HPCs (D6–14). Following UM171 supplementation of ESC-derived HPCs from D6-14, Li *et al.* observed a significant increase in the frequency of $CD34^+CD43^+$ and $CD45^+CD34^+$ cell populations [166]. However, when Li *et al.* subsequently repeated the study in iPSC-derived HPCs, they observed no significant changes in the frequency of $CD34^+CD43^+$ and $CD45^+CD34^+$ cell populations [166]. Similar to Li *et al.,* the highest yield of phenotypic HPCs was generated when SCACs were supplemented throughout the entire hematopoietic differentiation period (D0-12). These observations further demonstrate that there are fundamental differences in the effects of UM171 between ESC and iPSC cell sources.

To assess whether SCAC supplementation during differentiation and expansion would have a combined additive effect on iHPC yields, I transitioned the iHPCs into expansion media supplemented with SCAC for an additional 7-14 days. In my study, the supplementation of iHPCs with UM171 did not demonstrate a statistically significant fold increase beyond the initial 4 days of culture. Nonetheless, all the SCAC treatments significantly increased iHPC yields, emphasizing that the enhanced cell expansion is a result of the combined effects of the SCACs. Notably, X2A and X2A+GAS6 showed significantly superior expansion of TNCs compared to UM171-treated controls, while SM6 and SMA demonstrated a trend towards superior TNC expansion compared to UM171-treated controls. Thus, the use of SCAC during iHPC differentiation and expansion represents a notable improvement over existing SCA approaches as summarized in Supplementary Table 1.

4. 5 The impact of SCACs on multilineage potency of iHPCs

Since iHPC expansion can lead to a decrease in the progenitor pool and potential loss of multilineage differentiation capability, I sought to evaluate the multilineage potency of the iHPCs at the different stages of SCAC treatments. Following 12 days of differentiation, controls iHPCs contained a higher frequency of CFU-myeloid progenitors compared to CFU-erythroid progenitors, with little to no multipotent CFU-GEMM progenitors. This predominance of CFU-myeloid progenitors is in line with many studies differentiating iHPCs from iPSCs [76,78,187,188,190]. By D14 of SCAC-supplemented expansion, there was a notable bias towards CFU-myeloid progenitors compared to CFU-erythroid progenitors, with little to no multipotent CFU-GEMM progenitors. This observation aligns with the increased frequency of $CD45^{+}CD14^{+}$ myeloid committed-like cells and the decreased frequency of $CD45^{-}CD235a^{+}$ erythroid progenitor-like cells. The reduction of CFU-erythroid progenitors after SCAC treatment is in line with the repression of erythroid differentiation programs in X2A-mediated expansion of CB-HSPCs [156,160–162]. UM171 supplementation during 7 and 14 days of expansion did not lead to a significant difference in the number of colonies. Assessing the timing of colony formation, higher numbers of CFU colonies were observed at day 7 compared to day 14 of expansion. However, the number of CFU colonies decreased substantially in the second week of expansion suggesting potential exhaustion or loss of potency with increased proliferation. This observation is consistent with the progressive differentiation of iHPCs in extended cultures [76]. Nevertheless, all SCAC-expanded iHPCs trended towards higher numbers of CFU colonies compared to non-treated controls and UM171-expanded cultures. Similar to the increased CFU colony formation observed with SCAC supplementation, the Pineault lab demonstrated in CB-HSPCs that SCAC supplementation resulted superior short- and long-term engraftment[156].

4. 6 The combinatorial effect of SCACs during differentiation and expansion on the multilineage potency of iHPCs

To investigate the potential combinatorial additive effects of SCAC supplementation during both differentiation and subsequent expansion, I transferred the iHPCs to expansion media supplemented with SCAC for an additional 7-14 days. In my studies, UM171 Diff+Exp iHPC after 7 and 14 days of expansion were composed of only CFU-myeloid progenitors, with a complete loss of CFU-erythroid and CFU-GEMM progenitors. I observed no significant difference in total CFU or any CFU types between UM171 Diff+Exp iHPC and non-treated control cultures after 7 days of expansion. However, there was a significantly higher number of CFU-myeloid progenitors in UM171 Diff+Exp iHPC compared to non-treated controls after 14 days of expansion. This is similar to observations by Mesquitta *et al.* in ESC-derived hematopoietic cells, which demonstrated a significant increase in CFU-G colonies in UM171 compared to DMSO-expanded cells at day 5 and 7 of expansion [137]. However, in iPSC-derived hematopoietic cells, there was no significant difference in CFU-G between UM171- and DMSO-expanded cells. My findings indicate the possibility of iHPC exhaustion and reduced potency after prolonged UM171 supplementation and increased proliferation. However, UM171 supplementation during differentiation and expansion may delay iHPC exhaustion and loss of potency.

Similar to UM171, SCAC Diff+Exp iHPC after 7 and 14 days of expansion were composed of only CFU-myeloid progenitors, with a loss of CFU-erythroid and CFU-GEMM progenitors. All

SCAC Diff+Exp iHPC showed significantly higher expansion of CFU-myeloid progenitors compared to UM171 Diff+Exp iHPC after 7 and 14 days of expansion. The number of CFU colonies decreased by day 14 of expansion suggesting potential iHPC exhaustion and loss of potency following prolonged SCAC supplementation and culture. Similar to UM171, SCAC supplementation during differentiation and expansion may delay iHPC exhaustion and loss of potency; however, this would require further investigation and a higher number of biological replicates. Overall, these results indicate that the supplementation of SCACs during iHPC differentiation and expansion did not negatively affect the differentiation efficiency or multilineage potential while increasing TNC yields.

In conclusion, a reoccurring observation across all conditions and timepoints is that there is a predominance of CFU-myeloid progenitors. It has been demonstrated that iHPCs from multiple iPSC lines recapitulated DNA methylation (DNAm) patterns of the monocytic lineage seen in primary hematopoietic cells [78]. Epigenetic alterations indicating monocytic differentiation may contribute to the bias of iHPCs towards the myeloid lineage however, this would require further investigation. With respect to the expansion of iHPCs, an important observation is that the potency of iHPCs appears to decline after 14 days of expansion. Our results highlight that there is a limited expansion window for iHPCs, with a timeframe of 7 days showing better results compared to 14 days of expansion. This suggests that there is a finite period during which iHPCs can be successfully expanded before a loss of potency and spontaneous differentiation into more committed HPCs. These findings underscore the importance of optimizing the expansion protocols for iHPCs and emphasize the need to carefully consider the duration of expansion. Future studies should aim to identify the underlying mechanisms that contribute to the limited

expansion window of iHPCs and explore strategies to extend their expansion potential while preserving their potency.

4. 7 Mechanisms of SCAC-mediated iHPC expansion

The mechanism underlying HSPC expansion induced by SCAs and SCACs is not fully understood. Among the various SCAs incorporated in SCACs, two notable ones are SR1, functioning as an AHR antagonist, and UM171, a pyrimidoindole derivative. UM171 plays a crucial role in maintaining the equilibrium between pro- and anti-inflammatory pathways and achieves this by suppressing the LSD1/RCOR1 suppressor complex through proteosomal degradation, preserving crucial epigenetic marks within HSPCs, which are diminished in *ex vivo* human HSC cultures [134,138,225,226]. HDAC inhibitor VPA maintains an open chromatin configuration to facilitate the expression of pluripotency and self-renewal genes [142–144]. Vit-C, recognized for global hypomethylation induction [227], is paralleled by its derivative, AA2P, which has been demonstrated to induce DNA demethylation works synergistically with SCACs to enhance the expansion of HSPCs through the activation of the GAS6/AXL signaling axis. It is becoming increasingly recognized that SCAC-mediated HSPC expansion involves the suppression of differentiation programs mediated, in part, by epigenetic reprogramming [161]. The collective epigenetic activities induced by SCACs could provide an explanation for the observed heightened efficacy of SCACs in fostering HSC-like phenotypes when introduced during the initial phases of iPSC cultivation, prior to the reinforcement of lineage-specific epigenetic marks.

The Pineault lab demonstrated that X2A-mediated expansion of CB-HSPCs was partially driven by the repression of erythroid and megakaryocytic differentiation programs, but not SM6 [156,160–162]. Similarly, I saw that SCAC supplementation resulted in a reduction of CFU-erythroid progenitors and trends towards a loss of MEP-like cells, erythroid progenitor-like cells, and myeloid committed-like cells. In contrast, SCAC-mediated expansion increased the population of megakaryocyte committed-like cells and increased the expression of hematopoietic marker CD45, suggesting a push towards hematopoietic commitment. However, additional experiments are required to confirm whether SCAC-mediated expansion suppresses differentiated immunophenotypes as in CB-HSPCs. Additionally, further investigation is warranted to dissect how lineage-specific differentiation programs are affected and to confirm the phenotype and function of the hematopoietic cells generated by SCAC-mediated expansion.

To build on these observations, epigenetic regulation plays a crucial role across the spectrum of stem cell development, HSC generation, and HSC differentiation. In the iPSC state, the epigenetic state of DNA remains open, allowing for minimal expression of lineage-specific genes without repression by epigenetic rearrangement [228,229]. This open chromatin configuration sustains iPSC self-renewal and pluripotency, facilitating rapid differentiation through nuclear organization, DNAm, and histone modifications [230]. During HSC development via endothelial-to-hematopoietic transition, HSC-specific chromatin loops strengthen progressively, accompanied by pre-established active histone modifications, which are further enhanced in LT-HSCs [231]. Previous studies have demonstrated that the initial stages of *bona fide* hematopoietic development are also characterized by dynamic alterations in DNAm patterns, with a continuous gain or loss of DNAm occurring during the differentiation process[232,233]. Epigenetic

modifications, specifically DNAm of CpG islands, are integral for normal development[234,235]. Subsequently, as HSCs mature into distinct blood lineages, the lineage-mediating protein BAP1 assumes a pivotal role, orchestrating histone modifications on promoters of pro-hematopoietic and myelopoiesis-promoting factors, regulating the delicate balance between lymphopoiesis and myelopoiesis [236,237].

iHPCs have been shown to exhibit similar epigenetic patterns to their primary cell counterparts, suggesting that the epigenetic landscape is faithfully, but incompletely, recapitulated during *in vitro* differentiation[78]. Prior studies have shown that many SCAs in SCACs, including VPA, UM171 and AA2P, promote HSPC expansion via epigenetic reprogramming[138,149]. Given that SCACs contain potent epigenetic modifiers, it is probable that SCAC-mediated expansion of iHPCs in this study can be attributed, in part, to epigenetic reprogramming. Thus, it will be important to understand the epigenetic modifications that accompany iHPC differentiation to better inform SCAC formulations to more selectively target the proper epigenetic reprogramming events.

4. 8 Conclusions and Future Directions

Considering the current evidence, the SCAC strategy, employing the additive and synergistic effects of multiple small molecules, represents a promising approach for enhancing iHPC expansion and functionality compared to single SCAs, such as UM171 and SR1. Since the SCACs were developed and optimized for the CB-HSPCs, further optimization in formulations is required to achieve the same outcomes in iHPCs. This is due to the fundamental differences

between CB-HSPCs and iHPCs. CB-HSPCs exhibit a more HSC-like phenotype, possessing long-term repopulating capability and demonstrating higher expansion rates *in vitro* when stimulated with SCACs. In contrast, iHPCs are more committed and represent a more advanced stage of hematopoietic differentiation and hence are lacking an HSPC equivalent cell population that may more positively respond to the SCACs. The observed differences in expansive capacities, phenotype, and multilineage potential between CB-HSPCs and iHPCs suggest that novel SCAC formulations may be necessary to optimize the expansion and functionality of iHPCs.

The findings from my study revealed an important limitation in the current monolayer approach for iHSC generation, which warrants further investigation. One possible reason for the limited success in generating iHSCs could be the timing of SCAC supplementation. Initiating the expansion at D12 of iPSC differentiation might be too late to efficiently generate iHSCs. Since SCACs are potent epigenetic modifiers, commencing SCAC supplementation at an earlier stage of iPSC differentiation may be crucial to promote the development of iHSCs with enhanced self-renewal and differentiation capabilities.

Another aspect that needs careful consideration is the understanding of the underlying mechanisms governing iHSC generation. Despite robust differentiation protocols for various hematopoietic lineages, the generation of true iHSCs has proven to be elusive. A better comprehension of the signaling pathways and transcriptional regulators that promote HSC fate specification is necessary to overcome this major limitation. By targeting these key factors

through small molecules or gene-based approaches, we may be able to steer the differentiation process more effectively towards the generation of functional iHSCs.

In conjunction to generating iHSCs and iHPCs, it will also be important to identify accurate markers for iHPCs and iHSCs. Employing single-cell RNA sequencing can refine the identification of precise markers for iHPCs and iHSCs. This will validate the flow cytometry data in this study, reveal upregulated genes during expansion, and guide the formulation of SCACs targeting these genes. The integration of single-cell RNA sequencing data into SCAC design promises to enhance iHPC/iHSC production efficiency and quality.

In conclusion, the use of SCACs in enhancing iHPC expansion shows promise as an approach to improve iHPC potential compared to single SCAs. However, further optimization of SCAC formulations is required for iHPCs, given their fundamental differences from CB-HSPCs. The timing of SCAC supplementation during iPSC differentiation should be carefully examined to maximize iHSC generation. Additionally, a deeper understanding of the molecular mechanisms governing iHSC specification and identifying precis markers for iHPCs and iHSCs is crucial for developing more effective small molecules or gene-based approaches strategies. By addressing these challenges, we can advance towards the goal of generating and expanding functional iHSCs for regenerative medicine applications.

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Contributions of Collaborators

Casey Wong (C.W.) designed, conducted, and analyzed the experiments, interpreted the experimental data, created the figures, and wrote the thesis. Junzhuo Huang (J.Z.), Ewa Baumann (E.B.), and Claudie Charlebois (C.C.) provided assistance with iPSC differentiation and maintenance, while J.Z. also aided in phenotypical and expansion imaging analyses and offered guidance on data analysis in Figure 3.1, Figure 3.2, Figure 3.4, Figure 3.6, and Figure 3.8. Chelsea McGregor (C.M.) conducted phenotypical analysis and provided advice on data analysis in Figure 3.6 and Table 3.1. E.B. conducted MGG staining in Figure 3.5. Nicolas Pineault (N.P.) provided valuable scientific feedback and insights and contributed to the writing of the thesis. Lastly, Anna Jezierski (A.J.) developed the concept, led, and supervised the studies, analyzed and interpreted the data, and contributed to thesis writing.

Appendices

Chapter 5 Supplemental Results

5. 1 SCAC supplementation during iHPC expansion does not change the frequency of CD235a^{lo}CD41a^{+} MEP-like cells

CD235a^{+}CD41a^{+} cells have been shown to be enriched in bipotent MEPs which have the ability to differentiate into platelet-producing megakaryocytes, as well as hemoglobinized erythroblasts exhibiting primitive and fetal characteristics[207]. In my analysis, the expression of CD235a and CD41a was classified into two cell subsets: CD235a^{hi}CD41a^{-} and CD235a^{lo}CD41a^{+}. Overall, non-treated iHPCs (without expansion) had the highest frequency of CD235a^{hi}CD41a^{-} cells (34.2%), whereas frequencies of CD235a^{hi}CD41a^{-} cells decreased with expansion [day 7 (2.87%) and day 14 (6.71%, Supplementary Figure 3)]. The frequencies of CD235a^{lo}CD41a^{+} cells in non-treated iHPCs remained unchanged at 0 (29.2%) and 7 (30.6%) days of expansion (Supplementary Figure 3); however, these decreased significantly by day 14 of expansion (11.6%) (Supplementary Figure 3).

Focusing on CD235a^{lo}CD41a^{+} MEP-like cells, SCAC supplementation during expansion for 7 days decreased frequencies of CD235a^{lo}CD41a^{+} cells compared to non-treated controls (Supplementary Figure 3). iHPCs supplemented with SCACs for 14 days of expansion showed CD235a^{lo}CD41a^{+} frequencies of 14.4% (SMA), 6.24% (X2A+GAS6), 4.40% (X2A), and 3.13% (SM6); UM171 supplementation had a frequency of 10.8% (UM171) CD235a^{lo}CD41a^{+} cells (Supplementary Figure 3). Although there was a trend that SCAC supplementation decreased the

frequency of MEP-like cell, there were no statistically significant changes between SCAC supplementation and non-treated controls.

5. 2 SCAC supplementation during iHPC expansion does not change the frequency of erythroid progenitor-like cells

I also investigated the impact of SCACs on the generation of phenotypic erythroid-progenitor-like cells, characterized by expression of CD45 and CD235a[98,238–242]. Since the expression of CD45 and CD235a on erythroid progenitors varies[238–242], the cells were separated into two subsets: $CD45^{+}CD235a^{+}$ and $CD45^{-}CD235a^{+}$ erythroid progenitor-like cells. In both non-treated and SCAC-supplemented cultures, only a very small (0.72% to 2.00%) percentage of cells were positive for $CD45^{+}CD235a^{+}$ after 0 and 7 days of expansion (Supplementary Figure 4). By day 14 of expansion, non-treated (7.83%), UM171- (10.1%) and SCAC- (14.7% for X2A+GAS6, 11.1% for SM6, 8.97% for X2A, 1.02% for SMA) supplemented iHPC demonstrated increased frequencies of $CD45^{+}CD235a^{+}$ cells compared to non-treated controls on day 0 of expansion (Supplementary Figure 5). Most notably, SCAC supplementation with X2A+GAS6 produced iHPCs with the highest frequency of $CD45^{+}CD235a^{+}$ cells (Supplementary Figure 5). Overall, while there is a trend that the frequencies of $CD45^{+}CD235a^{+}$ erythroid progenitor-like cells increased during expansion, there were no statistically significant differences between days of expansion. Supplementation of iHPC cultures with X2A+GAS6 during expansion for 14 days trends towards bolstering the population of $CD45^{+}CD235a^{+}$ erythroid progenitor-like cells.

Based on $CD45^{-}CD235a^{+}$ expression, non-treated control iHPCs harvested without expansion had the highest frequency of $CD45^{-}CD235a^{+}$ cells (38.3%), whereas continued expansion of control iHPCs for 7 (7.12%) and 14 days (2.24%) resulted in decreased frequencies of $CD45^{-}CD235a^{+}$ cells (Supplementary Figure 4 and Supplementary Figure 5). SCAC supplementation during expansion for 7 days showed a decreased frequency of $CD45^{-}CD235a^{+}$ cells compared to non-treated controls, with 2.92% (X2A), 2.34% (SM6), 2.16% (X2A+GAS6), and 1.56% (SMA) and 2.35% (UM171) (Supplementary Figure 4). iHPCs supplemented with SCACs or UM171 for 14 days of expansion showed negligible frequencies of $CD45^{-}CD235a^{+}$ cells (Supplementary Figure 5). In summary, expansion of iHPC and SCAC supplementation shows a trend towards the loss of $CD45^{-}CD235a^{+}$ erythroid progenitor-like cells.

5. 3 SCAC supplementation during iHPC expansion does not change the frequency of megakaryocyte committed-like cells

I also investigated the impact of SCAC on the generation of phenotypic megakaryocyte-like cells, characterized by expression of CD41a and CD45[80,98]. Given that Mesquitta *et al.* identified HPCs enriched in G-CFCs with a $CD34^{+}CD41a^{lo}CD45^{+}$ phenotype[80], I separated harvested cells into two subsets: $CD45^{+}CD41a^{hi}$ and $CD45^{+}CD41a^{lo}$. Looking at non-treated control iHPC cultures, $CD45^{+}CD41a^{hi}$ cells appears to peak at day 7 of expansion (15.9%), compared to day 0 (4.13%) and 14 (11.4%) (Supplementary Figure 4 and Supplementary Figure 5). At day 7 of expansion, SCAC and UM171 supplementation decreased the frequencies of $CD45^{+}CD41a^{hi}$ compared to non-treated controls (7.79% for SMA, 7.36% for SM6, 5.95% for X2A, 5.41% for X2A+GAS6, and 6.36% UM171) (Supplementary Figure 4). Similarly, SCAC and UM171

supplementation decreased the frequencies of $CD45^{+}CD41a^{hi}$ compared to controls by day 14 of expansion (2.84% for SMA, 1.05% for X2A+GAS6, 0.54% for SM6, 0.47% for X2A, and 3.09% for UM171) (Supplementary Figure 5). It appears that the frequency of $CD45^{+}CD41a^{hi}$ megakaryocyte progenitor-like cells peaks at day 7 of expansion and SCAC supplementation trends towards the loss of $CD45^{+}CD41a^{hi}$ megakaryocyte committed-like cells.

Subsequently, I investigated $CD45^{+}CD41a^{lo}$ megakaryocyte committed-like cells. In non-treated control iHPC, iHPCs harvested without expansion demonstrated the lowest frequency of $CD45^{+}CD41a^{lo}$ cells (1.05%), where subsequent expansion of control iHPCs for 7 (5.54%) and 14 days (11.4%) resulted in increasing frequencies of $CD45^{+}CD41a^{lo}$ cells (Supplementary Figure 4 and Supplementary Figure 5). SCAC supplementation during expansion for 7 days further increased frequencies of $CD45^{+}CD41a^{lo}$ cells compared to controls, with $CD45^{+}CD41a^{lo}$ frequencies of 13.5% (SMA), 9.21% (X2A), 8.51% (SM6), and 8.36% (X2A+GAS6); UM171 had a frequency of 6.87% (UM171) $CD45^{+}CD41a^{lo}$ cells (Supplementary Figure 4). After 14 days of expansion with SCAC supplementation, the frequencies of $CD45^{+}CD41a^{lo}$ cells continued to be higher than controls, with $CD45^{+}CD41a^{lo}$ frequencies of 24.6% (SMA), 19.8% (X2A+GAS6), 17.4% (X2A), and 12.5% (SM6); UM171 had a frequency of 19.5% (UM171) $CD45^{+}CD41a^{lo}$ cells (Supplementary Figure 5). Based on these observations, it appears that expansion of iHPC and SCAC supplementation shows a trend towards increased frequency of $CD45^{+}CD41a^{lo}$ megakaryocyte committed-like cells.

5. 4 SCAC supplementation during iHPC expansion does not change the frequency of myeloid committed-like cells

The expression of CD45 and CD14 was investigated by focusing on the $CD45^{+}CD14^{+}$ subset of myeloid committed-like cells. Non-treated control iHPCs harvested without expansion demonstrated the lowest frequency of $CD45^{+}CD14^{+}$ cells (0.21%), whereas continued expansion of control iHPCs for 7 (6.15%) and 14 days (12.9%) resulted in increasing frequencies of $CD45^{+}CD14^{+}$ cells (Supplementary Figure 4 and Supplementary Figure 5). SCAC supplementation during expansion for 7 days decreased frequencies of $CD45^{+}CD14^{+}$ cells compared to non-treated controls, with $CD45^{+}CD14^{+}$ frequencies of 4.37% (X2A), 3.81% (X2A+GAS6), 2.66% (SM6), and 0.72% (SMA); UM171 had a frequency of 3.26% (UM171) (Supplementary Figure 4). After 14 days of expansion with SCAC supplementation, the frequencies of $CD45^{+}CD14^{+}$ cells continued to decrease compared to controls with frequencies of 9.81% (X2A), 7.68% (X2A+GAS6), 4.80% (SM6), and 4.82% (SMA); UM171 had a frequency of 7.91% (Supplementary Figure 4 and Supplementary Figure 5). Based on these results, it appears that expansion of iHPC trends towards increasing the frequency of $CD45^{+}CD14^{+}$ myeloid committed-like cells whereas SCAC supplementation decreases the frequency of $CD45^{+}CD14^{+}$ myeloid committed-like cells, suggesting SCAC may delay terminal differentiation in favour of iHPC expansion.

Chapter 6 Supplemental Data

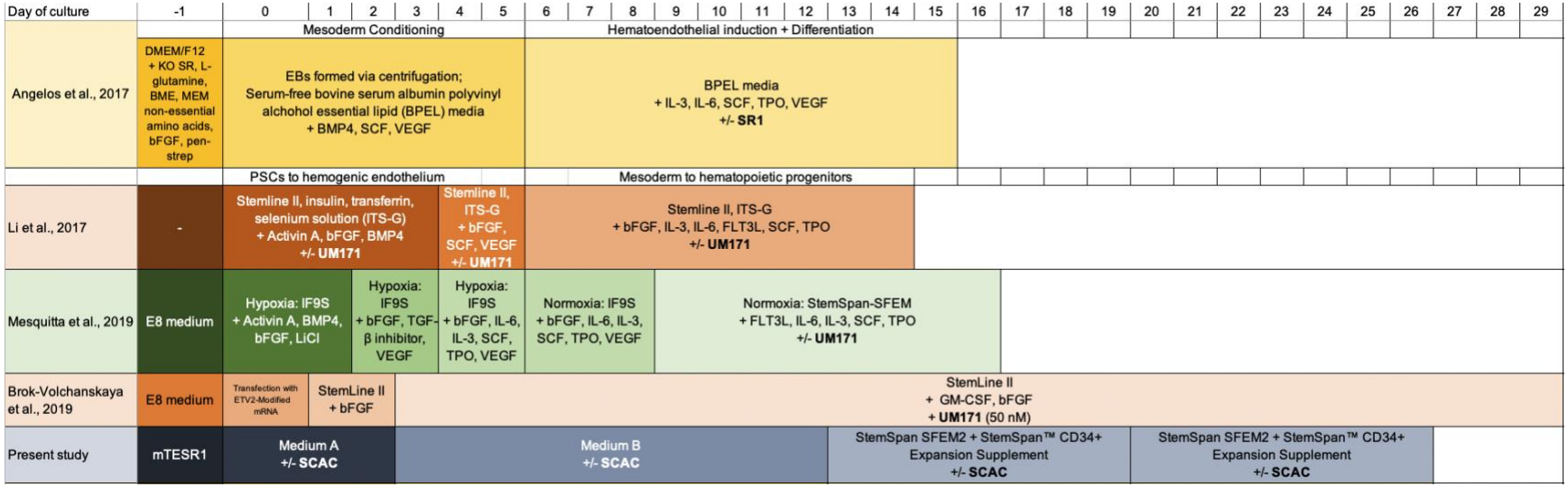
Supplementary Table 1. Studies expanding iPSC-derived HSPCs using single stem cell agonists and stem cell agonist cocktails. iPSC, human induced pluripotent stem cell; HPC, hematopoietic progenitor cell; SCA, stem cell agonist; SCAC, stem cell agonist cocktail.

Paper	Source	Cell line	SCA/SCAC	Conc.	Fold expansion	Significance	Day of harvest	HPC markers
Li *et al.*, 2017	Urine	UiPSC-012 hiPSC	UM171	100 nM	1.5	ns	Diff D14	CD34+CD43+
					1.9	ns	Diff D14	CD45+CD34+
Mesquitta *et al.*, 2019	Fibroblasts	DF19-9-7T hiPSC	UM171	35 nM	4.1	ns	Exp D5	CD34+CD43+
					3.5	ns	Exp D5	CD45+CD34+CD43+
					12.5	*	Exp D5	CD45+CD34+CD43+
Florencia *et al.*, 2019	Healthy donor	Healthy-hiPSC	UM171	Unspecified	1.9	*	Unspecified	CD34+
	Patients with aplastic anemia	AA-hiPSC	UM171	Unspecified	3.9	p<0.07	Unspecified	CD34+
Brok-Volchanskaya *et al.*, 2019	BM	IISH2i-BM9 hiPSC	UM171	50 nM UM171	1	ns	Diff D8, D15, and D24	TNC
Angelos et al., 2017	CD34+ umbilical CB–derived hiPSCs	UCBiPSC7 (validated in Knorr, 2013)	SR1	1 µM SR1	1.2	ns	Diff D9	CD34+CD43+
					2.1	ns	Diff D9	CD34+CD43+
					1.3	ns	Diff D9	CD34+CD43+
					1.7	ns	Diff D9	CD45+CD34+
		DUB7 (developed in-house and validated in Ferrell, 2015)			3.0	ns	Diff D9	CD34+CD43+
					4.6	ns	Diff D9	CD45+CD34+
			SR1	1 µM SR1	2.3	ns	Diff D12	CD34+CD43+
					3.0	ns	Diff D12	CD45+CD34+
					5.3	ns	Diff D15	CD34+CD43+
					4.1	ns	Diff D15	CD45+CD34+
Present Study	Human amniotic fluid cells	HAF-iPSC E188A (validated Ribecco-Lutkiewicz et al., 2018)	X2A	1X	2.1	*	Exp D7	TNC
			X2A	1X	3.5	****	Exp D14	TNC
			X2A+GAS6	1X, 10 ng/mL	3.8	***	Exp D14	TNC
			SM6	1X	2.8	*	Exp D14	TNC
			SM6	1X	2	***	Diff D0-12	CD34+CD43+

Paper	Source	Cell line	SCA/SCAC	Conc.	Fold expansion	Significance	Day of harvest	HPC markers
			SMA	0.25X	1.9	**	Diff D0-12	CD34+CD43+
			X2A	1X	2.5	****	Diff D5-12	CD34+CD43+
			X2A	1X	2.1	****	Diff D0-12	CD45+CD34+
			X2A	1X	2.3	****	Diff D5-12	CD45+CD34+
			X2A	1X	2.3	****	Diff D0-12	CD45+CD34+CD43+
			X2A	1X	2.6	****	Diff D5-12	CD45+CD34+CD43+
			Control	N/A	2.9	N/A	Diff D0-12 + Exp D7	TNC
			X2A	1X	9.3	**	Diff D0-12 + Exp D7	TNC
			SMA	0.25X, 0.25X	8.45	*	Diff D0-12 + Exp D7	TNC
			SMA	0.25X, 1X	9.15	*	Diff D0-12 + Exp D7	TNC
			Control	N/A	0.6	N/A	Diff D0-12 + Exp D14	TNC
			UM171	35 nM	6.8	*	Diff D0-12 + Exp D14	TNC
			X2A	1X	13.45	****	Diff D0-12 + Exp D14	TNC
			SM6	1X	19.1	****	Diff D0-12 + Exp D14	TNC
			SMA	0.25X, 0.25X	10.95	****	Diff D0-12 + Exp D14	TNC

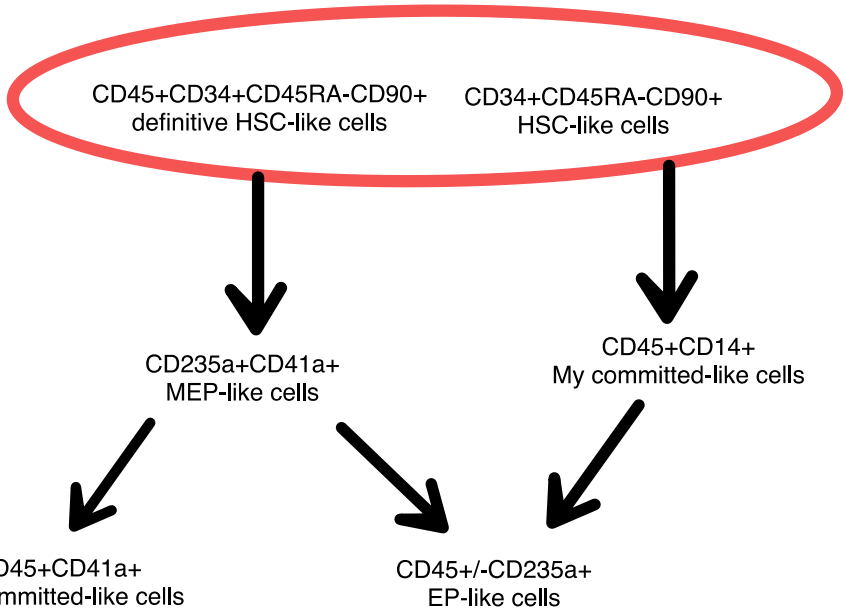
Supplementary Table 2. Experimental conditions of studies expanding human iPSC-derived HSPCs using SCAs compared to SCACs.

BME, β-mercaptoethanol; bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein 4; EB, embryoid body; FLT3L, FMS-like tyrosine kinase 3 ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-3, Interleukin-3; IL-6, Interleukin-6; KO SR, knockout serum replacement; LiCl, lithium chloride; pen-strep, penicillin-streptomycin; SCAC, stem cell agonist cocktail; SCF, stem cell factor; TPO, thrombopoietin; VEGF, vascular endothelial growth factor.

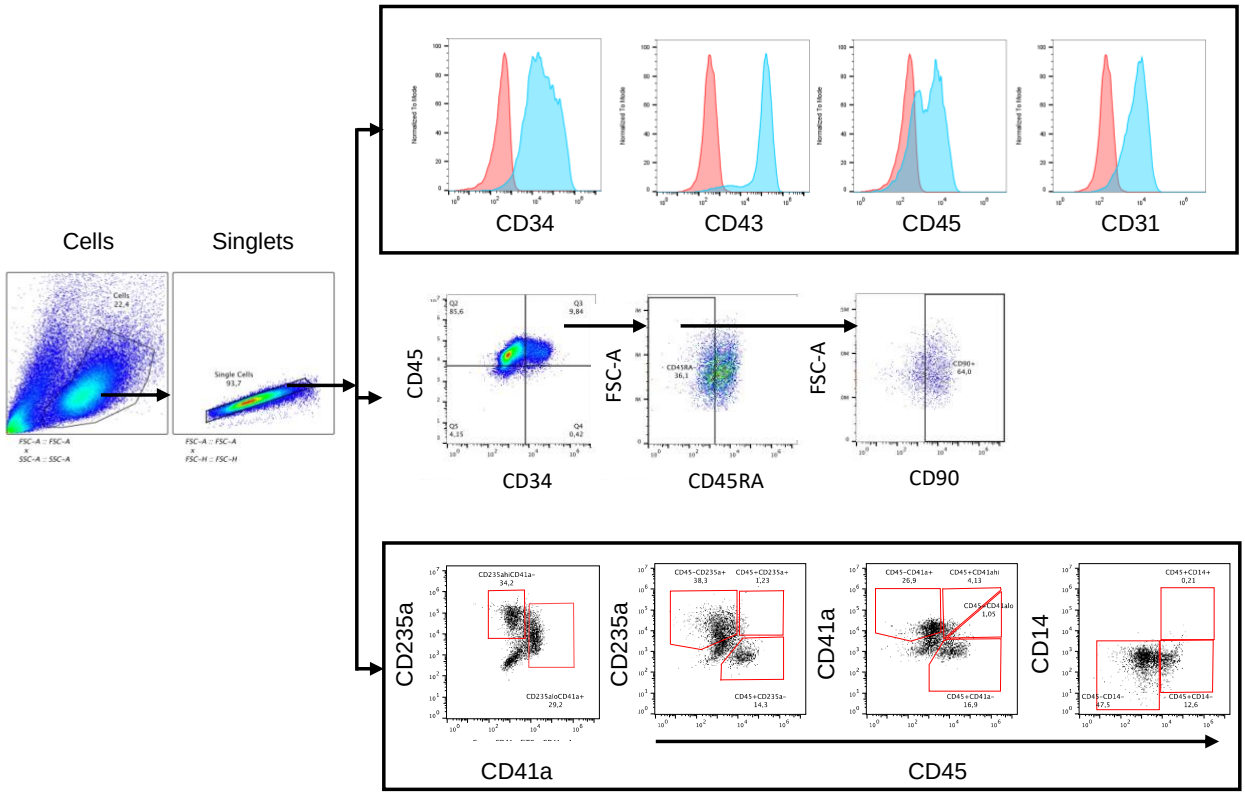


Supplementary Table 3. Markers used to classify cell populations from different cell sources.

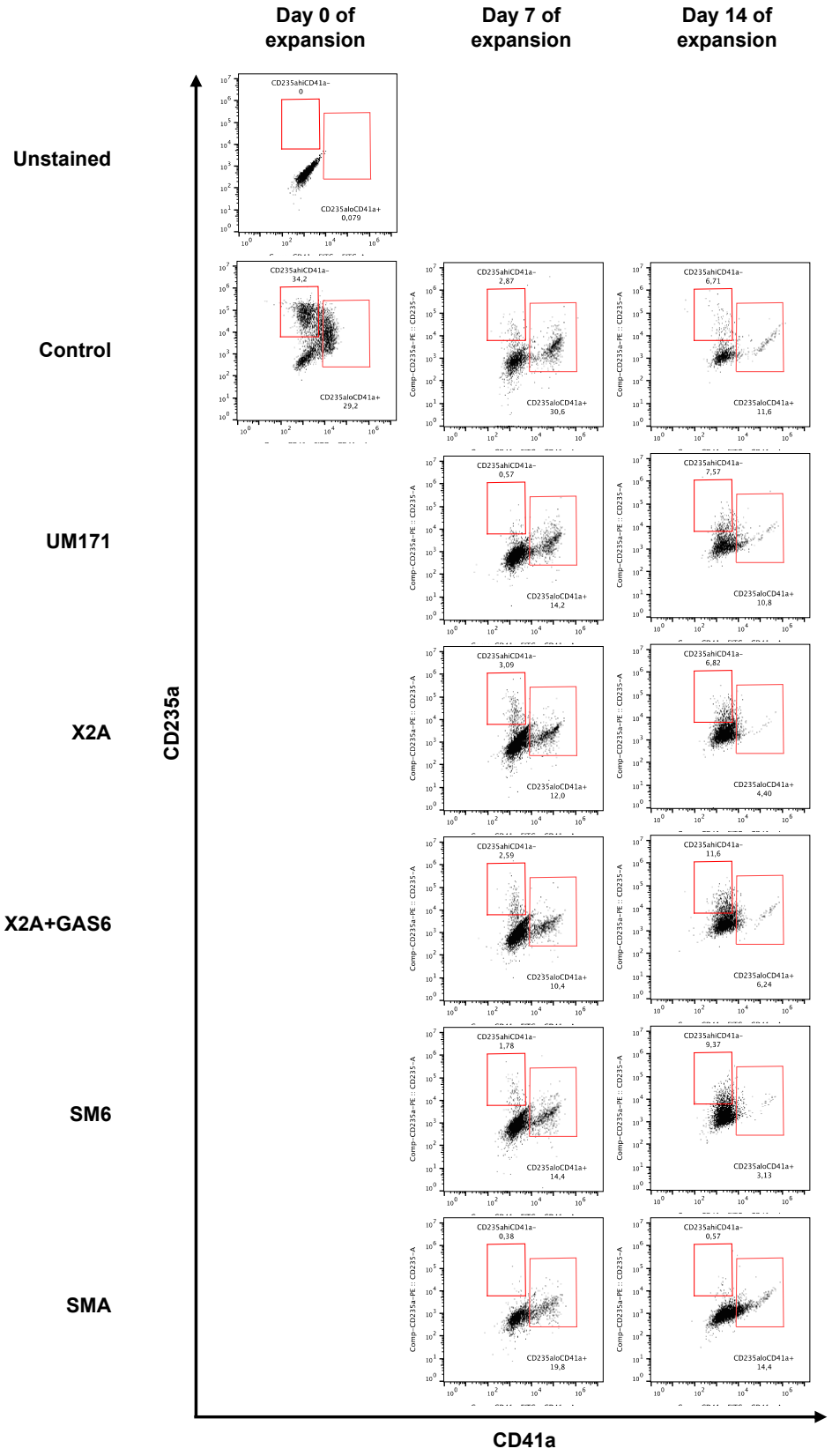
Markers	Cell source	Cell population	References
CD34+	iPSC	Hematopoietic progenitor marker	Li et al., 2017; Paes et al., 2021; Turksy et al., 2020
CD43+	ESC	Early hematopoietic progenitor marker	Vodyanik et al., 2006
CD45+	iPSC	Pan-hematopoietic marker	Paes et al., 2021
CD31+	iPSC	Endothelial cell marker	Netsrithong et al., 2020
CD34+CD43+	iPSC	Hematopoietic progenitors	Li et al., 2017; Mesquitta et al., 2019; Turksy et al., 2020
CD34+CD45+	iPSC	Hematopoietic progenitors	Choi et al., 2012; Li et al., 2017; Leung et al., 2018; Turksy et al., 2020; Paes et al., 2021
CD34+CD31+CD43-	iPSC	Endothelial cells	Choi et al., 2009
KDR+CD34+CD31+	iPSC	Multipotent hematoendothelial progenitors (HEPs)	Netsrithong et al., 2020
CD34+CD43+CD45-	iPSC	Multipotent hematopoietic progenitors	Li et al., 2017
Lin–CD34+CD43+CD45–	iPSC, ESC	Multipotent hematopoietic progenitors	Choi, Yu et al., 2009; Choi, Vodyanik et al., 2009
CD34+CD43+CD45-Lin-CD38-	iPSC	Multipotential hematopoietic progenitors	Choi et al., 2012
CD34+CD43+CD45+	iPSC	Hematopoietic progenitors	Ruiz et al., 2019; Li et al., 2017
Lin-CD34+CD43+CD45+	iPSC, ESC	Hematopoietic progenitors	Choi, Yu et al., 2009; Choi, Vodyanik et al., 2009
Lin-CD34+CD43+CD45+CD38-	iPSC, ESC	Hematopoietic progenitors	Choi et al., 2012
CD34+CD38−CD45RA−CD90+CD49f+	iPSC	Enriched in LT-HSCs	Ruiz et al., 2019
CD41a+	iPSC	Megakaryocytic lineage marker	Turksy et al., 2020
CD34+CD41aloCD45+	iPSC	HPCs enriched in G-CFCs	Mesquitta et al., 2019
CD235a+CD41a+	iPSC	Megakaryocyte–erythroid progenitor	Leung et al., 2018
CD235a+	iPSC	Erythroid lineage marker	Turksy et al., 2020



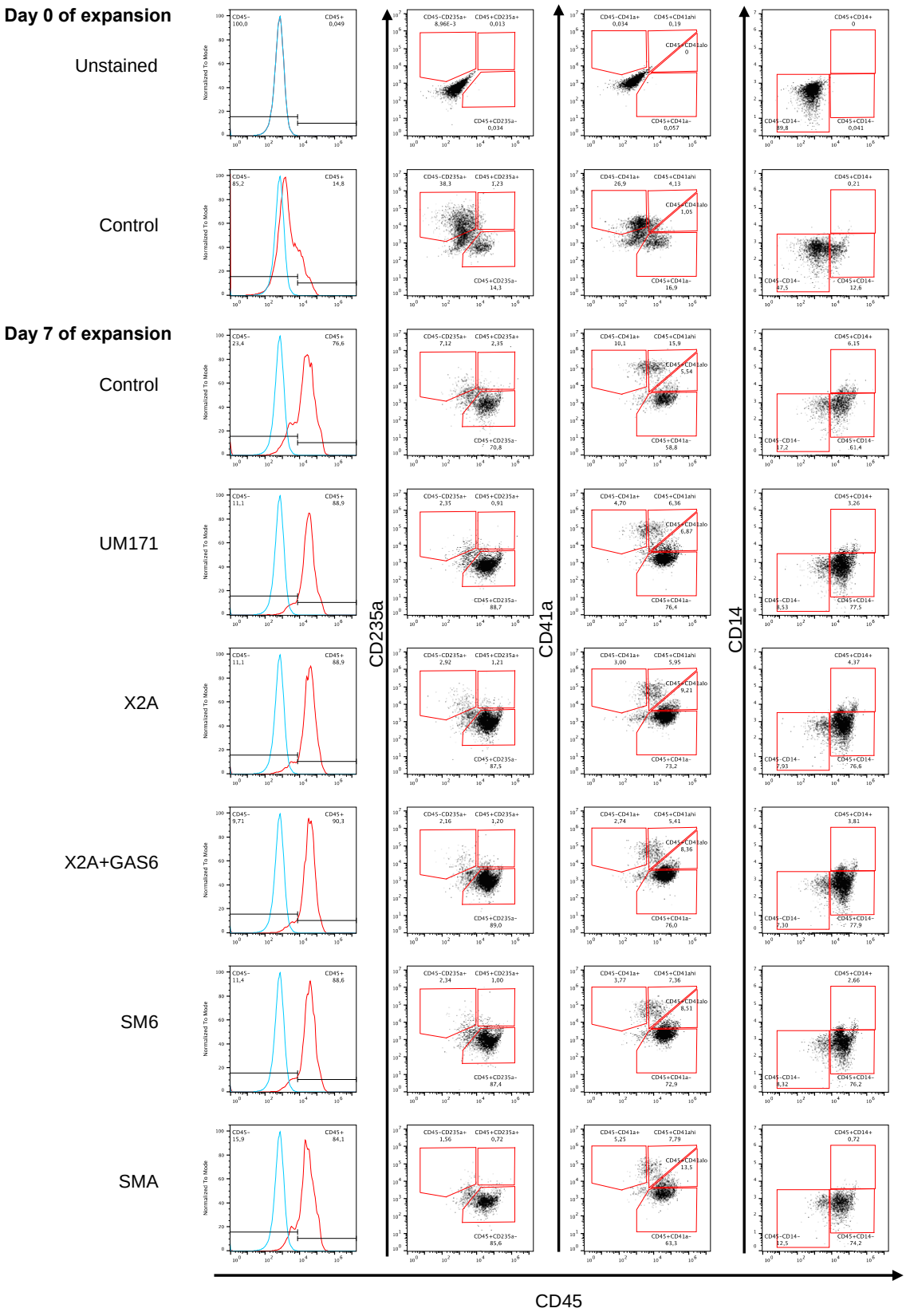
Supplementary Figure 1. Hematopoietic stem cell lineage cell surface markers
EP, erythroid progenitors; HSC, hematopoietic stem cell; MEP, megakaryocytic-erythroid progenitor; Mk, megakaryocyte; My, myeloid.



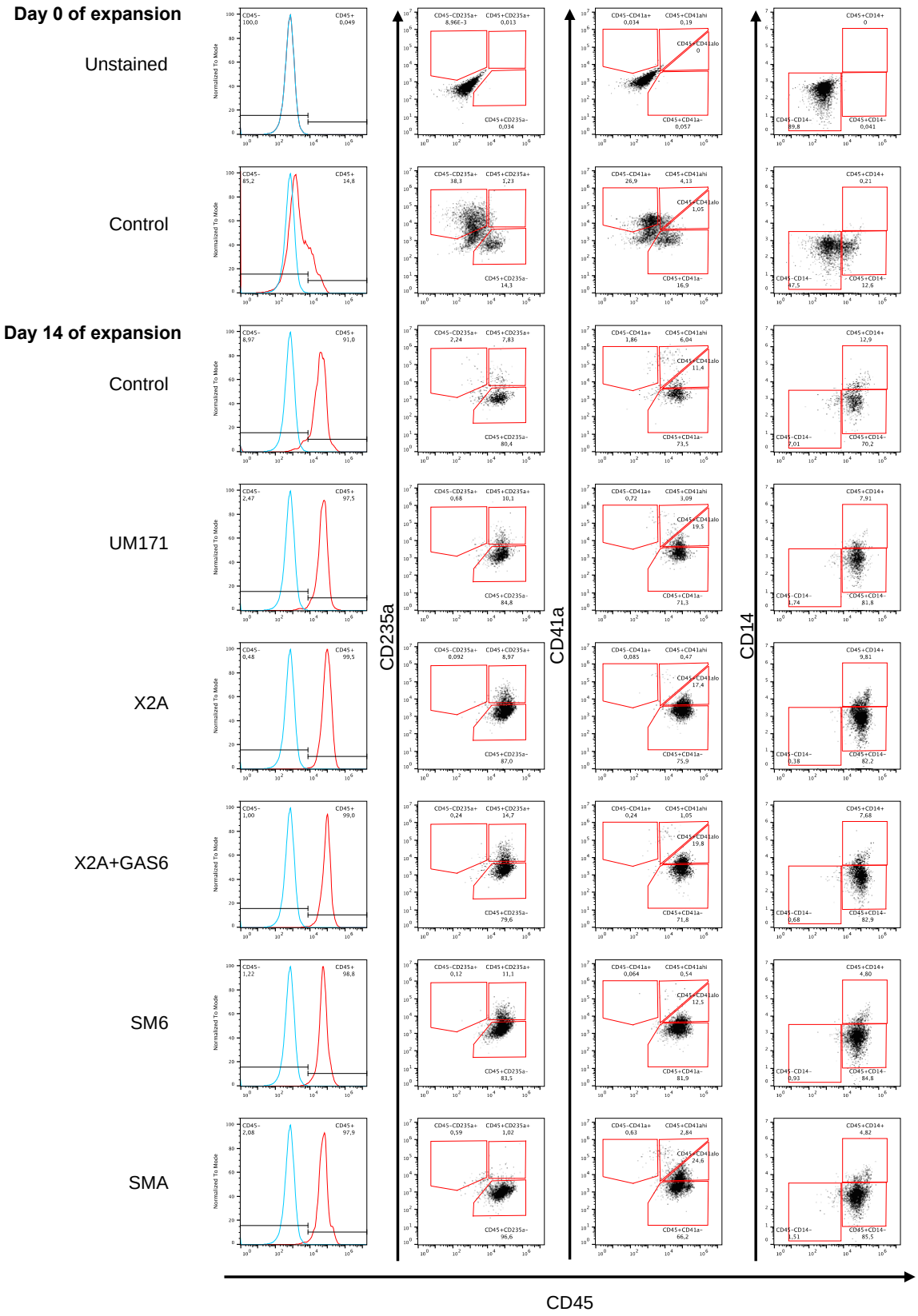
Supplementary Figure 2. Gating strategy for flow cytometry analysis of harvested iHPCs.


Day 0 of expansion
Day 7 of expansion
Day 14 of expansion
Unstained
Control
UM171
X2A
X2A+GAS6
SM6
SMA
CD235a
CD41a
CD235ahiCD41a-
0
CD235aloCD41a+
0,079
CD235ahiCD41a-
34,2
CD235aloCD41a+
29,2
CD235ahiCD41a-
2,87
CD235aloCD41a+
30,6
CD235ahiCD41a-
6,71
CD235aloCD41a+
11,6
CD235ahiCD41a-
0,57
CD235aloCD41a+
14,2
CD235ahiCD41a-
7,57
CD235aloCD41a+
10,8
CD235ahiCD41a-
3,09
CD235aloCD41a+
12,0
CD235ahiCD41a-
6,82
CD235aloCD41a+
4,40
CD235ahiCD41a-
2,59
CD235aloCD41a+
10,4
CD235ahiCD41a-
11,6
CD235aloCD41a+
6,24
CD235ahiCD41a-
1,78
CD235aloCD41a+
14,4
CD235ahiCD41a-
9,37
CD235aloCD41a+
3,13
CD235ahiCD41a-
0,38
CD235aloCD41a+
19,8
CD235ahiCD41a-
0,57
CD235aloCD41a+
14,4

Supplementary Figure 3. A fraction of expanded iHPCs co-express markers of MEP-like cells.
Representative flow cytometry analysis for MEP-like cell surface markers (CD235a and CD41a) of iHPCs after 0, 7 and 14 days of expansion supplemented with UM171 (35 nM) or SCACs (X2A, SM6 or SMA), and/or GAS6 (10 ng/mL) (n=1 independent differentiation).


Day 0 of expansion
Unstained
Control
Day 7 of expansion
Control
UM171
X2A
X2A+GAS6
SM6
SMA
CD235a
CD41a
CD14
CD45
Normalized To Mode

Supplementary Figure 4. A proportion of iHPCs expanded for 7 days co-express markers indicative of several lineages.
Representative flow cytometry analysis for hematopoietic cell surface marker (CD45) and lineage-specific markers (CD235a, CD41a, and CD14) of iHPCs after 0 and 7 days of expansion supplemented with UM171 (35 nM) or SCACs (X2A, SM6 or SMA), and/or GAS6 (10 ng/mL) (n=1 independent differentiation).


Day 0 of expansion
Unstained
Control
Day 14 of expansion
Control
UM171
X2A
X2A+GAS6
SM6
SMA
CD235a
CD41a
CD14
CD45
Normalized To Mode

Supplementary Figure 5. A proportion of iHPCs expanded for 14 days co-express markers indicative of several lineages.
Representative flow cytometry analysis for hematopoietic cell surface marker (CD45) and lineage-specific markers (CD235a, CD41a, and CD14) of iHPCs after 0 and 14 days of expansion supplemented with UM171 (35 nM) or SCACs (X2A, SM6 or SMA), and/or GAS6 (10 ng/mL) (n=1 independent differentiation).