



University of Tennessee Health Science Center
UTHSC Digital Commons

Theses and Dissertations (ETD)

College of Graduate Health Sciences

12-2012

Hematopoietic Stem Cell Threshold Sensing Controls Regulatory Pathways Facilitating Clinically Relevant Ex Vivo Expansion for Stem Cell Transplantation

Andrew D. Lasiter
University of Tennessee Health Science Center

Follow this and additional works at: <https://dc.uthsc.edu/dissertations>



Part of the [Medical Cell Biology Commons](#)

Recommended Citation

Lasiter, Andrew D. , "Hematopoietic Stem Cell Threshold Sensing Controls Regulatory Pathways Facilitating Clinically Relevant Ex Vivo Expansion for Stem Cell Transplantation" (2012). *Theses and Dissertations (ETD)*. Paper 150. <http://dx.doi.org/10.21007/etd.cghs.2012.0172>.

This Thesis is brought to you for free and open access by the College of Graduate Health Sciences at UTHSC Digital Commons. It has been accepted for inclusion in Theses and Dissertations (ETD) by an authorized administrator of UTHSC Digital Commons. For more information, please contact jwelch30@uthsc.edu.

Hematopoietic Stem Cell Threshold Sensing Controls Regulatory Pathways Facilitating Clinically Relevant Ex Vivo Expansion for Stem Cell Transplantation

Abstract

The ex vivo expansion of hematopoietic stem cells (HSCs) for transplantation has threefold importance: 1.) First, because of the rarity of stem cells there often isn't a sufficient supply obtainable from common sources for larger children and adults. 2.) Secondly, patient morbidity and time to hematopoietic reconstitution following myeloablative preconditioning is improved by administering a larger pool of HSCs. 3.) Lastly, gene therapies for hematological diseases still require a robust supply of HSCs to offset varying degrees of inefficiency in vector mediated transfection protocols. These reasons, and others, have been an impetus for many discoveries made within four primary subdivisions within the field of HSC expansion; culture media optimization, hematopoietic gene regulation, development of small molecular compounds, and use of induced pluripotent stem cells (iPSCs). This article is a review of the current trends in HSC expansion methodology and posits that the majority of the signaling mechanisms involved can be explained by the collective contribution of activating and inhibitory gene expression products interacting through regulatory homeostatic process mediated by HSC sensing of key pathway dependent thresholds.

Document Type

Thesis

Degree Name

Master of Science (MS)

Program

Biomedical Sciences

Research Advisor

Gerard P. Zambetti, Ph.D.

Keywords

HSC, expansion, hematopoietic, model, stem, cell

Subject Categories

Medical Cell Biology | Medical Sciences | Medicine and Health Sciences

**Hematopoietic Stem Cell Threshold Sensing Controls Regulatory Pathways
Facilitating Clinically Relevant Ex Vivo Expansion for Stem Cell Transplantation**

A Thesis
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science
From The University of Tennessee

By
Andrew D. Lasiter
December 2012

Copyright © 2012 by Andrew D. Lasiter.
All rights reserved.

ACKNOWLEDGMENTS

The author wishes to thank his committee, whose help made this degree possible. He would also like to thank Derek Persons and his lab for the opportunity to work among an excellent group of scientists.

ABSTRACT

The *ex vivo* expansion of hematopoietic stem cells (HSCs) for transplantation has threefold importance: 1.) First, because of the rarity of stem cells there often isn't a sufficient supply obtainable from common sources for larger children and adults. 2.) Secondly, patient morbidity and time to hematopoietic reconstitution following myeloablative preconditioning is improved by administering a larger pool of HSCs. 3.) Lastly, gene therapies for hematological diseases still require a robust supply of HSCs to offset varying degrees of inefficiency in vector mediated transfection protocols. These reasons, and others, have been an impetus for many discoveries made within four primary subdivisions within the field of HSC expansion; culture media optimization, hematopoietic gene regulation, development of small molecular compounds, and use of induced pluripotent stem cells (iPSCs). This article is a review of the current trends in HSC expansion methodology and posits that the majority of the signaling mechanisms involved can be explained by the collective contribution of activating and inhibitory gene expression products interacting through regulatory homeostatic process mediated by HSC sensing of key pathway dependent thresholds.

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION	1
HSC Expansion.....	1
Background.....	1
CHAPTER 2. MODELING PATHWAY SPECIFIC THRESHOLDS	3
CHAPTER 3. OPTMIZING MEDIA CONDITIONS.....	6
Automated Feeding Systems	6
Recapitulating the Bone Marrow Niche: Hypoxia	7
CHAPTER 4. GENES AND TRANSCRIPTION FACTORS	10
Hox Genes and HSC Expansion	10
Two Routes on the Wnt Pathway	11
Relevancy of SALL4	12
Relevancy of the Gatekeeper Notch	13
CHAPTER 5. HSC MOLECULAR THERAPIES	16
The Aryl Hydrocarbon Receptor Success Story	16
Chromatin Modifying Agents: Reestablishing Multipotency.....	17
Culture Media Copper Chelation.....	18
CHAPTER 6. IPSC: A POTENTIAL CURE-ALL	20
CHAPTER 7. CONCLUSION.....	22
LIST OF REFERENCES.....	23
VITA.....	33

LIST OF ABBREVIATIONS

AA2P	Ascorbic Acid 2-Phosphate
AhR	Aryl Hydrocarbon Receptor
APC	Adenomatous Polyposis Coli
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
ATP	Adenosine-5'-Triphosphate
bHLH	Basic-Helix-Loop-Helix
BIO	6-Bromoindirubin-3'-Oxime
BM	Bone Marrow
CBF-1	C-promoter Binding Factor-1
CDK	Cyclin-Dependant Kinase
CDKI	Cyclin-Dependent Kinase Inhibitor
CRUs	Competitive Repopulating Unit
DKK1	Dickkopf-Related Protein 1
DNA	Deoxyribonucleic Acid
ES	Embryonic Stem
Flt-3L	Fms-Like Tyrosine Kinase 3
Fmi	Flamingo
Fz	Frizzled
GAR	Garcinol
GSH	Glutathione
GSK-3 β	Glycogen Synthase Kinase 3 Beta
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
HMPB	Human Mobilized Peripheral Blood
HOXA10	Homeobox Protein Hox-A10
HOXB4	Homeobox Protein Hox-B4
HRE	Hypoxia Response Element
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplant
ICP-MS	Induction-Coupled Mass-Spectroscopy
IL-3/6	Interleukin 3/6
IP-10	IFN-Gamma-Inducible Protein 10
iPSC	Induced Pluripotent Stem Cell
ISO	Isogarcinol
KLF4	Krueppel-Like Factor 4
Lef	Lymphocyte-Enhancer Binding Factor
LRP-5/6	Lipoprotein Receptor-Related Proteins
LSK	Lin- Sca-1+ c-Kit+
LT-HSCs	Long-Term Hematopoietic Stem Cells
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein-1

MIP-1 α	Macrophage Inflammatory Protein 1 α
miRNA	Micro Ribonucleic Acid
mPB	Mobilized Peripheral Blood
MPP	Multipotent Progenitors
mRNAs	Messenger Ribonucleic Acid
N-cad	N-Cadherin
NCID	Notch Intracellular Domain
NOD	Non-Obese Diabetic Mice
NUP98	Nuclear Pore Complex Protein
OB	Osteoblasts
OCT4	Octamer-Binding Transcription Factor 4
PAS	Per-ARNT-Sim
PBx1	Pre-B-Cell Leukemia Transcription Factor 1
PCP	Planar Cell Polarity
QSAR	Quantitative Structure-Activity Relationship
ROS	Reactive Oxygen Species
SAHA	Suberoylanilide Hydroxamic Acid
SALL4	Sal-Like Protein 4
SCF	Stem Cell Factor
SCID	Severe Combined Immunodeficiency
SOX2	Sex Determining Region Y-Box 2
SR1	StemRegenin
ST-HSCs	Short-Term Self-Renewing Hematopoietic Stem Cells
TACE	Tumor Necrosis Factor Alpha Converting Enzyme
TAT	Transactivating Protein
TCDD	2,3,7,8-Tetrachlorodibenzo-P-Dioxin
Tcf	T-Cell Transcription Factor
TEPA	Tetraethylenepentamine
TF	Transcription Factors
TGF- β	Transforming Growth Factor Beta
TNC	Total Nucleated Cell
TPO	Thrombopoietin
TSA	Trichostatin
VPA	Valproic Acid
5azaD	5-Aza-2'-Deoxycytidine
5FU	Fluorouracil

CHAPTER 1. INTRODUCTION

HSC Expansion

While our knowledge of hematopoietic stem cell (HSC) regulatory systems is quickly becoming more advanced, the complex interplay between relevant signaling pathways has made development of HSC expansion regimes a challenge. Seminal accomplishments in overcoming HSC expansion difficulties have been achieved, albeit marginally, by optimizing media through the use of a multitude of cytokine combinations at very high doses or through the constitutive activation of key transcription factors and genes. While these approaches have generated invaluable knowledge about HSC regulatory mechanisms, they have not been overwhelmingly successful at providing clinically significant quantities of HSCs for use in hematopoietic stem cell transplants (HSCTs) without combining multiple sources. It is not lost on this author that hindsight affords an easy view of the shortcomings surrounding any particular method of HSC expansion, and that such discoveries carry great weight in our current understanding. However, at some point our culmination of knowledge and rapidly developing technology mandates that outmoded methods be challenged to quickly capture the possibilities that loom before us. Simply, there are numerous examples of worthwhile therapeutic developments that are producing markedly better HSC expansion than a half-decade ago. It is this author's contention that these methods should be appropriately integrated for maximal HSC expansion if the ultimate goal is to produce clinical quality stem cell treatments as quickly as possible so that patients as well as the science can benefit from these forward-looking strategies.

Background

Hematological disorders are routinely treated through chemotherapeutic ablation of the hematopoietic system and subsequent reconstitution with HSCTs. Currently, more than 25,000 HSCTs are performed annually utilizing HSCs from bone marrow (BM), umbilical cord blood (CB), and peripheral blood containing stem cells mobilized through granulocyte colony-stimulating factor (G-CSF) stimulation. However, matching an unrelated allogeneic donor with a recipient by human leukocyte antigen (HLA) can be challenging due to the lack of diverse race and ethnic makeup contained within many bone marrow registries.¹ Even so, when a patient does meet the requirements to be considered for a transplant, then the need to obtain as many HSCs as possible becomes paramount because patient outcomes have been shown to be correlated with administering a total nucleated cell (TNC) dose of greater than $3 \times 10^7/\text{kg}$.² Moreover, doses attaining a TNC of $5.2 \times 10^7/\text{kg}$ conferred better outcomes further highlighting the necessity for a robust means of obtaining the greatest amount of HSCs possible.³ Hematopoietic stem cell expansion for the purpose of reconstituting myeloablative treated patients receiving HSCTs has been an important goal for blood therapeutics since the pioneering work of Till and McCulloch in the 1960's as well as Nobel laureate Donall Thomas' research on blood transplants for treating leukemia in the 1970's.^{4,5}

Myeloablative preconditioning of a diseased hematopoietic system before a HSCT can reconstitute the space often compromises a patient's immune system and leaves him anemic. Only about .01% of whole bone marrow (WBM) is thought to contain multipotent HSCs. There needs to be quick and robust means of reconstituting the BM quickly so that the duration of morbidity is reduced. Inherent limitations to supplying patients with sufficient numbers of HSCs are compounded by the rarity of stem cells within the BM space as well as the multitude of signaling pathways that carefully regulate their proliferation.

Expanding HSCs would be inordinately easier if they were all created equal. Instead, HSCs are a heterogeneous mixture of cells that have been difficult to characterize based upon cell surface marker expression.⁶ The heterogeneity of HSCs within the bone marrow (BM) allows them to be divided into three main populations: long-term self-renewing HSCs (LT-HSCs), short-term self-renewing HSCs (ST-HSCs) and multipotent progenitors (MPPs). ST-HSCs and MPPs are only able to restore the ablated hematopoietic system of a lethally irradiated mouse for up to four months, after which failure of the hematopoietic system will occur. This being the case, much effort has been spent trying to expand and maintain LT-HSCs, which can fully restore the hematopoietic system of lethally irradiated BM when administered within a transplant. When LT-HSCs and ST-HSCs are extracted from their niche, either through G-CSF mobilized bone marrow or by bone marrow extraction, they can differentiate into MPPs through the progressive loss of self-renewal ability, which includes concordant increases in hematopoietic progenitor proliferation. Unless methods are in place to maintain HSC self-renewal capacity within specialized culturing conditions, they will undergo apoptosis or differentiate into progenitors, thereby jeopardizing their usefulness for conducting HSCTs. Current methods of promoting HSC self-renewal in favor of differentiation can be classified into four main categories: culturing conditions and factors (ex. hypoxia, automated bioreactor culture feeding, SCF, TPO, IL-3, IL-6); regulatory pathways and transcription factors (ex. HOXB4, Notch-1, Wnt, SALL4); small molecule therapeutics (ex. AhR antagonists, GSK-3 β inhibitors), and through hematopoietic lineage commitment of induced pluripotent stem cells (iPSCs).

CHAPTER 2. MODELING PATHWAY SPECIFIC THRESHOLDS

Many of the methods this article enumerates involve exogenous manipulations of the steady-state of HSC gene expression for various relevant signaling pathways. Granted, little detail is known about how the steady-state behaves under homeostatic processes for these pathways within the BM niche or how it changes under physiological stress. Moreover, when utilizing HSC expansion adjuvants, the interplay between genes that have beneficial effects on HSC expansion and the concordant increases in negative feedback regulatory genes can be extremely complex. Evidence of this complexity is documented within the literature as underwhelming HSC expansion and blocks in progenitor populations when constitutively expressed genes or over stimulation of important HSC regulatory pathways occurs.⁷⁻⁹ Paradoxically, moderation can stray too far in the opposite direction, failing to achieve adequate pathway stimulation. However, despite this level of complexity, optimal pathway stimulation is often achieved by promoting key gene products whose expression enhances expansion capacity. These groups of genes can be described for every repeating interval of time (t) within a single hematopoietic stem cell through hypothetical microarray analysis, and taking repeated snapshots of gene expression from the moment of administering a single targeted adjuvant through its duration. The collective continuum of upregulated and down-regulated genes can be placed into three broad categories for a particular point in time as: 1.) Activating (A) genes that maintain quiescence, deter differentiation, and promote self-renewal. 2.) Inhibitory (I) genes that serve a negative feedback role and ameliorate the effects of the expansion promoting set. 3.) And null (N) genes that are up or down regulated, but collectively and individually do not have any measurable direct or indirect impact on HSC expansion, and can therefore be ignored.

The collective interaction between A and I determines whether a particular stimulus or environmental cue at a particular time will promote expansion or favor differentiation. While it might be useful to imagine that for every interval of time (t) each upregulated or down regulated gene within A could be scored on its individual ability to expand HSCs, but this would be of little practical use because it would ignore the collective interactions between complementary genes. Therefore the interaction of A with I over a period of time can be represented by the sum of two logarithmic curves. The positive A curve represents the initial stimulation events and the subsequent increase in gene expression that favors maintaining stem cell properties. The negative value of the I curve, which subtracts from the A curve, represents the delayed response of the genes involved in ameliorating the initial signaling wave through negative-feedback. The smoothed out sum of these two curves ($A+I$) forms a sinusoidal biphasic response with potential (depending upon the signaling pathway) for repeating when the stimulation of a HSC pathway is strong enough to exceed the pathway specific threshold that induces upregulation of negative-feedback.

It is posited that stimulation that induces signaling, but does not cross the negative-feedback threshold range can induce expansion promoting gene expression, while optimally maintaining genes associated with negative-feedback at low homeostatic

levels. Preventing sharp biphasic swings between stimulation and feedback inhibition should therefore be the goal when using HSC expansion promoting adjuvants. To this extent automated culture feeding and dilution mechanisms can likely achieve the control necessary to mitigate excessive signaling crests and troughs. Examples of this reasoning can be observed within the Wnt and Notch pathways, which have been shown to respond in a biphasic manner to pathway stimulation.^{10,11} It is hypothesized that this biphasic response is mediated by two pathway specific thresholds. The first threshold, T_1 , is defined by the level of pathway stimulation needed to upregulate genes associated with negative-feedback control from a particular stimulus beyond baseline. A high T_1 , which is believed to occur in Hif-1 α mediated hypoxia signaling, allows stronger environmental stimulation before failure occurs (**Figure 1-1**). Conversely, canonical Wnt signaling within HSCs is posited to have a low T_1 , mandating moderate signaling regulation to achieve optimal expansion. The second threshold, T_2 , is defined by the range that the collective effects of negative-feedback genes are able to reduce the expansion capabilities by at least 50% of optimal max signaling. T_2 corresponds to the sensitivity of the pathway to its own negative-feedback. A low T_2 will mean that a pathway needs less negative-feedback gene expression over baseline to ameliorate expansion inducing gene expression. Likewise, a high T_2 means that the pathway stimulation has a greater range and likely requires less stringent regulation.

An important factor in determining the period of the biphasic response to stimulation is the time between T_1 being reached by A and when T_2 is crossed by I . This period determining interval (P) along with the rate and potency of negative-feedback inhibition will determine the decay of the stimulation. If the amplitude of a biphasic response isn't able to be compressed by adjusting the concentration of the signaling stimulus, so that the stimulatory signal can be maintained at a more steady level instead of fluctuating, then decreasing the best-fit slope of the falling wave will provide extended period of expansion promoting signaling. To this extent, the falling portion of the stimulatory wave corresponds to increasing permissiveness of HSCs to expansion inducing signaling. An example would be providing exogenous Notch signaling within HSC culture media to maintain permissiveness to concurrent canonical Wnt signaling, which has been shown to promote expansion.¹² By the same notion, delaying the rising slope of I , through the potential use of selective inhibitors or siRNA should permit a longer duration HSC promoting signal.

To this end, the synergistic effects of all expansion promoting genes on one another under the effects of a single targeted compound need to be quantitated. This should be done under the same stimulus for both A and I . While current technological limitations bar such comprehensive measurements, examples of ongoing optimization using induction-coupled mass spectrometry indicate potential access to comprehensive data across multiple signaling pathways.¹³

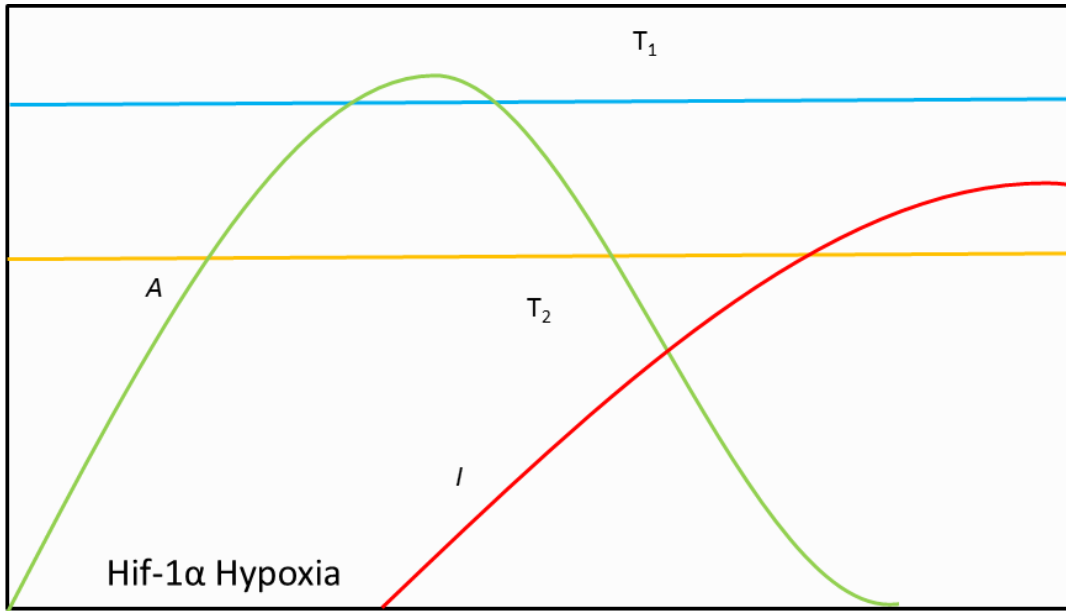


Figure 1-1. Location of theoretical regulatory gene thresholds T_1 and T_2 for HIF-1 mediated hypoxia

CHAPTER 3. OPTIMIZING MEDIA CONDITIONS

HSC cytokines have been optimized by different labs but have not worked well at expanding HSC cultures to the levels required for HSCT.¹⁴⁻¹⁶ While the administration of such cytokines is necessary for maintaining HSCs, they have not been shown to impart marked therapeutic potential on their own, necessitating other complimentary signals.¹⁴ Despite further identification of HSC culturing media factors and a greater understanding of the signaling mechanisms promoting HSC expansion, none of the cytokine-based culturing strategies has been successful at deterring cell cycling, apoptosis, disrupted bone marrow homing, or HSC differentiation.¹⁷

Automated Feeding Systems

Advances that attempt to rectify the issues with cytokine treatments have involved automated culture-feeding systems. It has been known that growing HSC cultures produce secreted factors that, if not quenched have an overall inhibitory effect upon growing cultures.¹⁸ Recently, efforts have been made to improve *ex vivo* culturing conditions to better favor self-renewal by using automated feeding methods to limit the effects of inhibitory molecules (TGF- β , MCP-1, MIP-1 α , MIP-1 β , and IP-10) that accumulate within growing HSC cultures.¹⁹⁻²² By continually adding media at an optimized rate, an automated feeding method is reported to dilute inhibitory factors below a threshold so that the negative feedback effects on HSC expansion are sufficiently reduced. To date, this method has led to an 80-fold increase in CD34⁺ cells and an 11-fold increase in blood stem cells, which was better than simple media exchange of the cultures.²³

The fed-batch method of media delivery that Csaazar *et al.* developed continually increases the volume of cultures so as to optimally expand cells and dilute away the inhibitory factors, which was better than perfusion driven media exchanges that maintain the same volume of the cultures by removing an equal amount of media that is added. The report claims that the fed-batch method simplifies HSC culturing conditions by eliminating the need for dosing the cultures with multiple compounds that independently target and inhibit the negative feedback ligands produced in culture. However, this benefit is offset by the increased complexity over basic culturing methods required with an automated feeding system. Moreover, the authors found that the fed-batch method works synergistically when combined with small molecular factors that have demonstrated successful HSC expansion capabilities, suggesting that the optimal means of expanding HSCs involves combining fed-batch with small molecular therapeutics and cytokines. For instance, both the small molecule aryl hydrocarbon receptor (AhR) antagonist StemRegenin (SR1) and the soluble TAT-HOXB4 were shown to expand HSCs more robustly when the inhibitory molecules were diluted away using the fed-batch culture feeding system.²³

Recapitulating the Bone Marrow Niche: Hypoxia

Strategies for *ex vivo* HSC expansion often rely on providing the essential HSC factors in the optimal stoichiometric ratios within the confines of a culture dish in order to promote robust self-renewal and survival. While efforts to understand and distill the multitude of intrinsic and extrinsic cues that control HSC biology have made great progress, determining what factors get labeled essential for *ex vivo* expansion can come down to a mixture of targeted discovery, serendipity and trial and error. So what role does providing hypoxic cues play in establishing an analogous physiological environment for HSC expansion? The answer isn't clear; however, the importance of *in vivo* low partial pressure oxygen (pO^2) for maintenance of HSC quiescence and reduced dependence upon mitochondrial oxidative phosphorylation is certain.²⁴

Under normal physiological conditions within the bone marrow, HSCs have been found to be concentrated in the less vascular endosteum regions of trabecular bone, regions that carry an oxygen tension between .1 to 4%.²⁵ Within this hypoxic BM niche, HSCs have adapted to the low pO^2 by utilizing glycolysis as the primary source of ATP instead of mitochondrial oxidative phosphorylation. Cellular adaptation to hypoxia through Hif-1 α -induced increases in vascular endothelial growth factor (VEGF) and Tie-1 have been found to play a role in maintaining HSC quiescence.²⁶ There is also evidence that hypoxia-induced stabilization of Hif-1 α plays a regulatory role within the canonical Wnt pathway of embryonic stem (ES) and neural stem cells that leads to β -catenin accumulation and downstream interaction with Lef/Tcf, which needs further investigation to determine if a similar function occurs within HSCs.²⁷

While translational processing of Hif-1 α is thoroughly understood, much is still unknown about regulation at the transcriptional level. In 2010, Hif-1 α was shown by Simsek *et al.* to be induced by Meis1, a member of the Hox gene family.²⁴ Interestingly Meis1 was found to be expressed in 96% of LT-HSCs while only 1.4% of WBM had expression. Moreover, Meis1, through a consensus binding sequence at the first intron of Hif-1 α , plays an important role in Hif-1 α preservation even in the absence of hypoxic cues. The ability of Meis1 to prevent Hif-1 α degradation under normoxic conditions, along with diminishing Meis1 levels coinciding with increasing levels of HSC differentiation, suggests that creating a conditionally activated Meis1 could be used to help maintain HSC quiescence. However, there is evidence that such efforts won't work or would need precise regulation because very high levels of Hif-1 α expression, which a constitutively active Meis1 might induce, can lead to impairment of transplantation capacity.⁹

Additional observations from Eliasson *et al.*²⁸ were consistent with an independent study from Takubo *et al.*⁹ that found high-level expression of Hif-1 α accomplished through either a constitutively active Hif-1 α , culture treatment with HIF-stabilizing agent FG-4497, or through deletion of the Von Hippel-Lindau (VHL) E3 that mediates Hif-1 α degradation, ultimately led to a reduction in HSC reconstituting ability.^{9,28,29} This shouldn't dissuade investigation of Meis1 for HSC expansion purposes because markedly different dosage-dependent outcomes exist within other HSC-relevant

signaling pathways (canonical Wnt, Notch-1) wherein a narrow range of stimulation provides optimal HSC expansion, and activation straying too far outside these cellular-dependent ranges can lead to anemic HSC expansion or outright failure.³⁰ These common HSC dosage-dependent sensitivities highlight the usefulness of employing a gradient of expression levels such as that used by Luis *et al.* for determining optimal HSC expansion ranges within the canonical Wnt pathway.³⁰

Concurrent with HIF induction, hypoxic HSC culturing conditions have been shown to be advantageous by reducing reactive oxygen species (ROS) generation and preventing a quiescent to cycling influx.^{28,31} Under normoxic conditions of 20% O₂, ROS generation increases DNA damage as well as activates p38 MAPK, which is known to adversely affect HSC longevity.^{32,33} Hypoxic environments, such as those of the BM niche, have been shown to protect LT-HSCs from ROS-induced DNA damage and preserve LT-HSC quiescence and multilineage repopulation ability.²⁸ However, it should not be overlooked that ROS have important signaling functions throughout the cell that include inducing differentiation. In line with the signaling function of ROS is the finding that they have been found to play a synergistic role with thrombopoietin (TPO) and other HSC cytokines to promote HIF-1 mediated quiescence under normoxic conditions.³⁴ This suggests that the decision between utilizing hypoxic or normoxic culturing conditions can be muddled due to the complexity of interactions under differing cytokine combinations, which might very well obfuscate the utility for recapitulating the oxygen tension found within the BM niche.

That being said, hypoxic conditions for *ex vivo* culturing have demonstrated increased repopulating capacity and HSC numbers, despite reductions in total cell numbers when contrasted with normoxic conditions.^{26,28,35} Long-term FLT3⁻ CD34⁻ Lin⁻ Sca-1⁺ c-Kit⁺ (LSK) cells, when cultured under hypoxic conditions, had total cell numbers that were significantly reduced in comparison to normoxia.²⁸ However, these observations were shown to be due to decreases in progenitor proliferation and an efflux of cells from the cell cycle into G₀ due in part to upregulation of cyclin-dependent kinase inhibitor (CDKI) genes p21cip1, p27Kip1, and p57Kip2 within the hypoxic LSK population. Moreover, fewer hypoxic LSK cells were required to reconstitute lethally irradiated mice to the same degree as normoxic cultures, suggesting that despite both hypoxic and normoxic conditions' ability to preserve HSC populations; LT-HSCs are expanded more robustly under hypoxic conditions.

While using lowered oxygen tensions instead of normal atmospheric oxygen levels might be of benefit, it is known that certain stem cell factors such as thrombopoietin (TPO) can scavenge and inactivate ROS, which most likely provides some of the benefits of hypoxia while maintaining the cultures under non-hypoxic conditions.³⁶ It is hypothesized that quenching ROS formation using antioxidant culturing adjuvants, such as glutathione (GSH) and ascorbic acid 2-phosphate (AA2P), under normoxic conditions might preserve HSC multipotency and retard differentiation in human mobilized peripheral blood (mPB) slated for transplant.³⁷ Yet to be identified is whether such additions will make significant improvements in HSC maintenance, and it is assumed that such treatments would supplement more potent forms of HSC expansion

instead of being used in isolation. Paradoxically, quenching ROS production within HSC cultures to extremely low levels could have adverse effects on expansion efforts. This is because findings in 2008 suggest that TPO stabilizes HIF-1 α by generating mitochondrial ROS, that when quenched by electron transport chain (ETC) inhibitors or potent ROS scavengers mitigated downstream *Hif-1 α* expression.³⁴ Similar findings have been observed for proliferation-inducing cytokines such as stem cell factor (SCF), IL-3, erythropoietin (Epo) and granulocyte colony-stimulating factor (G-CSF), all of which generate and use ROS to promote proliferation.³⁸⁻⁴⁰ These are not incongruent findings, per se, but most likely highlight the multifunctional roles that cytokines and ROS can play within different cellular contexts.

CHAPTER 4. GENES AND TRANSCRIPTION FACTORS

Hox Genes and HSC Expansion

Hox genes that were first implicated in embryonic development and structural patterning are now known to play a function not just in embryonic cells, but also in somatic cells, such as those of the hematopoietic system.⁴¹ Among the Hox genes, which compose the homeobox gene family, are four clusters of highly conserved transcription factors (TF) that are classified as A, B, C, and D and have been found to have overlapping roles among the clusters.⁴² Of the 39 *Hox* mammalian genes, 16 are transcribed during hematopoiesis and encode TFs that are involved in regulating self-renewal and differentiation. Certain pivotal discoveries have shown that species of the A and B clusters are highly enriched in primitive hematopoietic subpopulations and play a positive regulatory role in HSC self-renewal.⁴³

HoxB4 is particularly enriched within the early hematopoietic subpopulations and, when overexpressed in murine bone marrow (BM) has led to a 50-fold increase in numbers of competitive repopulating unit (CRU). *Ex vivo* expansion of HoxB4-transduced cells resulted in a 40-fold increase in HSC compared to starting numbers after 14 days of culturing and over 1000-fold increase when compared to controls.⁴⁴ Moreover, retrovirally transduced HoxB4-expanded murine HSCs maintained lymphoid and myeloid repopulating potential without incurring blocks in development. Successful efforts have been made to improve upon the retroviral transduction of HoxB4 by concurrently suppressing expression of Hox cofactor PBx1 as well as generating a NUP98 nucleoporine gene fusion with HoxB4 and HoxA10.⁴⁵ With respect to the NUP98 fusion proteins, there was a 300-fold increase in CRUs among the NUP98-HoxB4 version and 2000-fold increase with the NUP98-HoxA10 version.

Furthermore, strategies have been pursued that remove the need for retroviral transduction, such as recombinant TAT-HoxB4 fusion using the transactivating protein (TAT) of HIV to permit protein delivery through the cell membrane. Use of the transduction-free TAT-HoxB4 protein for four days on *ex vivo* murine HSC cultures led to 6-fold increases over starting HSC numbers and upwards of 20-fold increases over control cultures.⁴⁶ However, problems have been reported with high ectopic expression of HoxB4 in human retrovirally transduced cord blood (CB) CD34⁺ cells, causing a presumptive block in myeloerythroid progenitor differentiation while promoting an *in vivo* growth advantage of primitive CD34⁺ populations.⁴⁷ It has been suggested that there isn't a complete block, but instead a delay in differentiation commitment that is potentially due to a dosage-dependent response, wherein an upper threshold, when crossed, increases myeloerythroid abnormalities and within an undetermined range optimal HSC expansion occurs without skewing production of certain progenitor subpopulations.^{48,49} Means of addressing the commitment delay encountered by constitutively high expression of HoxB4 have used inducible systems such as fusion of *HoxB4* with tamoxifen responsive oestrogen receptor serving as a way to eliminate exogenous HoxB4 expression after expansion has occurred.⁵⁰ Current studies are using

such systems to investigate HoxB4 target gene expression and reaction kinetics necessary for clinically relevant HSC expansion.

Two Routes on the Wnt Pathway

Maintenance of HSC self-renewal and inhibition of differentiation is known to depend upon the presence of the canonical Wnt signaling pathway, which has received focused research efforts.^{51,52} While there are other noncanonical Wnt pathways, namely the planar cell polarity (PCP) and Wnt-Ca²⁺ pathway, neither directly promotes β -catenin accumulation, which has been shown to play an important role in HSC self-renewal.⁵¹

Despite the lack of direct induction of β -catenin within the noncanonical pathways, recent research has found that *in vivo* Wnt signaling through the noncanonical pathways maintains LT-HSC quiescence within the BM niche populated by N-cadherin positive osteoblasts (N-cad⁺OBs).⁵³ Sugimura *et al.* report that N-cad⁺OBs within the BM microenvironment interact with HSCs expressing Flamingo (Fmi) at the N-cad⁺OB interface, which hydrophobically recruits the noncanonical Wnt receptor Frizzled 8 (Fz8) to maintain quiescence. This localized noncanonical Wnt signaling, which antagonizes canonical Wnt signaling, occurs predominately under homeostatic processes, but under physiological stress, such as when fluorouracil (5FU) is administered for myeloablative purposes, dynamic changes occur in the Wnt ligands of N-cad⁺OBs, leading to a shift in increased canonical Wnt signaling due to loss of the antagonistic actions of the noncanonical Wnt pathway.⁵³ These observations suggest that noncanonical and canonical Wnt signaling within the BM microenvironment work in opposition to balance between HSC quiescence and proliferation.

The canonical wingless-type (Wnt) pathway consists of secreted palmitoylated morphogens (Wnt3a) that bind the Frizzled family of receptors along with lipoprotein receptor-related proteins 5 and 6 (LRP-5/6) and prevent ubiquitin-driven degradation of β -catenin.⁵⁴ Upon canonical Wnt pathway stimulation, glycogen synthase kinase (Gsk-3 β) phosphorylation of β -catenin is prevented, thereby promoting accumulation of β -catenin, which is imported into the nucleus where it can interact with T-cell transcription factor (Tcf)/lymphocyte-enhancer binding factor (Lef) family to induce changes in target gene expression. Concomitant with a whole host of Wnt-induced gene changes are notable increases in HoxB4 and Notch1 expression, which have been shown to promote self-renewal and likely play a role in the increased self-renewal capacity of Wnt-stimulated HSCs.^{44,55} As of this writing, expansion through canonical Wnt stimulation has led to an 8-80 fold expansion in functional HSC numbers.⁵¹ Despite these achievements, apparently incongruous results within the field have made HSC expansion using canonical Wnt stimulation unclear.⁵⁶ In several papers there has been demonstration of exhaustion and blocks in myeloid differentiation within the HSC pool following constitutive expression of stabilized forms of β -catenin^{7,57} whereas other reports from different labs have shown enhancement of HSC function and maintenance.^{51,54,58} A complete understanding of such inconsistencies will likely need further research; however, it does appear likely that some of the inconsistencies can be

attributed to differences in degree of Wnt pathway stimulation.³⁰ Recent work by Luis *et al.* used five different mutations in the adenomatous polyposis coli (APC) gene to generate a gradient of *in vivo* Wnt signaling to show how intermediate levels of Wnt stimulation led to increased myeloid differentiation potential and clonogenicity, whereas only mildly increased Wnt signaling led to enhancement of HSC repopulation capacity.³⁰ Notably, higher levels of Wnt pathway activation led to total failure of repopulation capacity and were supported by a strong reduction of CFU colonies in methylcellulose assays. Reductions in HoxB4 and Cdkn1a (p21) gene expression were also observed with concomitant increases in Wnt stimulation, extending the evidence that optimal maintenance of HSC self-renewal capacity is achieved through minimal canonical Wnt stimulation.³⁰ It is possible that low-dose Wnt stimulation may smooth out a sharp biphasic response and thereby may limit exaggerated expression of negative regulators such as Dickkopf-related protein 1 (DKK1).^{52,59} While some studies have shown constitutively high Wnt activation to be beneficial in HSC expansion, it now appears that regimented Wnt signaling offers the best HSC expansion outcomes.^{51,57}

Achieving optimal levels of Wnt stimulation in a dynamic system can be challenging; however, small molecular compounds that can be removed facilitate a level of control that can be very useful for *ex vivo* expansion. Targeting Gsk-3 β , which functions as a hub for crosstalk among many other signaling pathways, doesn't indicate precise Wnt stimulation. This is because preservation of cytoplasmic β -catenin is one of the primary means of changing target gene expression for the canonical Wnt, Hedgehog, and Notch signaling pathways.⁶⁰ Overlap of signaling mechanisms and indirect regulatory feedback by downstream target genes can make it challenging to tease apart the exact mechanism of HSC expansion drugs.

Regardless, most small molecule inhibitors of Gsk-3 β attributed to the Wnt pathway affect the ATP binding pocket, thereby preventing phosphorylation of β -catenin on Thr41, Ser37, and Ser33.⁶¹ Several constituents of Tyrian purple, a dye produced from mollusks, have been successfully tested for their selective ability to inhibit Gsk-3 β and expand the HSC pool through preventing β -catenin degradation.⁶² Chemical modifications to these compounds, in particular, 6-bromoindirubin-3'-oxime (BIO), a synthetic indirubin analog which is readily cell permeable, have shown a modest ability to promote HSC expansion and prevent loss of HSCs when grown in culture.⁶³ However, recent insights suggest that Wnt stimulation alone isn't sufficient to expand HSC *ex vivo*. Notch signaling appears integral to regulating HSC permissiveness to Wnt induced self-renewal. This interdependence upon multiple signaling pathways is likely common and will necessitate expanding our continued understanding of HSC biology to connect once-disparate pathways for development of better treatments.

Relevancy of SALL4

The zinc-finger transcription factor, SALL4 functions as a regulator of embryonic stem (ES) cell pluripotency through Oct4 and Nanog and has been recognized for its connections between leukemia and self-renewal in HSCs.⁶⁴ Recent investigations of

SALL4 HSC expansion properties have used lentiviral transduction to constitutively express two isoforms (SALL4A, SALL4B) in human bone marrow (BM) CD34⁺ cells which produced a 130-fold expansion of the CD34⁺/CD38⁻ population after 7 days while cultured in cytokines (SCF, TPO, Flt-3L).⁶⁵ Extending the culturing time to 14 days led to a 368-fold and 384-fold increase for SALL4A and SALL4B isoforms respectively, with further increases in culturing leading to over 10,000-fold expansion. However, it appears that SALL4 expansion is cytokine dependent and limiting levels of SCF and TPO impacted SALL4 HSC expansion capabilities. An effort to test a more clinically relevant means of using SALL4 without transducing cells led to the production of a cell-permeable fusion SALL4B-TAT protein. When SALL4B-TAT was administered at 200nM twice daily for four days to human CD34⁺ cells, it showed a modest 10-fold expansion. The authors noted that further optimization will ultimately be necessary to achieve more robust *in vitro* HSC expansion with the SALL4-TAT fusion protein. These future optimizations will be important because the possibility exists for the latent development of leukemia when transduction is used to achieve a constitutively active SALL4.⁶⁶

Relevancy of the Gatekeeper Notch

The Notch signaling pathway is an evolutionary conserved mechanism involved in development and cell-fate specification along with regulatory functions that have been identified in a multitude of stem cell systems.⁶⁷ While there are four type I receptors (Notch-1, -2, -3, and -4) with extracellular domains that can interact with a neighboring cell's membrane-bound ligands (Delta1, -3, -4, Jagged1, -2), determination of which Notch receptor-ligand interactions are most efficacious within HSCs at promoting *ex vivo* expansion is still ongoing.¹¹ Mechanistically it is clear, however, that Notch receptor-ligand interactions between HSCs and HSCs-to-stromal cells (adipocytes, endothelial cells, macrophages and fibroblast) within the BM niche precipitate Notch pathway activation. This induces TACE and γ -secretase mediated proteolytic cleavage of the Notch intracellular domain (NICD), freeing it to translocate into the nucleus whereupon it can interact with the transcription repressor CBF-1 and upregulate Notch target genes. Elucidation of particular target genes that have been found to have significant roles in maintaining HSC quiescence is still ongoing, but the Hes family of genes along with GATA-2 is notable.⁶⁸

Relevance of the Notch signaling pathway to hematopoietic tissues was first made when Milner *et al.* identified Tan-1, a Notch homologue, within human Lin⁻CD34⁺ BM cells based upon shared consensus sequences within other Notch homologues.⁶⁹ Notch activity within the hematopoietic system was further supported through the identification of increased reconstitution ability when soluble human Jagged-1 was administered to HSC cultures by Karanu *et al.*⁷⁰ Furthermore, it was found that signaling between HSCs expressing Notch-1 and BM stromal cells expressing Jagged-1 led to inhibition of HSC differentiation, which was successfully abrogated through inhibition of γ -secretase mediated Notch activation.^{71,72} Once relevance of Notch signaling was established within the hematopoietic system, further investigations were conducted into HSC expansion

using various forms of Notch signaling, including Notch-2.⁷³ Within murine models, it was conclusively shown that constitutively active expression of the intracellular domain of Notch-1 (IDN1) within HSCs resulted in cytokine-dependent immortalization that permitted both myeloid and lymphoid lineage differentiation.⁵⁵ Despite moderate success of promoting Notch-mediated increases in HSC progenitors within murine models, successes within human HSCs initially proved to be hit or miss. In 2007, Chadwick *et al.* reported that when human umbilical cord blood CD34⁺ cells were transduced with a constitutively activated form of Notch, it led to unexpected altered cell-cycle kinetics, resulting in apoptotic reductions in the absolute number of CD34⁺ cells.⁸ These observations are in line with other reports corroborating altered cell cycle kinetics when a constitutively active form of Notch is used.⁷⁴

Interestingly, no impairment of HSC self-renewal or differentiation was observed when a Cre-LoxP mediated conditional inactivation of Jagged1 within BM stromal cells was performed.⁷⁵ These findings led Mancini *et al.* to conclude that Notch-1-Jagged-1 signaling is dispensable for human HSC self-renewal and differentiation under physiological conditions. Further investigations will likely need to be performed to ascertain whether other Notch pathway paralogs with overlapping functionality compensated for the conditional knock out of Notch-1-Jagged-1 signaling. In light of the dispensable nature of certain Notch signaling pathways *in vivo* it is interesting to see marked success when an immobilized instead of soluble Delta-1 was used within CD34⁺CD38⁻ human cord blood cultures.⁷⁶ Ohish *et al.* from the Bernstein lab demonstrated that an immobilized Delta-1 was able to provide upwards of 100-fold increase in the CD34⁺ cell population relative to controls.⁷⁶ This observations led to the publication of a 2010 phase I clinical study by Delaney *et al.* that successfully demonstrated *ex vivo* Notch activation can be used to increase the number of HSCs and progenitors while rapidly reconstituting myeloid engraftment in patients receiving HSCTs.⁷⁷ This is noteworthy because studies that had previously used soluble Notch ligands within their investigations potentially did not induce endocytosis of the Notch receptor-ligand complex necessary for pathway activation.⁷⁸ Within the same lab, a dosage dependent effect was observed for immobilized Delta-1 ligand stimulation of the Notch pathway.⁷⁹ In that study, lower levels of immobilized Delta-1 led to increased CD34⁺ precursors, whereas higher levels were associated with apoptosis and inhibition of myeloid differentiation. Moreover, lethally irradiated NOD/SCID mice ability to reconstitute using CD34⁺ cells was better when lower Delta-1 densities were used.

It bears noting that these dosage-dependent Notch findings are similar to recent discoveries made within the canonical Wnt pathway.³⁰ Within the Wnt pathway, relatively minimal stimulation provided better HSC expansion than higher doses did. While the level of stimulation is important, the bigger picture is that determining the optimal concentration range for HSC expansion regimens is critical. In fact, studies utilizing a constitutively activated Notch or Wnt, while providing important data, haven't achieved ideal HSC expansion levels.

What would be the outcome if optimal levels of selected signaling pathways were determined and used simultaneously? A more advanced understanding of the complex interplay is finally facilitating better optimization. For instance, it has been known that canonical Wnt stimulation promotes self-renewal; however, it has now been found that Notch plays an essential gatekeeper function that controls the Wnt pathway's ability to promote self-renewal.¹² Interestingly, as Notch stimulation was inhibited, canonical Wnt stimulation's self-renewal properties were diminished and led to increasing rates of differentiation. The integration of these two pathways fits our current understanding of the BM niche because increasing levels of HSC differentiation show concordant drops in Notch signaling. However, it is too early to determine whether differentiation itself leads to lowered Notch signaling or whether decreases in Notch expression precede differentiation events leading to the self-renewal effects of Wnt driving further differentiation. Regardless, this study has implications outside understanding the physiology of the BM niche because it can be used for better promoting the self-renewal properties of small molecular therapeutics, such as GSK-3 β inhibitors, and further optimization efforts regarding optimal HSC density within starter cultures. While, it had been known that high starting HSC cell densities are necessary within cultures to prevent HSCs from prematurely differentiating, it is now assumed that high cellular densities provide greater HSC-to-HSC Notch signaling that maintains HSC permissiveness to concomitant Wnt self-renewal cues. It still remains to be seen exactly how other signaling pathways integrate with one another within the HSC niche, but elucidation of such interactions will improve upon and possibly lead to the development of new *ex vivo* expansion techniques.

Furthermore, just as has been observed in the Wnt pathway, Notch stimulation leads to the upregulation of genes that have a negative feedback regulatory roles.⁸⁰ For the canonical Wnt pathway within ES cells, it was shown that a slow ramping on and off, biphasic response was typical.¹⁰ It is possible that the biphasic response described for the Wnt pathway might very well be truncation of a proposed oscillation model within neural stem cells that has yet to be applied to HSCs.^{68,81} Nonetheless, the oscillation model is interesting because HSCs need to function in two primary roles: self-renewal of LT-HSCs and proliferation of progenitors. However, governing between these two roles during homeostasis at the cellular level is relatively slow. The cellular cycling likely belies a much more dynamic oscillating response that actively balances between homeostasis and promoting proliferation under physical duress. More detailed single-cell analysis will provide better insights into whether the oscillation model applies to HSCs. If it does, then the information gleaned from such studies will enable better control of the both the Notch and Wnt pathways.

CHAPTER 5. HSC MOLECULAR THERAPIES

Development of small-molecule compounds to alter existing signaling pathways in order to induce HSC expansion has been of interest to researchers since the identification of the first HSC factors. The ability to expand stem cells with an easily administered adjuvant is particularly tantalizing because it can be withdrawn from the culture relatively easily. Moreover, challenges faced by purifying proteins and mitigating risks associated with vector-mediated gene integration approaches can make small-molecule development appear tantalizing.⁸² Of course, small-molecule development has its own set of hurdles that can necessitate screens of large chemical libraries, which are often difficult or costly to access. However, advances in cheminformatics have permitted *in silico* screening of large virtual chemical libraries, which enable target genes to be computationally docked with databases of millions of small molecular compounds for reduced lead-development costs.⁸³ Regardless of how they are discovered, the following efforts have led to chemical compounds that can induce HSC self-renewal, control lineage commitment of progenitors, and revert lineage-committed cells back to multipotent states. Some of the primary means of facilitating HSC expansion include, but are not limited to, canonical Wnt stimulation, AhR antagonism, Notch-1 stimulation, chromatin modification, and copper chelation. While each of these has demonstrated its own measure of success at HSC expansion, StemRegenin (SR1) has come the closest so far of achieving the idealized goal of a single molecular compound that robustly expands HSCs.⁸⁴ This does not diminish the usefulness of other molecular compounds or pathways, but highlights the challenge associated with finding a single compound that can achieve expansion in a highly regulated and complex process.

The Aryl Hydrocarbon Receptor Success Story

The aryl hydrocarbon receptor (AhR) is a basic-helix-loop-helix (bHLH) transcription factor that is involved in metabolizing xenobiotics and belongs to the PAS (Per-ARNT-Sim) superfamily, which includes genes (*Hif-1 α*) that play a role in cellular sensing of hypoxia and oxidation-reduction status.⁸⁵ The function of AhR in hematopoiesis is not fully understood, but it is thought that one of its roles is as a negative regulator of hematopoiesis by relaxing quiescence constraints and promoting HSC proliferation when AhR is ablated or under the influence of an antagonist.^{85,86} Searches for endogenous AhR ligands that function to regulate hematopoiesis have not yielded any tangible leads; however, studies have shown that exogenous constitutive activation of AhR by the endocrine toxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) leads to a 4-fold expansion of HSC numbers, albeit with a diminished long-term repopulation capacity.⁸⁷ In 2010, Boitanio *et al.* performed a confocal microscopy-aided screen of 100,000 heterocyclic compounds for changes in human mobilized peripheral blood (mPB) cell surface marker expression of CD34 and CD133 after *ex vivo* culture for five days.⁸⁴ StemRegenin1 (SR1) is one of the more successful purine analogs that were identified to function as an antagonist of AhR, which was verified by reversibly blocking with TCDD. Furthermore, SR1 went on to expand primary human CD34⁺ cells 73-fold

after culturing with cytokines (TPO, SCF, FLT3, IL-6) for 3 weeks, which has been the most successful *ex vivo* HSC expansion method to date, save the >10,000-fold month-long expansion reported in the Aguila *et al.* SALL4 study.^{65,84}

However, failure of the SR1 heterocycle to expand HSC in murine models highlights the possibility that the < 20% amino acid shared homology between mouse and human AhR confers species specific ligand binding.⁸⁸ These differences between mice and human AhR could limit the potential of developing therapeutic leads for humans based on drug screens using murine models. Additionally, improvements in HSC expansion using AhR as a target for docking of virtual ligands cannot occur since no crystal structure of human AhR exists. However, there appear to be reports of successful use of receptor mapping or quantitative structure-activity relationship (QSAR) for lead development that might be applicable to *in silico* screens in the future if a crystal structure is still unavailable.^{89,90} The discovery of SR1 and its robust expansion of HSCs has the potential to be an effective clinical therapeutic for successful HSCTs.

Chromatin Modifying Agents: Reestablishing Multipotency

Concomitant with HSC differentiation are epigenetic changes in critical gene expression networks that arise from global changes in chromatin methylation and acetylation status.^{91,92} The hematopoietic genes that are regulated through these networks ultimately direct progenitor fate and restrict HSC self-renewal potential. Interest in reversing or mitigating changes in chromatin structure that promote differentiation so that HSC self-renewal capacity can be preserved has led to the use of hypomethylating agents as well as histone deacetylase (HDAC) and histone acetyltransferase (HAT) inhibitors. Of three known HDAC inhibitors including suberoylanilide hydroxamic acid (SAHA), trichostatin (TSA), and valproic acid (VPA), VPA has been observed to have the most potent epigenetic reprogramming efficiency.⁹³ The antiepileptic medication VPA was identified as an HDAC inhibitor that can induce differentiation of cancer cells by promoting hyperacetylation.⁹⁴ It has been shown that VPA functions in a dose-dependent manner to prevent differentiation and promote proliferation of human CD34⁺ BM cells when cultured for 7 days.⁹⁵ Valproic acid induction of proliferation appears to be linked to down-regulation of the cyclin-dependent kinase (CDK) p21^{cip-1/waf-1}, which regulates HSC quiescence and entry into the cell cycle.^{95,96} Additional VPA effects include inhibition of Gsk-3 β , leading to downstream Wnt target gene expression, including HoxB4.⁹⁵

Additional efforts to chemically mediate epigenetic programming to facilitate expansion of HSCs have involved the sequential treatment of cultures with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5azaD) followed by TSA.⁹⁷ Araki *et al.* posit that the sequential nature of the treatment is necessary to remove the histone methylation modifications that sterically restrict access of the deacetylation inhibitor, TSA. The addition of the compounds to cord blood (CB) CD34⁺ cells led to a 5-fold to 3.5-fold expansion in CD34⁺ CD90⁺ cells that was dependent on the cocktail of cytokines used within the culture. However, these modest increases were not sustainable past 9

days of culture and corresponded with a significant decrease in p21 expression, suggesting that the HSC pool became exhausted.⁹⁷

Other studies on epigenetic modifiers have resulted in a screen of 92 natural compounds that led to the isolation of Garcinol (GAR), a plant benzophenone derivative from the mangosteen family *Garcinia indica*, which functions as a HAT inhibitor.⁹⁸ A synthetic derivative of GAR, Isogarcinol (ISO) led to a 7.4-fold expansion of CD34⁺CD38⁻ cells when cultured with cytokines (SCF, TPO) for 7 days. Microarray analysis of the ISO cultured cells showed upregulation of AMICA1, BTG2, and HLF; however, upregulation of genes associated with HSC self-renewal (HoxB4, BMI1, GATA2, Notch-1) was not observed. Possible alternative mechanisms for the HSC expansion besides epigenetic modifications were suggested to be attributable to suppressed acetylation of p53 at K382, which leads to reduced targeted DNA binding by p53.^{98,99} Using the GAR and ISO observations as an example, it would be prudent to consider that other chromatin-modifying compounds could also be concurrently affecting post-translational modifications of proteins that play a role in HSC expansion, like p53. Consistent with the multiple mechanisms through which chromatin-modifying agents can function, further work will need to be done to understand and take advantage of their therapeutic possibilities.

Culture Media Copper Chelation

Copper (Cu) is a dietary trace mineral that is necessary for a fully functional hematopoietic system. When there is a copper deficiency the resulting effects on HSC differentiation can manifest in a pathology consisting of anemia, neutropenia, and thrombocytopenia.¹⁰⁰ Efforts to understand the role that inadequate absorption of dietary Cu plays in hematological abnormalities have led to investigations of how Cu promotes HSC differentiation and whether limiting Cu availability is beneficial for therapeutic expansion of HSCs. There isn't a complete understanding of the role Cu plays in HSC differentiation, but it is known that retinoic acid, a known promoter of differentiation, induces uptake of Cu during the early-stage differentiation in human myeloid leukemia HL-60 cells.¹⁰¹ Additionally, it has been found that cellular-free Cu in cultures promotes generation of reactive oxygen species (ROS), which leads to HSC exhaustion and can hinder maintenance of multipotent hematological pools for therapeutic purposes.^{31,102}

Based on these observations, investigators have successfully slowed HSC differentiation by attenuating copper levels using copper-specific chelators such as tetraethylenepentamine (TEPA).^{100,102,103} In one study, TEPA was administered to *ex vivo* cultures of purified CD34⁺ cord blood (CB), which led to a robust enrichment of early progenitor subpopulations (CD34⁺CD38⁻ and CD34⁺Lin⁻) along with increased capacity to repopulate NOD/SCID mice.¹⁰⁰ These studies set the stage for a phase I/II clinical trial that was established at MD Anderson Cancer Center to test the effectiveness of TEPA (StemEx) at expanding a single CB unit that was split into a treated and untreated arm, then was recombined right before patient administration.¹⁰³ After culturing a portion of the CB unit with TEPA and cytokines (Flt-3 ligand, IL-6, and TPO) for 21

days, the untreated CB arm was infused and administered within 24 hours to each of the respective ten patients, of whom the average age was 21. Efforts to use a single CB unit have historically run into the problem of not being able to provide an adult patient with an adequate supply of TNC for successful engraftment. In this case, the TEPA (StemEx) treated CB units led to an average TNC expansion of 219-fold and a 6-fold increase in CD34⁺ cells over starting numbers, but did not result in faster neutrophil or platelet reconstitution.¹⁰³ However, even with the 219-fold expansion, the mean 1.8×10^7 /kg TNCs administered per adult patient fell below the 3×10^7 /kg dose recommended for engraftment, with higher levels of 5.2×10^7 /kg providing even better success.^{2,3} Despite the low TNC dose administered, nine of the ten patients engrafted with a 70% survival reported at the end of the 180-day study.¹⁰³ At the conclusion of the StemEx clinical study, de Lima *et al.* deemed that treatment of *ex vivo* cultures with TEPA is feasible and has led to the establishment of a phase II/III study that will further address safety and efficacy when expanding a single CB unit for treatment of hematological cancers.

Considering the modest, albeit successful, expansion that StemEx attained, it would be interesting to see how hypoxic conditions affect *ex vivo* HSC expansion since 5% CO₂ in humidified air (20% O₂) was used for the respective study. Reasons for making such suggestions stem from evidence that administering CuCl₂ can stabilize nuclear Hif-1 α under normoxic conditions and that by chelating copper away for upwards of 3 weeks, while ultimately shown to benefit expansion, could be mitigating known benefits of hypoxia response element (HRE) mediated gene expression.^{9,104} Whether additions of certain stem cell factors, such as TPO, under normoxic conditions can compensate for hypoxic effects would also need investigation.¹⁰⁵ Regardless, it's possible that TEPA-induced copper chelation could lead to decreased HIF-1 target gene expression and negatively impact HSC numbers. While speculative, the effects of hypoxia would be assumed to lead to decreases in TNC in favor of more LT-HSCs versus controls. While copper chelation has been shown to be a viable adjuvant to HSC expansion protocols, further optimizations will be needed if copper chelation is to serve as an alternative to more robust HSC expansion regimens, such as immobilized Delta-1 and SR1. That being said, copper chelation is most suitable as a potential adjuvant instead of as a primary HSC expansion therapeutic.

CHAPTER 6. IPSC: A POTENTIAL CURE-ALL

The seminal discovery by Takahashi and Yamanaka that over-expression of key transcription factors (OCT4, SOX2, KLF4, c-MYC) in fully differentiated somatic cells can induce embryonic stem (ES) cell-like pluripotency by reprogramming the genomic methylation signature has ushered in a wave of therapeutic potential for induced pluripotent stem cell (iPSC) biology.¹⁰⁶⁻¹⁰⁸ For hematological disorders, iPSCs offer the possibility to produce a scalable supply of patient-derived HSCs by successfully recapitulating an ES-like pluripotent phenotype and inducing directed differentiation of progenitors.¹⁰⁹⁻¹¹¹ Recent advances reveal that reprogrammed iPSCs are amenable to gene-targeting strategies that can be used to treat diseases such as β -thalassemia and sickle cell anemia.¹¹²⁻¹¹⁵ However, before such methods or iPSC-based HSC expansion protocols can be used in a clinical setting, a number of issues will first need to be rectified. Foremost, factor-based reprogrammed cells do not fully recapitulate the epigenetic state of ES cells despite having similar (95%) global gene expression profiles and the ability of iPSCs to form all three germ layers: the endoderm, mesoderm, and ectoderm.^{116,117} Moreover, the current intractability of iPSCs to be homogeneously and consistently reprogrammed leads to autochthonous lineage-derived methylation signatures that can foster differentiation that is more congruent with the tissue of origin.¹¹⁶

Granted, more thorough reprogramming has been achieved through concomitant use of chromatin-modifying adjuvants such as DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-AZA) and the HDAC inhibitor valproic acid.^{93,116} The drugs appear to ameliorate incomplete reprogramming by removing latent methylation when used in conjunction with current reprogramming protocols. Nonetheless, somatic nuclear transfer was shown to be better than iPSCs at achieving ES-cell-like properties even when treated with chromatin-modifying compounds. Determining what role these variable methylation differences play in driving differentiation of different cell types and finding ways to produce consistent epigenetically stable iPSCs will be imperative to future iPSC-derived HSC expansion.¹¹⁸

There have also been concerns regarding the ability to recapitulate ES-cell-like telomeres, which conceivably contributed to early senescence and limited expansion of iPSC-derived hemangioblasts.¹¹⁹ Conversely, recent reports have shown that iPSCs have upregulated telomerase expression along with elongated telomeres with ES-like epigenetic marks.¹²⁰ It appears that the recalcitrant nature of centromeric and telomeric DNA to remodeling along with observed iPSC stochastic methylation mechanisms, highlights the need to further understand what, if any, downstream differentiation effects exist.¹¹⁷ Additional iPSC concerns center on increasing reprogramming efficiency as well as discovering stringent clinical strategies for delivering the necessary transcription factors without utilizing genome-integrating vectors. While genomic safe-harbor locations have been identified for targeted integration for transgene expression in thalassemia iPSCs and it is known that exogenous transcription factors are silenced,

nonetheless the risk of oncogenic integration events arising should be reasonably mitigated for iPSC-derived HSCTs.¹¹⁴

An interesting use of synthetically modified mRNAs containing 5-methylcytidine for cytidine and pseudouridine in place of uridine among other structural changes has achieved an optimized reprogramming efficiency that surpasses .001-3% while also eliminating the need for vector integrations.¹²¹ While synthetic mRNAs presumably offer a reduced oncogenic profile, daily stoichiometric administration of factors can be prohibitively time consuming. Other non-integrating efforts have used cell-penetrating proteins and miRNA, but optimizations to achieve higher reprogramming efficiency are still necessary.^{122,123} With emerging higher-efficiency reprogramming conditions including hypoxia, MEK inhibitor PD0325901, GSK-3 inhibitor CHIR99021, kenpaullon, and the TGF- β inhibitor RepSox the possibility exists for iPSCs to be reprogrammed entirely by a cocktail of small molecular therapeutics.¹²⁴⁻¹²⁶

The resounding recognition that iPSCs and the 2012 Nobel Laureates Shinya Yamanaka and John Gurdon have received for their respective work in 2006 and 1962 is clear. The potential for iPSCs to revolutionize not just HSC expansion protocols, but also therapeutics that will benefit from patient-tailored therapeutics is substantial. Moreover, the vigor with which iPSC issues are being investigated is nothing short of remarkable. It doesn't take a great leap of imagination to see how progressing optimizations, along with our ever-increasing understanding of somatic-to-pluripotent reprogramming, is a keystone in a looming revolution of tailored cures for hematological diseases. Also, the inevitable integration of iPSCs with other successful HSC strategies will offer an exciting supply of clinically relevant HSC expansion opportunities.

CHAPTER 7. CONCLUSION

Insofar as multiple signaling pathways can be justifiably separated from one another through focused pathway research, there comes a time to unite our understanding of the combination of direct and indirect mechanisms that interact to produce HSC expansion. Although there is still much that needs to be elucidated, there is evidence that regulatory pathways can reinforce one another to produce better results than when isolation of treatments is adopted. These statements should in no way downplay the challenges of teasing apart complex interactions divided among multiple pathways because such a thorough understanding will likely necessitate HSC system-wide investigations to fully comprehend. However, the beginning of such efforts is under way with the exciting use of induction-coupled mass-spectroscopy (ICP-MS), which permits many cellular signatures to be analyzed simultaneously in responses to therapeutic modifications within a single cell.¹³ The surge in information that such advances in technology will provide, coupled with automated feeding systems, will facilitate precise control of potential negative feedback systems when expansion-promoting TFs are upregulated or HSC epigenetic reprogramming is desired. While still in its relative infancy with regard to the number of measurements that can be taken concurrently, the impressive amount of data that has been gathered at this early stage suggests that future optimizations will provide very detailed snapshots of HSC signaling under varying conditions.

With the increasing ability to precisely identify the cellular modifications attributable to HSC regulatory pathways comes the key to synergizing the multitude of modern clinical strategies in order to expand HSCs for performing HSCTs. It is my view that not just through solely synergizing complementary pathways together, but also through a thorough gaining understanding of the indirect and direct negative feedback effects concordant with upregulation of key regulatory pathways, will the opportunity be realized to tailor a cocktail of small molecular compounds that can generate clinically significant quantities of HSCs. It is this author's opinion that detailed identification of signaling mechanisms within HSCs will be imperative to extracting the best results successful for many forms of HSC expansion, as since any efforts to induce constitutive activation of expansion-promoting genes will likely be mitigated by a concordant increase in inhibitory regulatory pathways. Once these regulatory pathways are identified, they can be targeted through small molecular compounds which that will facilitate achieving the best results possible for HSCT-based therapies.

LIST OF REFERENCES

1. Harris, D. T. et al. Phenotypic and functional immaturity of human umbilical cord blood T lymphocytes. *Proc. Natl. Acad. Sci. USA* 89, 10006–10010 (1992)
2. Rocha, V. & Gluckman, E. Improving outcomes of cord blood transplantation: HLA matching, cell dose and other graft- and transplantation-related factors. *Br. J. Haematol.* 147, 262–274 (2009)
3. Michel, G. et al. Unrelated cord blood transplantation for childhood acute myeloid leukemia: a Eurocord Group analysis. *Blood* 102, 4290–4297 (2003)
4. Thomas, E., Storb, R. & Clift, R. Bone-marrow transplantation. *N. Engl. J. Med.* 292 (17), 895–902 (1975)
5. Till, J. E. & McCulloch, E. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* 14, 213–222 (1961)
6. Goodell, B. M. A., Brose, K., Paradis, G., Conner, A. S. & Mulligan, R. C. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* 183, 1798–1806 (1996)
7. Kirstetter, P., Anderson, K., Porse, B. T., Jacobsen, S. E. W. & Nerlov, C. Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat. Immunol.* 7, 1048–1056 (2006)
8. Chadwick, N. et al. Notch signaling induces apoptosis in primary human CD34+ hematopoietic progenitor cells. *Stem Cells (Dayton, Ohio)* 25, 203–210 (2007)
9. Takubo, K. et al. Regulation of the HIF-1 α level is essential for hematopoietic stem cells. *Cell Stem Cell* 7, 391–402 (2010)
10. Ueno, S. et al. Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc. Natl. Acad. Sci. USA* 104, 9685–90 (2007)
11. Bolós, V., Grego-Bessa, J. & de la Pompa, J. L. Notch signaling in development and cancer. *Endocr. Rev.* 28, 339–363 (2007)
12. Duncan, A. W. et al. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat. Immunol.* 6, 314–322 (2005)

13. Bendall, S. C. et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science* 332, 687–696 (2011)
14. Hofmeister, C. C., Zhang, J., Knight, K. L., Le, P. & Stiff, P. J. Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. *Bone Marrow Transplant.* 39, 11–23 (2007)
15. Shpall, E. J. et al. Transplantation of ex vivo expanded cord blood. *Biol. Blood Marrow Transplant.* 8, 368–376 (2002)
16. Murray, L. J. et al. Thrombopoietin, flt3, and kit ligands together suppress apoptosis of human mobilized CD34+ cells and recruit primitive CD34+ Thy-1+ cells into rapid division. *Exp. Hematol.* 27, 1019–1028 (1999)
17. Zhang, C. & Lodish, H. Cytokines regulating hematopoietic stem cell function. *Curr. Opin. Hematol.* 15, 307–311 (2008)
18. Madlambayan, G. J. et al. Dynamic changes in cellular and microenvironmental composition can be controlled to elicit in vitro human hematopoietic stem cell expansion. *Exp. Hematol.* 33, 1229–1239 (2005)
19. Broxmeyer, H. E. & Kim, C. H. Regulation of hematopoiesis in a sea of chemokine family members with a plethora of redundant activities. *Exp. Hematol.* 27, 1113–1123 (1999)
20. Cashman, J. D., Eaves, C. J., Sarris, A. H. & Eaves, A. C. MCP-1, not MIP-1 α , is the endogenous chemokine that cooperates with TGF- β to inhibit the cycling of primitive normal but not leukemic (CML) progenitors in long-term human marrow cultures. *Blood* 2338–2344 (1998)
21. Fortunel, N. O., Hatzfeld, A., Hatzfeld, J. A. & Dc, W. Transforming growth factor- β : pleiotropic role in the regulation of hematopoiesis. *Blood* 2022–2036 (2000)
22. Zhang, C. C., Kaba, M., Iizuka, S., Huynh, H. & Lodish, H. F. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. *Blood* 111, 3415–3423 (2008)
23. Csaszar, E. et al. Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling. *Cell Stem Cell* 10, 218–229 (2012)
24. Simsek, T. et al. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. *Stem Cell* 7, 380–390 (2010)

25. Chow, D. C., Wenning, L. A., Miller, W. M. & Papoutsakis, E. T. Modeling pO₂ distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. *Biophys. J.* 81, 685–696 (2001)
26. Danet, G. H., Pan, Y., Luongo, J. L., Bonnet, D. A. & Simon, M. C. Expansion of human SCID-repopulating cells under hypoxic conditions. *J. Clin. Invest.* 112, 126-135 (2003)
27. Mazumdar, J. et al. O₂ regulates stem cells through Wnt/β-catenin signaling. *Nat. Cell Biol.* 12, 1007–1013 (2010)
28. Eliasson, P. et al. Hypoxia mediates low cell-cycle activity and increases the proportion of long-term-reconstituting hematopoietic stem cells during in vitro culture. *Exp. Hematol.* 38, 301–310.e2 (2010)
29. Pollard, P. J. & Kranc, K. R. Hypoxia signaling in hematopoietic stem cells: a double-edged sword. *Cell Stem Cell* 7, 276–278 (2010)
30. Luis, T. C. et al. Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. *Cell Stem Cell* 9, 345–356 (2011)
31. Jang, Y.-Y. & Sharkis, S. J. A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. *Blood* 110, 3056–3063 (2007)
32. Roy, S., Tripathy, M., Mathur, N., Jain, A. & Mukhopadhyay, A. Hypoxia improves expansion potential of human cord blood-derived hematopoietic stem cells and marrow repopulation efficiency. *Eur. J. Haematol.* (2012)
33. Ito, K. et al. Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat. Med.* 12, 446–451 (2006)
34. Yoshida, K. et al. Thrombopoietin (TPO) regulates HIF-1α levels through generation of mitochondrial reactive oxygen species. *Int. J. Hematol.* 88, 43–51 (2008)
35. Ivanovic, Z. et al. Simultaneous maintenance of human cord blood SCID-repopulating cells and expansion of committed progenitors at low O₂ concentration (3%). *Stem Cells* 22, 716–724 (2004)
36. Kaushansky, K. Thrombopoietin and the hematopoietic stem cell. *Ann. N. Y. Acad. Sci.* 1044, 139–141 (2005)
37. Hao, Y., Cheng, D., Ma, Y., Zhou, W. & Wang, Y. Antioxidant intervention: a new method for improving hematopoietic reconstitution capacity of peripheral blood stem cells. *Med. Hypotheses* 76, 421–423 (2011)

38. Iiyama, M., Kakihana, K., Kurosu, T. & Miura, O. Reactive oxygen species generated by hematopoietic cytokines play roles in activation of receptor-mediated signaling and in cell cycle progression. *Cell. Signalling* 18, 174–182 (2006)
39. Pyatt, D. W., Stillman, W. S. & Irons, R. D. Reactive oxygen species mediate stem cell factor synergy with granulocyte/macrophage colony-stimulating factor in a subpopulation of primitive murine hematopoietic progenitor cells. *Mol. Pharmacol.* 49, 1097–1103 (1996)
40. Zhu, Q.-S. et al. G-CSF induced reactive oxygen species involves Lyn-PI3-kinase-Akt and contributes to myeloid cell growth. *Blood* 107, 1847–1856 (2006)
41. Sorrentino, B. P. Clinical strategies for expansion of haematopoietic stem cells. *Nat. Rev. Immunol.* 4, 878–888 (2004)
42. McGinnis, W. & Krumlauf, R. Homeobox genes and axial patterning. *Cell* 68, 283–302 (1992)
43. Sauvageau, G. et al. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. *Genes Dev.* 9, 1753–1765 (1995)
44. Antonchuk, J., Sauvageau, G. & Humphries, R. K. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell* 109, 39–45 (2002)
45. Ohta, H. et al. Near-maximal expansions of hematopoietic stem cells in culture using NUP98-HOX fusions. *Experimental Hematology* 35, 817–830 (2007). *Exp. Hematol.*
46. Krosl, J. et al. In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. *Nat. Med.* 9, 1428–1432 (2003)
47. Schiedlmeier, B. High-level ectopic HOXB4 expression confers a profound in vivo competitive growth advantage on human cord blood CD34+ cells, but impairs lymphomyeloid differentiation. *Blood* 101, 1759–1768 (2002)
48. Klump, H., Schiedlmeier, B. & Baum, C. Control of self-renewal and differentiation of hematopoietic stem cells: HOXB4 on the threshold. *Ann. N. Y. Acad. Sci.* 1044, 6–15 (2005)
49. Milsom, M. D. et al. Overexpression of HOXB4 confers a myelo-erythroid differentiation delay in vitro. *Leukemia* 148–153 (2004).doi:10.1038/sj.leu.2403554

50. Jiang, J. et al. Hemgn is a direct transcriptional target of HOXB4 and induces expansion of murine myeloid progenitor cells. *Blood* 116, 711–719 (2010)
51. Reya, T. et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature* 423, 409–414 (2003)
52. Fleming, H. E. et al. Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. *Cell Stem Cell* 2, 274–283 (2008)
53. Sugimura, R. et al. Noncanonical Wnt signaling maintains hematopoietic stem cells in the niche. *Cell* 150, 351–365 (2012)
54. Willert, K., Brown, J. D., Danenberg, E. & Duncan, A. W. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423, 448–452 (2003)
55. Varnum-Finney, B. et al. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat. Med.* 6, 1278–1281 (2000)
56. Staal, F. J. T., Luis, T. C. & Tiemessen, M. M. WNT signalling in the immune system : WNT is spreading its wings. *Nat. Rev. Immunol.* 8, 581–593 (2008)
57. Scheller, M. et al. Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. *Nat. Immunol.* 7, 1037–1047 (2006)
58. Baba, Y., Garrett, K. P. & Kincade, P. W. Constitutively active beta-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors. *Immunity* 23, 599–609 (2005)
59. Niida, A. et al. DKK1, a negative regulator of Wnt signaling, is a target of the beta-catenin/TCF pathway. *Oncogene* 23, 8520–8526 (2004)
60. Trowbridge, J. J., Xenocostas, A., Moon, R. T. & Bhatia, M. Glycogen synthase kinase-3 is an in vivo regulator of hematopoietic stem cell repopulation. *Nat. Med.* 12, 89–98 (2006)
61. Wu, G. & He, X. Threonine 41 in beta-catenin serves as a key phosphorylation relay residue in beta-catenin degradation. *Biochemistry* 45, 5319–5323 (2006)
62. Meijer, L. et al. GSK-3-selective inhibitors derived from Tyrian purple indirubins. *Chem. & Biol.* 10, 1255–1266 (2003)
63. Holmes, T. et al. Glycogen synthase kinase-3beta inhibition preserves hematopoietic stem cell activity and inhibits leukemic cell growth. *Stem Cells (Dayton, Ohio)* 26, 1288–1297 (2008)

64. Yang, J. et al. Genome-wide analysis reveals Sall4 to be a major regulator of pluripotency in murine-embryonic stem cells. *Proc. Natl. Acad. Sci. USA* 105, 19756–19761 (2008)
65. Aguila, J. R. et al. SALL4 is a robust stimulator for the expansion of hematopoietic stem cells. *Blood* 118, 576–585 (2011)
66. Ma, Y. et al. SALL4, a novel oncogene, is constitutively expressed in human acute myeloid leukemia (AML) and induces AML in transgenic mice. *Blood* 108, 2726–2735 (2006)
67. Chiba, S. Concise review: Notch signaling in stem cell systems. *Stem Cells* 24, 2437–2447 (2006)
68. Bigas, A. & Espinosa, L. Hematopoietic stem cells: to be or Notch to be. *Blood* 119, 3226–3235 (2012)
69. Milner, B. L. A., Kopan, R., Martin, D. I. K. & Bernstein, I. D. A human homologue of the *Drosophila* developmental gene, Notch, is expressed in CD34+ hematopoietic precursors. *Blood* 83, 2057–2062 (1994)
70. Karanu, F. N. et al. The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. *J. Exp. Med.* 192, 1365–1372 (2000)
71. Li, L. et al. The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. *Immunity* 8, 43–55 (1998)
72. Calvi, L., Adams, G., Weibrecht, K. & Weber, J. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425, 841–846 (2003).doi:10.1038/nature02041.1.
73. Varnum-finney, B. et al. Notch2 governs the rate of generation of mouse long- and short-term repopulating stem cells. *J. Clin. Invest.* 121, 1207–1216 (2011)
74. Carlesso, N., Aster, J. C., Sklar, J. & Scadden, D. T. Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. *Blood* 93, 838–848 (1999)
75. Mancini, S. J. C. et al. Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood* 105, 2340–2342 (2005)
76. Ohishi, K., Varnum-Finney, B. & Bernstein, I. D. Delta-1 enhances marrow and thymus repopulating ability of human CD34 + CD38 – cord blood cells. *J. Clin. Invest.* 110, 1165–1174 (2002)

77. Delaney, C. et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nat. Med.* 16, 232–236 (2010)
78. Varnum-Finney, B. et al. Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. *J. Cell Sci.* 113 Pt 23, 4313–4318 (2000)
79. Delaney, C., Varnum-Finney, B., Aoyama, K., Brashem-Stein, C. & Bernstein, I. D. Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. *Blood* 106, 2693–2699 (2005)
80. Bray, S. J. Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.* 7, 678–689 (2006)
81. Yoshiura, S. & Ohtsuka, T. Ultradian oscillations of Stat, Smad, and Hes1 expression in response to serum. *Proc. Natl. Acad. Sci. USA* 104, 11292–11297 (2007) at <<http://www.pnas.org/content/104/27/11292.short>>
82. Baum, C. et al. Side effects of retroviral gene transfer into hematopoietic stem cells. *Blood* 101, 2099–2114 (2003)
83. Bissantz, C., Folkers, G. & Rognan, D. Protein-based virtual screening of chemical databases. 1. Evaluation of different docking/scoring combinations. *J. Med. Chem.* 43, 4759–4767 (2000)
84. Boitano, A. E. et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science (New York, N.Y.)* 329, 1345–1348 (2010)
85. Gasiewicz, T. A., Singh, K. P. & Casado, F. L. The aryl hydrocarbon receptor has an important role in the regulation of hematopoiesis: implications for benzene-induced hematopoietic toxicity. *Chem.-Biol. Interact.* 184, 246–251 (2010)
86. Singh, K. P., Casado, F. L., Opanashuk, L. A. & Gasiewicz, T. A. The aryl hydrocarbon receptor has a normal function in the regulation of hematopoietic and other stem/progenitor cell populations. *Biochem. Pharmacol.* 77, 577–587 (2009)
87. Sakai, R. TCDD treatment eliminates the long-term reconstitution activity of hematopoietic stem cells. *Toxicol. Sci.* 72, 84–91 (2003)
88. Hankinson, O. The aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.* 307–340 (1995)
89. Piparo, E. L. et al. Virtual screening for aryl hydrocarbon receptor binding prediction. *J. Med. Chem.* 5702–5709 (2006)

90. Bisson, W., Koch, D. & O'Donnell, E. Modeling of the aryl hydrocarbon receptor (AhR) ligand binding domain and its utility in virtual ligand screening to predict new AhR ligands. *J. Med. Chem.* 52, 5635–5641 (2009)
91. Ivanova, N. B. et al. A stem cell molecular signature. *Science (New York, N.Y.)* 298, 601–604 (2002)
92. Cui, K. et al. Chromatin signatures in multipotent human hematopoietic stem cells indicate the fate of bivalent genes during differentiation. *Cell Stem Cell* 4, 80–93 (2009)
93. Huangfu, D. et al. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat. Biotechnol.* 26, 795–797 (2008)
94. Göttlicher, M. et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J.* 20, 6969–6978 (2001)
95. Bug, G. et al. Valproic acid stimulates proliferation and self-renewal of hematopoietic stem cells. *Cancer Res.* 65, 2537–2541 (2005)
96. Cheng, T. Hematopoietic stem cell quiescence maintained by p21^{cip1/waf1}. *Science* 287, 1804–1808 (2000)
97. Araki, H. et al. Cord blood stem cell expansion is permissive to epigenetic regulation and environmental cues. *Exp. Hematol.* 37, 1084–1095 (2009)
98. Nishino, T. et al. Ex vivo expansion of human hematopoietic stem cells by garcinol, a potent inhibitor of histone acetyltransferase. *PLoS One* 6, e24298 (2011)
99. Sakaguchi, K. et al. DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev.* 12, 2831–2841 (1998)
100. Peled, T. et al. Chelatable cellular copper modulates differentiation and self-renewal of cord blood-derived hematopoietic progenitor cells. *Exp. Hematol.* 33, 1092–1100 (2005)
101. Bae, B. & Percival, S. S. Copper uptake and intracellular distribution during retinoic acid-induced differentiation of HL-60 cells. *J. Nutr. Biochem.* 5, 457–461 (1994)
102. Prus, E. & Fibach, E. The effect of the copper chelator tetraethylenepentamine on reactive oxygen species generation by human hematopoietic progenitor cells. *Stem Cells Dev.* 16, 1053–1056 (2007)

103. de Lima, M. et al. Transplantation of ex vivo expanded cord blood cells using the copper chelator tetraethylenepentamine: a phase I/II clinical trial. *Bone Marrow Transplant.* 41, 771–778 (2008)
104. Martin, F. et al. Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. *Blood* 105, 4613–4619 (2005)
105. Kirito, K., Fox, N., Komatsu, N. & Kaushansky, K. Thrombopoietin enhances expression of vascular endothelial growth factor (VEGF) in primitive hematopoietic cells through induction of HIF-1 α . *Blood* 105, 4258–4263 (2005)
106. Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872 (2007)
107. Patel, M. & Yang, S. Advances in reprogramming somatic cells to induced pluripotent stem cells. *Stem Cell Rev.* 6, 367–380 (2010)
108. Yamanaka, S. Strategies and new developments in the generation of patient-specific pluripotent stem cells. *Cell Stem Cell* 1, 39–49 (2007)
109. Vodyanik, M. a & Slukvin, I. I. Hematoendothelial differentiation of human embryonic stem cells. *Curr. Protoc. Cell Biol.* Chapter 23, Unit 23.6 (2007)
110. Sakamoto, H., Tsuji-Tamura, K. & Ogawa, M. Hematopoiesis from pluripotent stem cell lines. *Int. J. Hematol.* 91, 384–391 (2010)
111. Rao, M., Ahrlund-Richter, L. & Kaufman, D. Concise review: Cord blood banking, transplantation and induced pluripotent stem cell: success and opportunities. *Stem Cells* 55–60 (2012) at <http://onlinelibrary.wiley.com/doi/10.1002/stem.770/full>
112. Wang, Y. et al. Genetic correction of β -thalassemia patient-specific iPS cells and its use in improving hemoglobin production in irradiated SCID mice. *Cell Res.* 22, 637–648 (2012)
113. Hanna, J. et al. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science (New York, N.Y.)* 318, 1920–1923 (2007)
114. Papapetrou, E. P. et al. Genomic safe harbors permit high β -globin transgene expression in thalassemia induced pluripotent stem cells. *Nat. Biotechnol.* 29, 73–78 (2011)

115. Ye, L. et al. Induced pluripotent stem cells offer new approach to therapy in thalassemia and sickle cell anemia and option in prenatal diagnosis in genetic diseases. *Proc. Natl. Acad. Sci. USA* 106, 9826–9830 (2009)
116. Kim, K. et al. Epigenetic memory in induced pluripotent stem cells. *Nature* 467, 285–290 (2010)
117. Lister, R. et al. Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature* 471, 68–73 (2011)
118. Hanna, J. H., Saha, K. & Jaenisch, R. Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. *Cell* 143, 508–525 (2010)
119. Feng, Q. et al. Hemangioblastic derivatives from human induced pluripotent stem cells exhibit limited expansion and early senescence. *Stem Cells* 28, 704–712 (2010)
120. Marion, R. M. et al. Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells. *Cell Stem Cell* 4, 141–154 (2009)
121. Warren, L. et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* 7, 618–630 (2010)
122. Kim, D. et al. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell* 4, 472–476 (2009)
123. Miyoshi, N. et al. Reprogramming of mouse and human cells to pluripotency using mature microRNAs. *Cell Stem Cell* 8, 633–638 (2011)
124. Ichida, J., Blanchard, J., Lam, K., Son, E. & Chung, J. A small-molecule inhibitor of Tgf-Beta signaling replaces Sox2 in reprogramming by inducing Nanog. *Cell Stem Cell* 5, 491–503 (2009)
125. Li, W. & Ding, S. Small molecules that modulate embryonic stem cell fate and somatic cell reprogramming. *Trends Pharmacol. Sci.* 31, 36–45 (2010)
126. Yoshida, Y., Takahashi, K., Okita, K., Ichisaka, T. & Yamanaka, S. Hypoxia enhances the generation of induced pluripotent stem cells. *Cell Stem Cell* 5, 237–241 (2009)

VITA

Andrew Douglas Lasiter was born in Chattanooga, Tennessee in 1977 to John and Mary Margaret Lasiter. He graduated high school from Cheltenham High in Wyncote, Pennsylvania in 1995 and subsequently obtained his bachelor's degree in biology from Middle Tennessee State University. He enrolled in the University of Tennessee: Health Science Center in 2009 where he joined Derek Persons lab at St. Jude Children's Research Hospital and studied Wnt-pathway related strategies for hematopoietic stem cell expansion.