BLOOD MATURATION INDUCED BY HEDGEHOG INHIBITION

MECHANISM OF BLOOD MATURATION INDUCED BY HEDGEHOG INHIBITION IN PLURIPOTENT SOURCES

By

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ABSTRACT

The generation of hematopoietic progenitors from human pluripotent cell sources for use in personalized medicine is an attainable goal for the ease of clinical intervention using these cells. Furthermore, generated platelets and mature red blood cells are enucleated which allows for the use of induced pluripotent stem cells as a starting source or other sources of genetic manipulation. Generating these cells has proven difficult as the cells appear to be stuck in a primitive state of differentiation and do not mature into an adult phenotype. This thesis shows that inhibition of the hedgehog signaling pathway early in the differentiation of pluripotent stem cells induces a maturation towards definitive hematopoiesis. Generated erythroid cells were shown to express beta globin at the transcript as well as protein level. This maturation effect was confirmed to occur through central hedgehog repressor, Gli3R, through genetic manipulation. Further interrogation of this mechanism showed that globin regulation was not mediated by chromatin methylation by the polycomb repressive complex. Finally, Gli3R was also shown to not act as a transcription factor influencing globin expression directly and is therefore engaging separate regulatory mechanisms. This data provides great strides towards the generation of clinically relevant hematopoietic populations from pluripotent sources, however Gli3R's direct mechanism of action remains to be determined.

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LIST OF ABBREVIATIONS

AGM – Aorta Gonad Mesonephrous
bFGF – Basic Fibroblast Growth Factor
BMP4 – Bone Morphogenic Protein 4
CFU – Colony Forming Unit
ChIP – Chromatin Immunoprecipitation
Co-IP – Co-immunoprecipitation
Dhh – Desert Hedgehog
EB – Embryoid Body
Eed – Ectodermal Development
EPO – Erythropoietin
Ezh2 – Enhancer of Zeste 2
FBS – Fetal Bovine Serum
Flt3L – FMS-like Tyrosine Kinase 3 Ligand
GCSF – Granulocyte Colony Stimulating Factor
Gli1 – Glioblastoma 1 Transcription Factor
Gli2 – Glioblastoma 2 Transcription Factor
Gli3 – Glioblastoma 3 Transcription Factor
Gli3R – Glioblastoma 3 Transcriptional Repressor
H2AK119ub – Ubiquitination of Lysine 119 on Histone 2A
H3K27me2 – Dimethylation of Lysine 27 on Histone 3
H3K27me3 – Trimethylation of Lysine 27 on Histone 3
HBB – Beta Globin
HBD – Delta Globin
HBE – Epsilon Globin
HBG – Gamma Globin
hCB – Human Cord Blood
HDAC – Histone Deactylase
hESC – Human Embryonic Stem Cell
hFL – Human Fetal Liver
Hh – Hedgehog Signaling Pathway
Hhip – Hedgehog Interacting Protein
hMPB – Human Mobilized Peripheral Blood
hPSC – Human Pluripotent Stem Cell
HSC – Hematopoietic Stem Cell
Ihh – Indian Hedgehog
IL3 – Interleukin 3
IL6 – Interleukin 6
iPSCs – Induced Pluripotent Stem Cell
KODMEM – Knockout Dulbecco's Minimal Essential Media
MEFCM – Mouse Embryonic Feeder Conditioned Media
MEL – Murine Erythroleukemia Cells

mESC – Mouse Embryonic Stem Cell
MHC – Major Histocompatibility Complex
NEAA – Non-Essential Amino Acids
PBS – Phosphate Buffer Saline
PHO – Pleiohomeotic Transcription Factor
PHOL – Pleiohomeotic Like Transcription Factor
PRC1 – Polycomb Repressive Complex 1
PRC2 – Polycomb Repressive Complex 2
PRE – Polycomb Response Element
Ptch1 – Patched Cell Surface Receptor
qRT-PCR – Quantitative Real-Time Polymerase Chain Reaction
Rbap46/48 – Retinoblastoma Associated Protein 46/48
RIKEN CDB – RIKEN Center for Developmental Biology
SCF – Stem Cell Factor
Shh – Sonic Hedgehog
Smo – Smoothened Cell Surface Receptor
Suz12 – Suppressor of Zeste 12
TPO – Thrombopoietin
UTR – Un-translated Region
VEGF-165 – Vascular Endothelial Growth Factor 165
Wnt – Wingless Signaling Pathway
YY1 – Yin Yang 1 Transcription Factor

DECLARATION OF ACADEMIC ACHIEVEMENT

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CHAPTER 1

INTRODUCTION

1.1 SOURCES TO GENERATE HEMATOPOIETIC CELLS FOR CLINICAL USE

Bone marrow transplantation was initially used as a last resort to provide healthy cells to patients with life-threatening blood disorders such as leukemias. Unknown at the time, it would be hematopoietic stem cells (HSCs) residing in the bone marrow that would reconstitute the blood system of recipients (Figure 1.1), making it the first and only approved stem cell therapy. However, Bone marrow transplantation is limited by the necessity of finding a donor with matching genetics to the recipient. The procedure may also induce further complications such as graft vs. host disease, reasons for which are not fully understood. Because of this it has long been a goal of research groups to generate methods of enhancing the applicability and efficiency of this therapy. For example, many groups attempt to culture HSCs *in vitro* to expand the population or to learn how

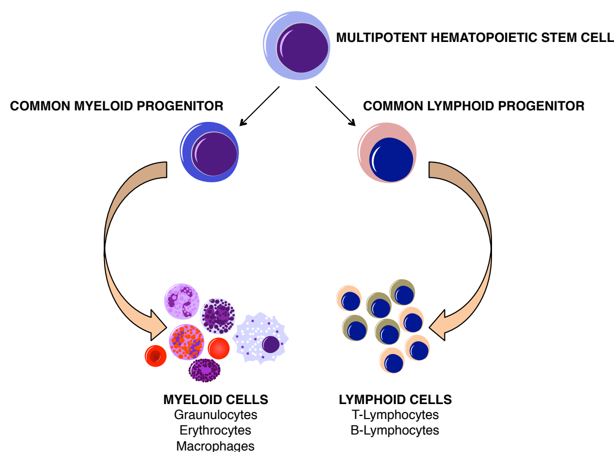

Figure 1.1 – The multilineage potential of a hematopoietic stem cell. HSCs have the potential to differentiate into any cell of the lympho-myeloid series. An HSC must be able to simultaneously generate cells of both myeloid (eg. macrophage) and lymphoid (eg. thymocyte) to be considered a true multipotent hematopoietic progenitor.

chemicals may improve their potency to increase proliferation after transplantation. However, these advancements would not be beneficial to patients whom cannot find donor matched cells. To overcome that barrier, another source of HSCs must be derived.

In 1998, Jamie Thomson reported the isolation of embryonic stem cells from human blastocysts (hESCs) and their long-term maintenance in culture[1]. These cells, which are derived from the inner cell mass of the blastocyst, are pluripotent and hold the potential to form any cell of the three germ layers of the adult body. While the experiment to generate a human from hESCs cannot be performed for ethical reasons, murine embryonic stem cells (mESCs) have been shown to generate an entire animal through tetraploid complementation[2]. More specifically mESCs have been successfully directed towards hematopoietic differentiation through *in vitro* protocols[3,4]. Based on this previous work, hESCs can be used as a source to generate HSCs for clinical use. However, even if HSCs could be generated from hESCs the issue of genetic incompatibility would remain. To answer this, major histocompatibility complex (MHC) molecules were shown to be expressed at low levels in hESCs, however these molecules are upregulated through differentiation which could pose a threat to mismatched donors[5]. In 2007, the first generation of human induced pluripotent stem cells (iPSCs) was reported[6]. Mature adult cells such as skin fibroblasts could be reprogrammed to a pluripotent state, essentially reverting back to embryonic potency of multi-lineage differentiation. Because these cells could be taken from a patient through a skin biopsy or even peripheral blood sources[7], iPSCs could be generated for individuals with an exact donor match and push towards personalized medicine.

Pluripotent cells provide an excellent source for generating potentially unlimited supplies of HSCs for transplantation purposes. Furthermore, the use of iPSCs can completely overcome donor-matching inaccessibilities. However, the production of HSCs from pluripotent cells has proven difficult, although great strides have recently been achieved towards this goal.

1.2 Generating Hematopoietic Cells From Pluripotent Sources

In humans, sites of hematopoiesis switch as development proceeds, and with these switches the phenotype and potency of the cells produced are altered. Measuring the potency and maturation of blood cells can be performed through assaying multilineage differentiation potential and by their hemoglobin expression profile. Multilineage differentiation can be assayed through colony forming unit (CFU) experiments where blood cells are cultured in semi-solid medium with differentiation inducing cytokines. Cells that produce colonies with multiple lineages of cells can be considered early progenitors with multilineage potential. However, a cell can only be experimentally considered a true HSC if it can reconstitute the hematopoietic system of an irradiated immunocomprimised mouse[8,9]. Alternatively, the globin profile of blood cells can act as a measure of maturation because of two predictably timed genetic switches in beta globin genes through ontogeny[10]. The hemoglobin molecule is a heterotetramer composed of two alpha peptide chains and two beta peptide chains. The beta chain genes are aligned in order of expression on chromosome 16, as epsilon (HBE), gamma (HBG) (of which there are two almost identical genes), beta (HBB) and delta (HBD)[10] (Figure 1.2). The

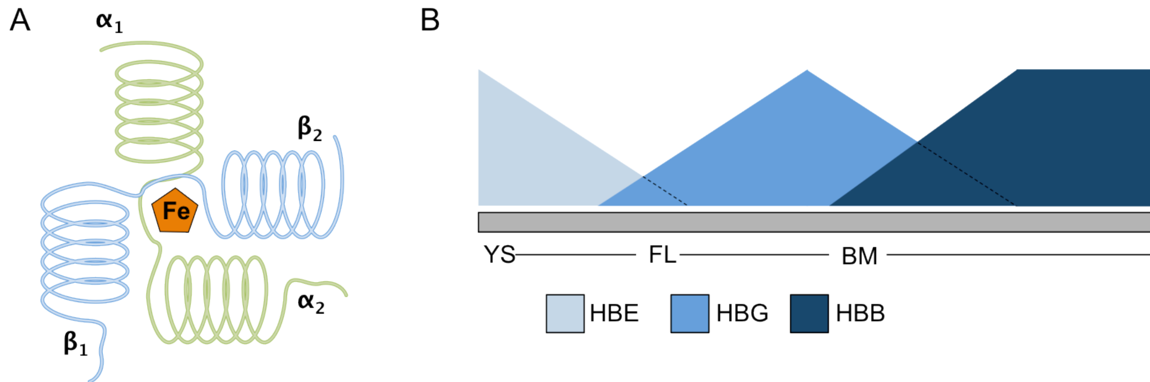

Figure 1.2 – The hemoglobin molecule and expression of the different beta globin genes through differentiation. The hemoglobin molecule is a heterotetramer made up of two alpha and two beta chains that will bind an iron molecule to capture oxygen for transport through the body(A). The alpha genes are located on chromosome 11 and do undergo an initial switch very early in ontogeny. The beta genes are located on chromosome 16 and are ordered as they are expressed (B).

regulation of this locus is discussed in section 1.5.

The early embryo yolk sac forms blood islands that consist of blood cells surrounded by endothelial cells, and is the first source of hematopoiesis in human development[11]. The primary site switches from the blood islands to the aorta gonad mesonephrous (AGM) after it is formed and is the first site to produce true HSCs capable of full hematopoietic reconstitution[12]. Hematopoietic progenitors can be identified by the expression of cell surface receptors CD34[13], a stem/progenitor marker, and CD45, a pan-hematopoietic marker[14]. The process of generating true HSCs is referred to as definitive hematopoiesis vs. primitive hematopoiesis which is incapable of generating blood cells with reconstituting potential[12]. The hematopoietic site migrates to the fetal liver following the AGM, and by this stage the cells will express HBG as the beta chain peptide of hemoglobin[15,16]. The fetal liver will remain the site of hematopoiesis through gestation, and near birth, HSCs will migrate to the bone marrow to form the final site of

hematopoiesis for the rest of the adult life[17]. Gradually after birth erythroid cells will begin to express HBB, and within 12 weeks it will be the primary beta globin peptide[16]. Therefore when differentiating pluripotent cells to the hematopoietic lineage, the true test for HSC generation are *in vivo* reconstitution of an irradiated hematopoietic system, while the monitoring of the beta globin profile can be used as a surrogate of hematopoietic maturity.

Hematopoietic differentiation from pluripotent sources can occur spontaneously through embryoid body (EB) formation, which are essentially cellular aggregates that result from shearing a monolayer of cells into suspension, and culturing in fetal bovine serum (FBS) containing media[18]. However, this method will only generate a small population of hematopoietic cells, while a large proportion of the cells will differentiate to other lineages such as neural and endoderm. The proportion of blood generated can be drastically improved if the cells are "directed" to differentiate towards hematopoiesis specifically, and this can be done through several methods including manipulating physical growth conditions, genetics or epigenetics, and developmental signaling pathways.

Culture manipulation was the first method reported for directed hematopoietic generation[19] by co-culture on a murine bone marrow stromal line, SP9. This protocol could generate multipotent progenitors that behaved similarly to human HSCs *in vitro* in CFU assays, however they could not reconstitute the hematopoietic system of an irradiated immunocomprimised animal indicating that these cells were not true HSCs. The generation of large populations of hematopoietic precursors through EB formation

and culture with a cocktail of differentiating cytokines was reported[20], but these cells still could not perform *in vivo*. The lack of *in vivo* potential soon became the shortfall of hematopoietic potential from pluripotent sources[21,22]. Human pluripotent stem cell (hPSC) derived hematopoietic cells were shown to be able to engraft fetal sheep at very low levels, but could be isolated and used to engraft a secondary recipient[23]. It would appear that cells generated through these protocols resemble yolk-sac derived hematopoietic cells in function, which also cannot reconstitute hematopoiesis in an adult mouse, although they do have this potential in newborn mice[24] where the main site of hematopoiesis is still the liver. Interestingly, once reconstituted in a newborn pup and the animal grows to an adult, HSCs can be extracted from the bone marrow and used to reconstitute another adult irradiated mouse[24]. This implies that there is a maturation effect in the transition between primitive and definitive hematopoiesis that allows the potency of HSCs, and that this maturation effect is missing in current protocols to differentiate hematopoietic cells from pluripotent sources *in vitro*. Progression of culture protocols have made strides in fine tuning hematopoietic differentiation[25-27], and one particular study managed to show *in vivo* engraftment of produced hematopoietic cells indicating the generation of HSCs[28]. Unfortunately this sole report has yet to be reproduced or followed-up.

Some groups have focused their efforts in generating HSCs from hPSCs using methods of genetic manipulation. In fact, mESCs can produce bona fide HSCs through overexpression of the developmental patterning gene HoxB4[29]. The overexpression of HoxB4 has been attempted in the human system but has not been able to overcome the developmental block in hPSC derived hematopoiesis[30,31]. It remains possible that other

genes will be able to accomplish HSC generation when manipulated in hPSCs. For example, a very recent report has shown that the overexpression of a particular isoform of Runx1, an important definitive hematopoietic regulator, was able to generate bona fide HSCs that expressed HBB and were able to reconstitute immunocomprimised animals[32]. This is exciting work and with mechanistic understanding could lead to replacement of Runx1 genetic alteration with small molecule intervention for clinical use. Another recent study of interest has shown that teratomas formed from hPSCs in mice can be collected, and the $CD34^{+}CD45^{+}$ population can be sorted and used to reconstitute hematopoiesis in an irradiated animal[33]. This study provides proof-of-principle evidence that hPSCs are capable of producing true HSCs and furthermore, since it was accomplished *in vivo,* it indicates that overcoming the block in differentiation to definitive hematopoiesis can be achieved with external factors and therefore could be produced in a dish. Environmental cues leading to developmental signaling could include cytokines released from physiological cues, cell-cell interactions, and patterning through developmental signaling.

1.3 The Hedgehog Signal Transduction Pathway in Hematopoiesis

The usual suspects involved in developmental patterning through signaling are the wingless (Wnt), hedgehog (Hh), and notch pathways, all of which have been implicated in some degree to affect hematopoiesis. Notch signaling, for example, has been implicated in lympho-hematopoietic generation through culture on a murine bone marrow stroma (OP9) that is altered for delta-like-ligand, an inducer of the pathway[25]. Notch has also been implicated in somatic generation and maintenance of early hematopoietic

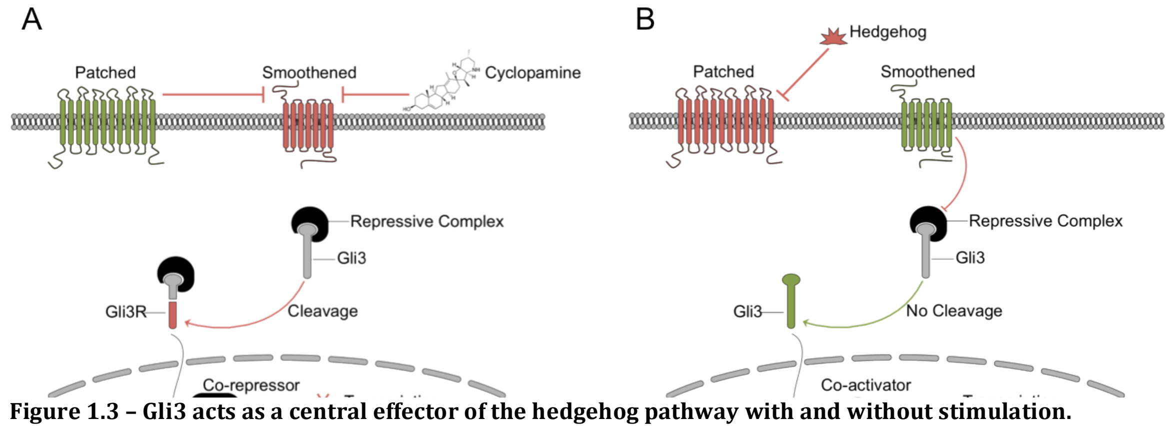

Figure 1.3 – Gli3 acts as a central effector of the hedgehog pathway with and without stimulation.

progenitors through self-renewal[34,35]. Wnt signaling *in vivo* has been shown to regulate almost all aspects of hematopoiesis including cell fate decisions, proliferation and self-renewal depending on the cell type signaling is affecting[36-38]. Our lab has shown that wnt signaling can be used to enhance hematopoietic differentiation by properly timing stimulation of the canonical vs. non-canonical pathways[39]. The two timepoints of stimulation are in line with the very first fate decisions made by pluripotent cells (Day 1 of differentiation) and after hematopoietic specification has been achieved (Day 10 of differentiation). This indicates that "directing" differentiation towards the production of bona fide HSCs is going to depend on understanding of fate decisions made at every step from pluripotency. For the purposes of this document, I will henceforth focus on the role of Hh signaling in hematopoiesis.

If the Hh pathway is not stimulated through extracellular signaling (Figure 1.3A), the cell surface receptor Patched (Ptch1) is acting to inhibit another membrane protein, Smoothened (Smo)[40]. An intracellular repressive complex binds and cleaves a transcriptional effector, Glioblastoma 3 (Gli3), into a truncated repressive form named

Gli3 repressor (Gl3R)[41]. In the nucleus, Gli3R will act in conjunction with co-repressive molecules to inhibit transcription of Hh target genes, which include the transcriptional activators Glioblastoma 1 (Gli1) and Glioblastoma 2 (Gli2)[41,42]. Signaling can be induced by one of three secreted hedgehog molecules: Sonic hedgehog (Shh), Indian hedgehog (Ihh) or Desert hedgehog (Dhh)[40,42]. In the presence of signaling (Figure 1.3B) the hedgehog protein will bind and repress Ptch, relieving repression of Smo which functions to inhibit the intracellular repressive complex that interacts with Gli3[42]. This inhibition prevents the cleavage of Gli3, and the full-length form will act to transcriptionally activate Hh target genes including Gli1 and Gli2[43]. Therefore, regardless of the activation state of the Hh pathway, Gli3 acts as a central effector of signaling and its expression is regulated independently of this pathway[44].

The role of Hh signaling in hematopoiesis is controversial. Initially, mESCs defective in Ihh production were reported to be deficient in blood generation from EBs[45,46]. However, Ihh knockout mice did not show obvious hematopoietic defects[46]. Other reports since have shown that active Hh signaling is essential for the onset of definitive hematopoiesis in zebrafish[47], and will expand human and murine hematopoietic progenitors *in vitro*[48,49]. However, efforts to elucidate Hh involvement in hematopoiesis *in vivo* using Smo knockout failed to find any hematopoietic defects in peripheral blood counts, HSC number, behavior *in vitro* or reconstituting potential in immunocompromised mice[50,51]. Other groups have reported the necessity of active Hh signaling in hematopoietic malignancies and that genetic or pharmacological inhibition of the pathway could eliminate cancer stem cell maintenance[52,53]. This discrepancy of Hh's

role in developmental hematopoiesis leaves the question of this pathway's involvement open ended, and manipulation of the pathway in differentiating hPSCs could help tease apart its human specific mechanisms.

1.4 The Polycomb Repressive Complex 2 - Function and Recruitment

The Polycomb Group (PcG) is a series of protein complexes that epigenetically repress expression of specific loci by catalyzing post-translational modifications of nucleosome histones. Originally identified in *Drosophila* as being required for spatial anterior-posterior regulation of the homeotic bithorax genes[54,55], it has been linked in mammals to regulate gene expression from a state of pluripotency to terminal differentiation[56-59]. The PcG is composed of two functional complexes, Polycomb Repressive Complex 1 (PRC1) which mono-ubiquitinates lysine 119 of histone 2A (H2AK119ub)[60] and Polycomb Repressive Complex 2 (PRC2) responsible for sequential histone 3 lysine 27 methylations (H3K27me2 / me3)[61,62]. Herein, I will focus on the PRC2 mechanism of action and recruitment as it relates to my hypothesis and aims.

The core subunits of PRC2 comprise Enhancer of Zeste 2 (Ezh2) the methyltransferase of the complex, Suppressor of Zeste 12 (Suz12), Embryonic Ectodermal Development (Eed) and Retinoblastoma-Associated Protein 46/48 (Rbap46/48)[63]. Together, the orthologue complex in fruit flies displays over 1000 fold greater methyltransferase activity on H3K27 in *in vitro* assays than Ezh2 alone[61]. The reason for this is not mechanistically understood, although target recognition and complex recruitment likely play a role[64]. Recruitment of PRC2 (and PcG as a whole) is not yet

defined in mammalian systems. In *Drosophila,* various DNA sequences, termed Polycomb response elements (PREs), recruit PRC2 via zinc-finger proteins Pleiohomeotic (PHO) and PHO-like (PHOL) to mediate silencing of the target gene[65] (Figure 1.4). PREs have yet to be discovered in mammals, and there are several other proposed mechanisms of PcG recruitment, one of which is mediated by transcription factors, and another (not discussed here) through non-coding RNA.

The murine PHO homologue Yin Yang 1 (YY1), originally associated with the discovery of PHO's role in recruitment[66], was shown to not only interact and co-localize with Ezh2 in mouse myoblasts, but its suppression also interfered with Ezh2 binding to

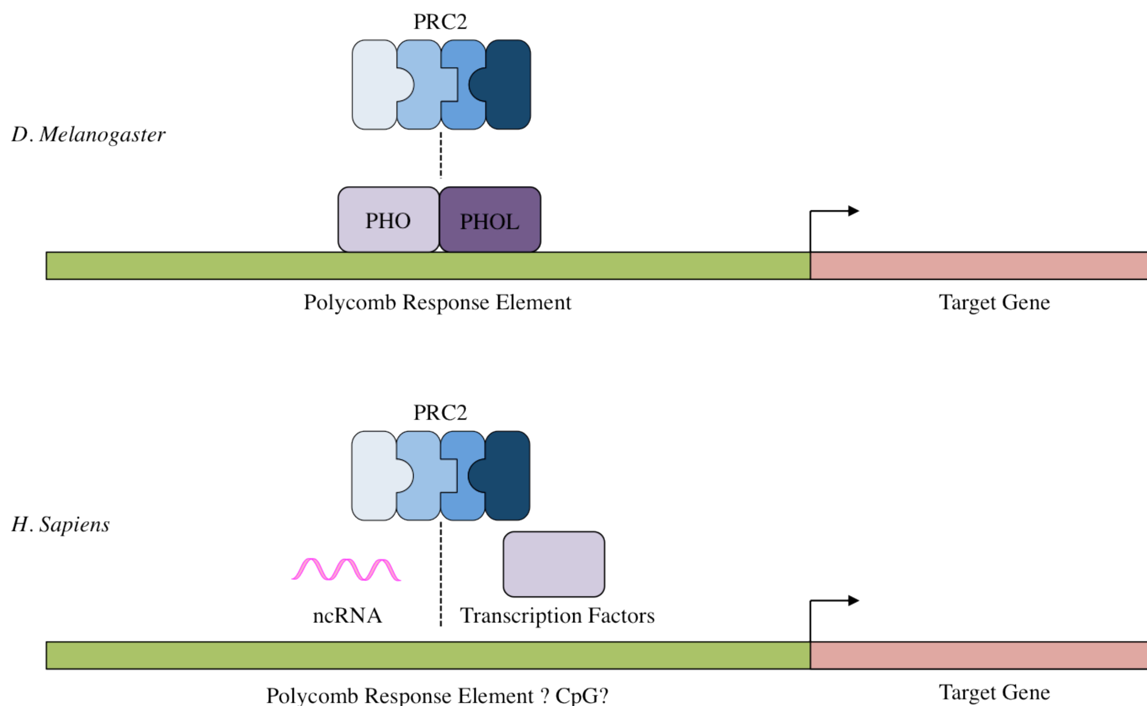

Figure 1.4 – PRC2 Recruitment in flies and man. PRC2 is recruited to a polycomb response element (PRE) in *Drosophila* by zinc finger proteins PHO and PHOL to repress target gene expression. The mechanism of recruitment in humans has yet to be elucidated. There may be undiscovered PREs or PRC2 may be recruited to CpG islands through ncRNA or transcription factors.

muscle gene loci which subsequently lost H3K27me3[67]. Further, some reports have shown evidence for the involvement of pluripotency factor Oct4 in recruiting PcG because of overlap of binding sites observed in ChIP-Seq experiments in mouse and human embryonic stem cells[56,57,68]. However biochemical interactions and genetic manipulation are required to provide support for this model. The specific example of YY1 in developing muscle portrays a *conserved* mechanism between mammals and insects and therefore justifies further inquiry of PRC2 recruitment by transcription factors.

1.5 Human Regulation of the Beta Globin Locus

The human beta globin genes were some of the first genes to be cloned[69], and the locus remains an important model for the study of gene regulation due to the two developmental switches that occur through ontogeny. The hemoglobin molecule is the functional unit of red blood cells, required for oxygen transport from the lungs to other tissues of the body[70]. As previously mentioned, the beta globin locus itself is a cluster of 5 beta-like globin genes located on chromosome 16 that are expressed sequentially and will form the beta chain of the hemoglobin molecule[10,16,71].

The developing embryo will initially express HBE peptide in the yolk sac, the primary site of hematopoiesis in the embryo. The first of two beta globin switches occurs with hematopoietic migration to the fetal liver at 3 months gestation, where HBE will quickly be silenced, followed by upregulation of HBG[72,73]. HBG will remain as the predominant beta chains expressed through fetal life, and upon birth will slowly decline as HBB is upregulated while hematopoiesis shifts once again, this time to the bone

marrow[74]. 12 months after birth HBB becomes the predominant beta chain, and HBG is present in only a minor fraction of red blood cells[74]. Another adult beta globin chain, HBD, is also upregulated at birth, however it retains very low levels in the adult[75,76] and is not as well studied as the other 4 genes in the locus.

Models for studying human regulation of this locus began by introducing human globin DNA vectors into mouse erythroleukemia cells (MEL) that can be chemically induced to perform the murine globin switch with DMSO, and then tracking the expression patterns of the human proteins produced[77,78]. However, MEL lines did not adequately recapitulate *in vivo* erythropoiesis and the field quickly switched to transgenic mouse models expressing segments of the human locus. Unfortunately, human proteins produced in these models follow mouse globin regulation which progress through a single globin switch as opposed to the two that occur in the human[79-82]. Transgenic mice with the entire human beta globin locus in yeast artificial chromosomes were not able to overcome murine specific regulation[83,84]. Among the first to question the full validity of these models in human beta globin regulation was a report attempting to understand why HBG was being treated as an embryonic globin in the mouse as opposed to a fetal globin, and showed that the function of an important HBG repressor (BCL11A[85]) has species divergent roles in mouse and human[86]. Data generated from these current models are difficult to interpret, laborious, and at times not overly convincing due to cross-species complications. The need for an optimal model to study novel roles of gene regulators such as PRC2 in human globin switching is being realized in the field.

1.6 Rationale

The differences in cellular programs between primitive and definitive hematopoiesis are poorly understood. It is likely that the induction of this change is triggered through conserved signaling pathways, of which Hh signaling is one. Further, this pathway has been implicated in hematopoiesis in previous studies of multiple model systems. The manipulation of this pathway in differentiating hPSCs provides a novel system to test the role of Hh in hematopoietic induction during early commitment steps as well as a novel approach in overcoming the developmental block that is observed in hPSC derived blood. Finally, preliminary data from our lab suggests that inhibition of the hedgehog pathway increases hematopoietic potential of hPSCs and may interact with PRC2.

1.7 Hypothesis

Hedgehog signaling controls the developmental switch from primitive to definitive hematopoiesis and recruits PRC2 for repression of early globins.

1.8 Experimental Objectives

1. Evaluate the role of the hedgehog pathway in hPSC hematopoietic differentiation through pharmacological inhibition and genetic overexpression. The effects of hematopoiesis will be assayed through blood generation, CFU potential and beta globin expression.

2. Evaluate the role of the hedgehog pathway in regulating the beta globin locus.

Chromatin Immunoprecipitation (ChIP) will be utilized to probe for Gli3R binding on globin promoters. To test if the hedgehog pathway is acting through the polycomb complex, further ChIPs for PRC2 will indicate if PRC2 recruitment is catalyzing the inhibition of primitive globins. Co-immunoprecipitations (Co-IP) will be used to probe for a Gli3R-PRC2 interaction.

CHAPTER 2

METHODS AND MATERIALS

2.1 Human Pluripotent Stem Cell Culture

Human embryonic stem cell lines H9[1] and CA2[87] and induced pluripotent cell line iPS1.2 were cultured on matrigel in mouse embryonic fibroblast conditioned media (MEFCM) supplemented with 8ng/uL of basic fibroblast growth factor (bFGF). hPSCs were formed into EBs by treating with collagenase IV followed by scraping the cells into clumps and changing culture media to EB Media (20% FBS, 80% KODMEM, 1% NEAA, 1mM L-glutamine, and 0.1mM B-mercaptoethanol). To direct EB differentiation to hematopoiesis SCF (300ng/mL), Flt3L (300ng/mL), IL3 (10ng/mL), IL6 (10ng/mL), GCSF (50ng/mL) and BMP4 (25ng/mL) were added to the culture media the day after EB formation as previously described[20]. Cyclopamine was added to indicated cultures at 10uM quantities on day 1 and 3 of differentiation culture, and removed at day 4.

2.2 Flow Cytometry

hEBs were collected at day 15 of differentiation and treated with collagenase B for 2 hours at 37°C, followed by 10-15 minute treatment with cell-dissociation buffer. EBs were then placed in 97%PBS, 3%FBS and 0.1mM EDTA and mechanically broken up by repeatedly pipetting up and down. Cells were passed through a 0.45um filter and subjected to conjugated antibodies at given concentrations in Table 1. Flow Cytometry was performed using a BD LSRII and analyzed using FlowJo software.

Table 1 – List of Antibodies Used

Antibody	Vendor	Purpose	Dilution/Concentration
CD34	Miltenyi Biotec	Flow Cytometry	1:100
CD45	Miltenyi Biotec	Flow Cytometry	1:100
CD235a	Miltenyi Biotec	Flow Cytometry	1:100
β-Globin	Santa Cruz	Flow Cytometry	1:50
H3K27me3	Abcam	ChIP	5μg/100μL
H3K4me3	Abcam	ChIP	5μg/100μL
Ezh2	Millipore	ChIP	5μg/100μL
Gli3	R&D Systems	WB/ChIP	5 μg/100μL (ChIP) 1μg/mL (WB)
V5	Abcam	IP/WB	5μg (IP) 1:1000 (WB)
Suz12	Abcam	WB/ChIP	5 μg/100μL (ChIP) 1μg/mL (WB)
Mouse IgG	Abcam	ChIP	5μg/100μL
Rabbit IgG	Abcam	ChIP	5μg/100μL
Goat IgG	R&D Systemss	ChIP	5μg/100μL
HRP-Anti Mouse	Abcam	WB	1:3000
HRP-Anti Goat	R&D Systems	WB	1:7500
HRP-Anti Rabbit	Abcam	WB	1:5000

2.3 Colony Forming Unit Assay

EBs were taken at 15 days of differentiation and processed as described for flow cytometry analysis. Cells were counted and plated at 10,000 live cells per well of a 12-well plate in Methocult H4434 media (Stem Cell Technologies). Colonies were counted after 14 days in culture. Cells that would be analyzed by flow cytometry were plucked from the media and prepared for the analysis (See Section 2.2).

2.4 Hemangioblast Assay

Hemangioblasts were generated as described previously[88]. EBs were formed as normal (See 2.1) however Stemline II (Sigma-Aldrich) was used as the media supplemented with BMP4 (50ng/mL) and VEGF-165 (50ng/mL) (R&D Systems) for 48 hours and bFGF (20ng/mL) added after this point. At day 4 EBs were dissociated with Collagenase B and 5000 cells were plated in hemangioblast medium: 100μL Stemline II, 500μL Methocult H4436 (Stem Cell Technologies), BMP4 (50ng/mL), VEGF-165 (50ng/mL), TPO (50ng/mL) and Flt3L (50ng/mL). 6 days following this culture cells were collected and replated in 100μL Stemline II, 1.5mL Methocult H4436, BMP4 (50ng/mL), VEGF-165 (50ng/mL), TPO (50ng/ml), Flit3L (50ng/mL) and EPO (3U/mL) 3 times every 6 days. At day 28, cells were collected and plated in 2mL Stemline II, EPO (30U/mL) and SCF (100ng/mL) for 4 days. Analysis of β-globin expression was performed at the end of this last 4 day culture.

2.5 RNA Extraction, cDNA Synthesis and Quantitative RT-PCR

Cellular RNA was extracted using Total RNA Isolation Kits from Norgen Biotechnology according to their provided protocol. RNA was quantified using Nanodrop2000 and 200-1000ng was allocated to cDNA synthesis. cDNA synthesis was performed using rtPCR Master Mix from Quanta. qRT-PCR was performed using Promega GoTaq SYBR Green qPCR Master Mix on BioRad CFX96 or Chromo4 qRT-PCR machines. PCR protocols ran as follows: 95°C for 5min, followed by 40 cycles of 95°C for 30sec, 62°C for 1min. Melt curve was created by recording SYBR fluorescence

every 0.5°C from 55°C to 95°C. All data was normalized to GAPDH alone or GAPDH and TBP averages using $\Delta\Delta C_T$. See Table 2 for primer sequences used.

Table 2 – List of PCR Primers Used

Target Gene		DNA Sequence
GAPDH	F	CGACGACTTCAAGCTCA
	R	CTGTGAGGAGGGGAGATTCA
TBP	F	CCACAGCTCTTCCACTCACA
	R	CTGCGGTACAATCCCAGAAC
18S	F	TAGAGGGACAAGTGGCGTTC
	R	TCCTCGTTCATGGGGAATAA
HBE	F	CAACAAAAAAGAGCCTCAGG
	R	GATTACTGAAGAAAATGTAC
HBG	F	CACTCGCTTCTGGAACGTC
	R	GTGATCTCTTAGCAGAATAG
HBB	F	ACATTTGCTTCTGACACAAC
	R	GCAATGAAAATAAATGTTTTT
Gli1	F	AGCTGAAGTCTGAGCTGGACA
	R	GATCCTGTATGCCTGTGGAGT
Gli2	F	GACATTCGGCTAAGGAGGGATT
	R	CCAAATGCTCCCTACCATCTTTC
Gli3	F	GCACTTTTGAAGGTTGCACA
	R	CCTTCGTGCTCACAGACGTA
Smo	F	AAGGCCTGCACGAATGAGGT
	R	CAGCTCTTGGGGTTGTCTGT
Hhip	F	AGGCCATATTCCAGGTTTCC
	R	GAAAGCACAACCCACCATCT
Ptch1	F	AGTGTCGCACAGAACTCCACT
	R	GCATAGGCGAGCATGAGTAAG
Lin28	F	AGGAAAGAGCATGCAGAAGCGC
	R	GTAGGTTGGCTTTCCCTGTGCA
Runx1	F	CCGAGAACCTCGAAGACATC
	R	GCTGACCCTCATGGCTGT
CD45	F	GGAGGACACAGCACATTGGA
	R	CCCTGAGCAGCAATCATCAC
Sox6	F	ACAGCCACATGGTGTTGACGGGAA
	R	AGTGGCACCTCCCTCTGCATCTTC

BCL11A	F	CCTCGTCGGAGCACTCCTCGG
	R	CGCCGAATGCGGGTGTGTGAAGAA
Myb	F	TGTTCACGCAGACCTCGCCTGT
	R	GGGCTCGCCAGGGACCTGTTTT
HBE UTR	F	GGCCTGAGAGCTTGCTAGTG
	R	GTCCATCCATCACTGCTGAC
HBG UTR	F	TGGCTAAACTCCACGCATGGGTTG
	R	CCAGAAGCGAGTGTGTGGAACTGCT
HBB UTR	F	AGGGAGGGCTGCGGGTTTGA
	R	CAGCGTGAGGTCTAAGTGATGACA
Pax6 Promoter	F	AAAACCCCAACCAAACAAAA
	R	GCAATAAAAATAAAGCGAGAAGAAA
HBE Promoter Tile 1	F	TGAGGCTTTCTTGGAAAAGG
	R	TCCTCATCTATCTGCAACACAAA
HBE Promoter Tile 2	F	TTTCTTTTCCTTGGCCCTGT
	R	GGAGCAAATCTCAAAATAGGAAAA
HBE Promoter Tile 3	F	TGTACGGTTTTTGTCTCCCTAGA
	R	CTGTTAAAACAGAATCTCACCCAGT
HBG Promoter Tile 1	F	AGCCTTGCCTTGACCAATAG
	R	GCTGAAGGGTGCTTCCTTTT
HBG Promoter Tile 2	F	AATCGGAACAAGGCAAAGG
	R	AGTTTAGCCAGGGACCGTTT
HBG Promoter Tile 3	F	AAACACATTTCACAATCCCTGA
	R	AGCTAGTTTCCTTCTCCCATCA
HBB Promoter Tile 1	F	CAGGTACGGCTGTCATCACTT
	R	TAGATGGCTCTGCCCTGACT
HBB Promoter Tile 2	F	CACTTGCAAAGGAGGATGTTT
	R	TCTGGCACTGGCTTAGGAGT
HBB Promoter Tile 3	F	ATTCTGGAGACGCAGGAAGA
	R	GGAATCACAGCTTGGTAAGCA

2.6 Co-Immunoprecipitation and Chromatin Immunoprecipitation

Cells were collected for Co-IP and subjected to Gentle Soft Cell Lysis Buffer (10mM NaCl, 0.5% NP-40, 0.05% 2-mercaptoethanol, 5mM EDTA, 20mM Pipes, pH 7.4) centrifuged and the protein containing supernatant was collected for Co-IP.

Dynabeads (Invitrogen) were coated with antibody of interest and incubated with the cell lysates for 1 hour at 4°C. The dynabeads were pulled down magnetically and the supernatant removed followed by heating of the beads to release bound protein. The supernatant was then collected for analysis by western blotting. Similarly for ChIP, cells were cross-linked using 1% formaldehyde and sonicated in 0.1% SDS to obtain approximately 1000bp fragments. Sonicated DNA was then immunoprecipitated using required antibodies coated on dynabeads and reverse cross-linked. DNA was purified and used for qRT-PCR to probe for immunoprecipitated areas of interest. See Table 2 for PCR primers used.

2.7 Western Blotting

Total protein was separated using 4-12% SDS-PAGE precast gels (NuPAGE, Life Technologies) and transferred semi-dry onto nitrocellulose using BioRad Transblot Turbo Transfer machine. Membrane was blocked in 5% Skim milk in TBST for 1 hour, and incubated with primary antibody overnight at 4°C. Membrane was rinsed 3x with TBST and then washed 3x with TBST for 10min and probed with secondary antibody conjugated with horseradish peroxidase for 1 hour and washed again. Chemiluminescence was detected on film and scanned to produce a digital file.

2.8 Generation of pHIV-V5Gli3R

pHIV-EGFP was obtained from Addgene and V5Gli3R was inserted into the vector after amplification from a previous in-house V5Gli3R construct. The V5Gli3R

open reading frame was amplified by PCR, and pHIV-EGFP vector was digested using XbaI and XmaI (Figure 3.8A). V5Gli3R was then ligated into the digested construct and transformed into competent *E.Coli*. Bacterial colonies were screened and then sequenced to ensure no mutations were acquired and then was produced in large scale by midi prep (Qiagen).

2.9 Preperation of pHIV-V5Gli3R Lentivirus

pHIV-V5Gli3R and pHIV-EGFP were packaged in $20x10^6$ HEK293FT cells using psPAX2 and pMD2.G (Second generation packaging plasmids) by transfection with lipofectamine. 72 hours following transfection, virus containing media was collected and concentrated by ultracentrifugation for 2 hours at 4°C.

2.10 Mass Spectrometry

Bulk hemangioblast cultures were washed twice in PBS and lysed in 100mM (NH4)HCO3 containing 8M urea and 2mM DTT. Samples were reduced in an equal volume of 100mM (NH4)HCO3 plus 20mM iodoacet- amide in the dark for 1 hour (all chemicals from Sigma-Aldrich). Protein tryptic digestion was performed with 400 ng of sequencing-grade trypsin (Promega) dissolved in an equal volume of 100mM (NH4)HCO3 plus 2mM CaCl2 overnight. Samples were then cleared by centrifugation for 20 minutes at 20 000g, snap-frozen in liquid N2, and lyophilized. Liquid chromatography-tandem mass spectrometry analysis of tryptic digests was performed using a combined setup consisting of an HTS-PAL autosampler (CTC Analytics), a

Paradigm MS4 HPLC (Michrom BioResources), and an LTQ (Thermo Finnigan) linear ion trap mass spectrometer. Tandem mass spectrometry data were analyzed using the MASCOT search engine against a nonredundant National Center for Biotechnology Information (NCBI) human protein database and a custom-made database consisting of se- quenced human globins (HBE, HBG, HBD, HBB, HBZ, and HBA2) verified via cloning and sequencing of corresponding transcripts from the hPSC lines used.

CHAPTER 3

RESULTS

3.1 Gli3R Augments Hematopoietic Differentiation of hPSCs

To test if the hedgehog-signaling pathway is involved in the differentiation of hematopoietic cells from pluripotent sources, expression levels of the pathway members were compared between hESCs, day 10 (D10) hEBs and human cord blood (hCB) as a somatic source of blood for reference. Every member of the pathway tested had higher levels of expression in pluripotent sources except Gli1 (Figure 3.1A), indicating the potential involvement of this pathway in regulating hematopoietic differentiation *in vitro*. Similarly, comparing pluripotent derived hematopoietic sources to somatic sources including human fetal-liver (hFL), hCB, and human mobilized peripheral blood (hMPB) by semi-quantitative-PCR for hedgehog genes confirmed differential expression of the pathway between these sources (Figure 3.1B). Surprisingly, hESCs were found to be expressing a basal level of the hematopoietic regulator Runx1, and hCB and hMPB contained some basal levels of Lin28 expression (Figure 3.1B). To test the involvement of the hedgehog signaling pathway in hematopoietic differentiation the small molecule cyclopamine[89] was used to inhibit the pathway through antagonism of the Smoothened cell-surface receptor. Differentiating hESCs were treated with cyclopamine during days 1-4 of differentiation and hematopoietic output was assayed at day 15 by flow cytometry. Immature ($CD34^{+}CD45^{+}$) blood output was increased by nearly 1.5-fold and mature blood

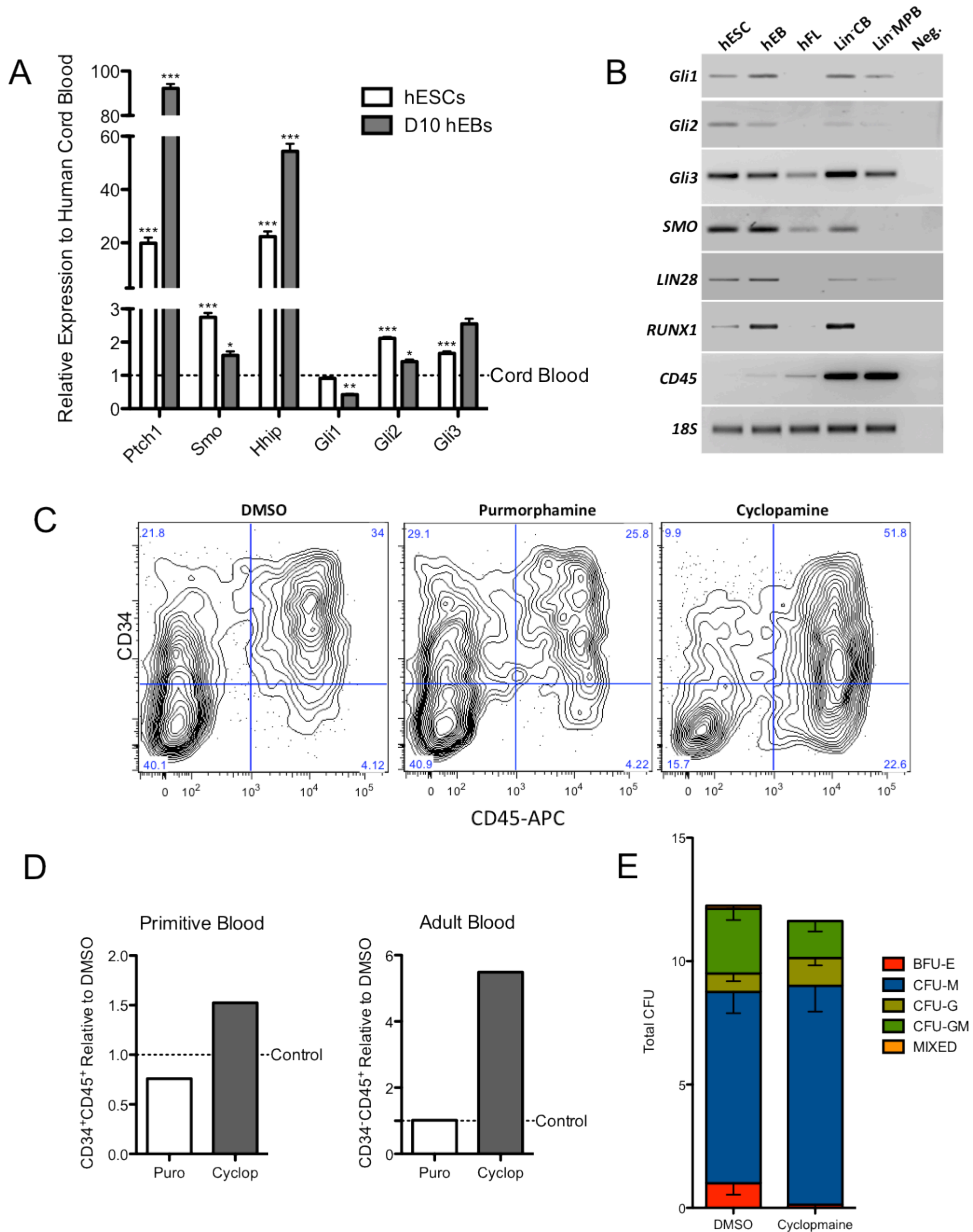

Figure 3.1 – Cyclopamine treatment augments hematopoietic differentiation from hESCs. qRT-PCR of hedgehog pathway members in hESCs, hEBs and CB shows higher expression in pluripotent sources than in somatic sources (A). Semi-quantitative PCR for hedgehog genes, Lin28 as pluripotent control and 18S as loading control showing differences in hedgehog pathway expression between pluripotent and somatic sources of blood (B). Manipulation of the hedgehog pathway through inhibition (cyclopamine) or activation (purmorphamine) shows that the pathway negatively regulates production of hematopoietic $CD34^+CD45^+$ and $CD34^-CD45^+$ hematopoietic cells (C, D). Manipulation of the hedgehog pathway through differentiation has no effect on CFU potential (E).

($CD34^-CD45^+$) output was increased by 5-fold in cyclopamine treated differentiation

cultures relative to control (Figure 3.1C-D), indicating that active hedgehog signaling may be antagonistic to hematopoietic differentiation *in vitro*. This was confirmed by similarly treating cultures with the hedgehog signaling inducer, purmorphamine[90], for the first 4 days of differentiation. Purmorphamine cultures showed inhibited $CD34^{+}CD45^{+}$ output at day 15 of differentiation (Figure 3.1C), but interestingly did not have an effect on CD45 single positive cells. It is therefore possible that hedgehog signaling inhibits the generation of early hematopoietic precursors and their ability to mature to $CD34^{-}CD45^{+}$ cells. However, there is no difference in CFU output between cells produced through hedgehog pathway inhibition (Figure 3.1E) indicating early hedgehog inhibition does not affect myeloerythroid progenitor potential.

Independent work from our lab has shown that the effect of cyclopamine treatment on hematopoietic differentiation is dependent on early treatment during the differentiation protocol. Cells were treated with cyclopamine for days 1-4, 7-10, or 1-10 of the 15 day differentiation protocol and percentage of mature $CD34^{-}CD45^{+}$ cells was measured by flow cytometry (Figure 3.2A)[91]. The effect of cyclopamine on blood augmentation was dependent on early treatment, while continued treatment was inhibitory to the early effects. Furthermore, late treatment of cyclopamine had no effect on blood generation relative to control (Figure 3.2A). This data indicates that the hedgehog pathway may be antagonistic to hematopoietic differentiation early in culture, before hematopoietic specification, and may need to be reactivated for optimal efficiency of blood generation. To test this I tracked expression of hedgehog pathway members throughout the 15-day differentiation protocol while treating with cyclopamine for the

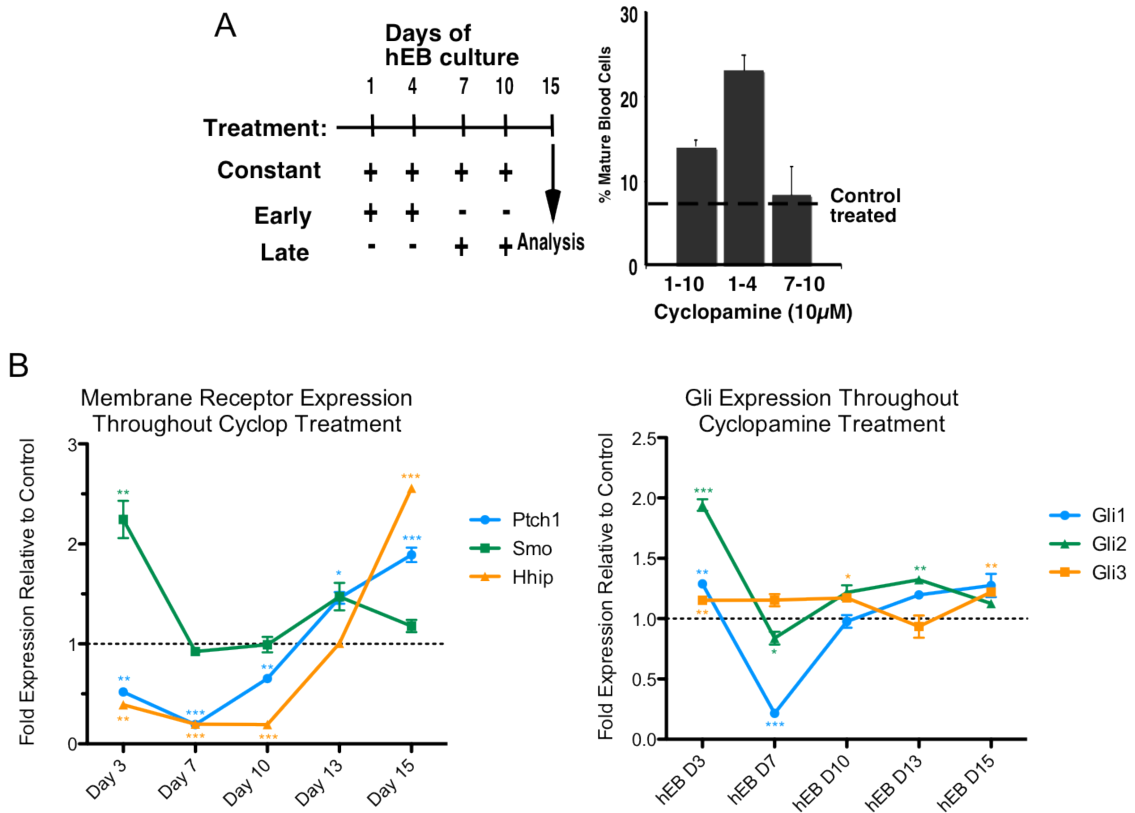

Figure 3.2 – Augmentation of blood generation from hESCs by cyclopamine is dependent on early treatment. Experiments performed by Dr. Vero Ramos-Meija show effects of continuous cyclopamine treatment for days 1-10, 1-4, 7-10 of a 15-day differentiation protocol. $CD34^{-}CD45^{+}$ cells were measured by flow cytometry. Treatment for days 1-4 yielded greatest increases in blood generation, while continuous treatment for days 1-10 increased blood generation at a lower level. Treatment late in differentiation at days 7-10 did not affect the yield of blood production1 (A). Early treatment (days 1-4) of cyclopamine causes a transient inhibition of the pathway through downregulation of Gli1, Gli2, Ptch1, Hhip and Smo (B).

first 4 days (Figure 3.2B). Indeed, there is an obvious antagonism of the pathway that occurs from days 3-10, after which the pathway is reactivated. Cell surface receptors Hedgehog Interacting Protein (Hhip) and Ptch respond acutely to cyclopamine treatment, while nuclear transcription factors Gli1 and Gli2 respond at a slower rate (Figure 3.2B). Expression of Gli3 is not regulated through the hedgehog pathway and as expected its transcript level is not affected by cyclopamine treatment (Figure 3.2B). Therefore

transiently inhibiting the Hh pathway produces a stimulatory effect on hematopoietic differentiation of hPSCs. The reactivation of the Hh pathway occurs around day 10 of differentiation following cyclopamine treatment during the first 4 days of differentiation.

Because the hedgehog signaling pathway is important in developmental patterning[42], it is possible that its manipulation will result in a change of the developmental state of the blood cells produced. Interestingly, we observed that the definitive hematopoietic genes BCL11A, Sox6 and Myb were all upregulated indicating that cyclopamine treated cells may be initiating developmental progression of hematopoietic programs (Figure 3.3). BCL11A and Sox6 are known regulators of the genetic globin switch that occurs through human ontogeny and their upregulation provides evidence of a switch to a mature phenotype. HBE and HBG were downregulated relative to the control indicating that a globin switch may have occurred at the level of transcript. Indeed, adult beta globin transcript was detected in cultures treated with cyclopamine at day 15 but not in control cultures (See figure 3.6B). This expression

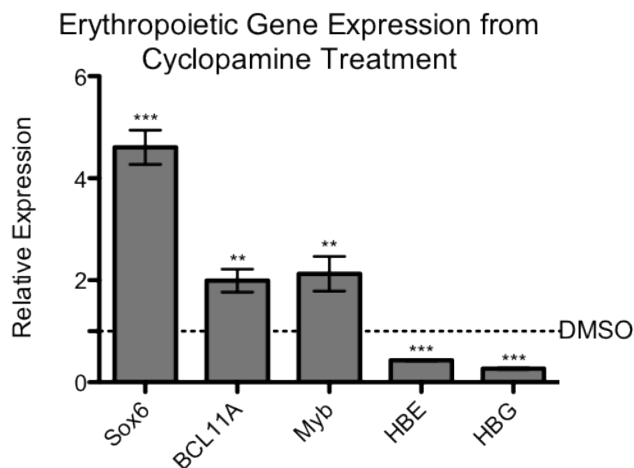

Figure 3.3 – Hematopoietic gene expression of cyclopamine treated cultures at day 15 shows definitive-like profile. Upregulation of important definitive hematopoietic genes BCL11A, Myb and Sox6 while embryonic (epsilon) and fetal (gamma) globin genes are downregulated.

profile is indicative of the production of hematopoietic cells of an adult developmental state.

I aimed to define the genetic role of the hedgehog pathway in the antagonism of early hematopoietic specification from hPSCs. Since the cleaved form of Gli3 (Gli3R) is the central repressor of hedgehog signaling, it is a prime target for genetic manipulation in differentiating cells. hESCs were transfected with an empty in house GFP vector (EGEL) or the same GFP vector expressing Gli3R under a second promoter (EGEL-Gli3R) 24 hours prior to embryoid body differentiation (Figure 3.4A). I failed to augment hematopoietic differentiation through EGEL-GLi3R transfection, although it has been

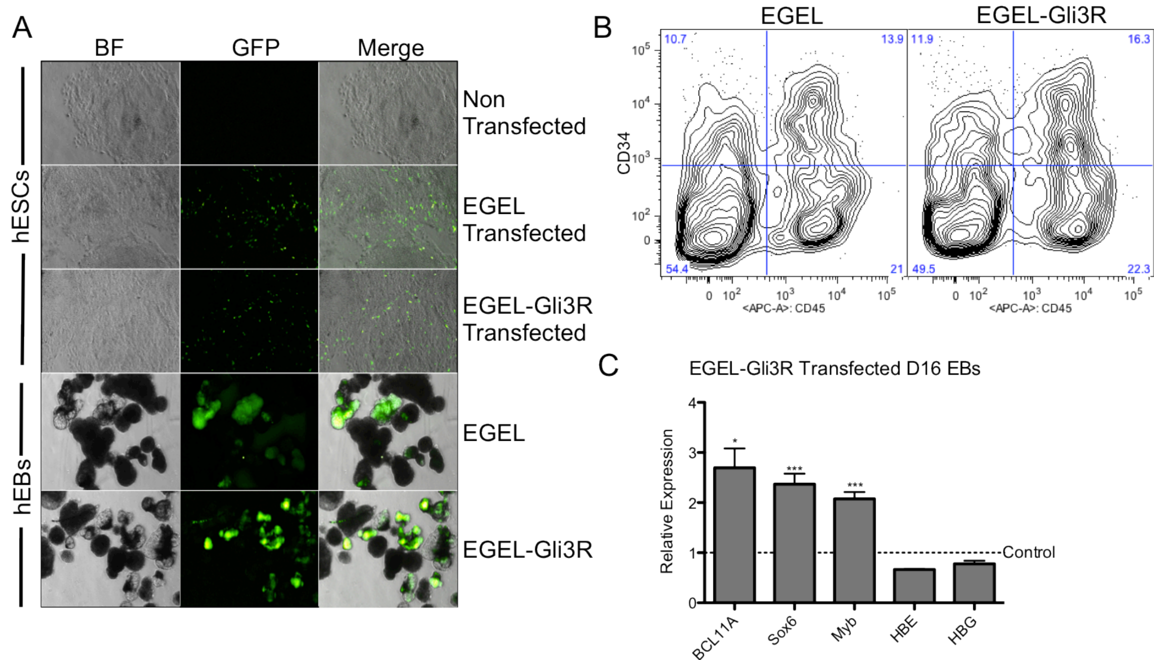

Figure 3.4 – Overexpression of Gli3R mimics cyclopamine treatment in gene regulation. hESCs are transfected with Gli3R expressing vector (EGEL-Gli3R) or empty backbone vector (EGEL) 24 hours before EB formation. GFP is detected at 24 hours in monolayer culture and is maintained in suspension EBs (A). CD45^{+} cell augmentation is not observed at day-15 in EGEL-Gli3R transfected samples (B), however genetic regulation of hematopoietic regulators and globins is similar to cyclopamine treatment (C).

accomplished in our lab to a significant degree[91] (Figure 3.4B). Interestingly upregulation of hematopoietic regulators BCL11A, Sox6 and Myb was detected by qRT-PCR and HBE and HBG were found to be downregulated similarly to cyclopamine treated samples (Figure 3.4C). This could indicate that levels of Gli3R upregulation affects the extent of hematopoietic augmentation.

To assess the maturation effect of hedgehog inhibition during hESC differentiation we further characterized the extent of changes in globin regulation previously observed (Figure 3.2). In collaboration with Dr. Guojun Sheng's group from the RIKEN Center of Developmental Biology (CDB), we characterized globin profiles of generated cells at the protein level through mass spectrometry[91]. DNA was extracted from hESCs and used to sequence HBE, HBG and HBB, all of which were wild type with reference to the NCBI genomic database. The sequencing data allowed for a custom database with shorter search terms in analyzing mass spectrometry data. From the sequences Dr. Sheng's group identified specific unambiguous peptides after trypsin digestion for HBE, HBG (data not shown) and HBB (Figure 3.5A). hESC-derived hematopoietic cells using a hemangioblast based differentiation protocol[88] were plated in methylcellulose to produce CFUs. Erythroid colonies from cyclopamine and control wells, as well as CB controls, were collected and lyophilized and shipped to RIKEN CDB for analysis. Consistent with previous expression data (Figure 3.2), erythroid cells produced from cyclopamine treated wells had initiated globin switching to produce adult HBB protein, where none was detected in control colonies (Figure 3.5B). Gamma globins were also found to be expressed at a higher level in colonies that originated from

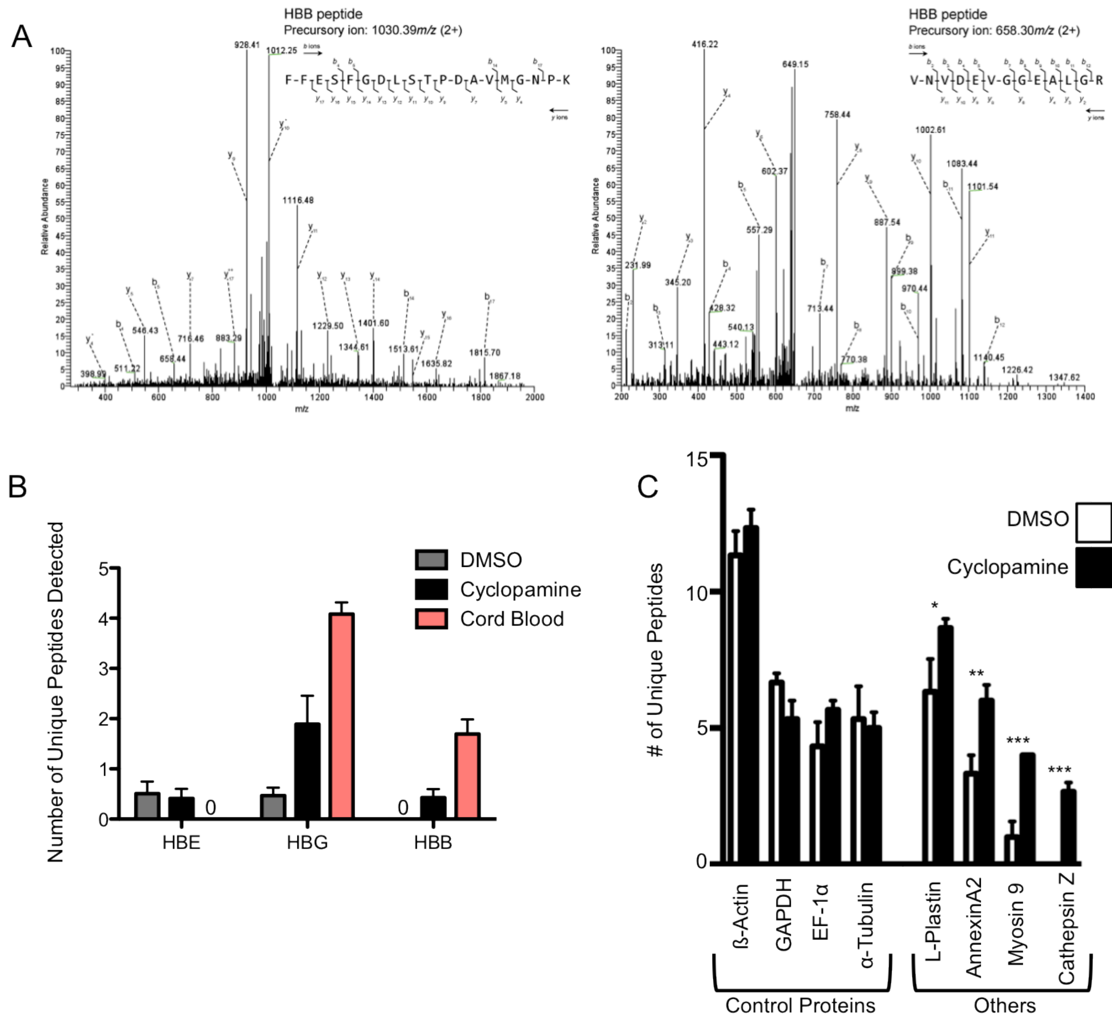

Figure 3.5 – Cyclopamine treated cells produce erythroid colonies that express beta globin protein. Spectra specific for beta globin that are unambiguous after protein digestion were determined by RIKEN CDB and used as search terms for erythroid colony lysates produced from hESC-derived hematopoietic cells (A). Unique peptides for beta globin (HBB) was found in cyclopamine treated samples and CB positive control, but not in DMSO control treated colonies (B). Proteins detected with differential expression between cyclopamine and DMSO treated colonies provide candidate proteins for determining mechanisms of globin switching (C).

cyclopamine treated cultures compared to controls (Figure 3.5B). This data demonstrates, for the first time, mature HBB production from hematopoietic cells derived from hESCs. This phenotype further implies that cyclopamine may be altering the developmental program of these cells to initiate a definitive hematopoietic fate vs. the primitive phenotype that is associated with hPSC hematopoiesis in the literature. Further probing by mass spectrometry revealed proteins that are differentially expressed between

cyclopamine and control treated colonies (Figure 3.5C). Interestingly, three of the four identified proteins with differential expression are actin-binding proteins (L-plastin, Annexin A2 and Myosin 9). The fourth, Cathepsin Z, is a proteinase that may have a role in development[92], and may be a target of hedgehog signaling considering another member of the cathepsin family (cathepsin B) is regulated by hedgehog in a pancreatic cancer context[93]. Collectively, this data confirms that transient early antagonism of the hedgehog pathway in differentiating hESCs produces hematopoietic cells that have initiated globin switching beyond what has been previously reported.

In an effort to track the dynamics of the observed globin switch, I attempted to measure expression of HBE, HBG and HBB through differentiation at multiple time points. In a series of experiments, cyclopamine treatment gave rise to cells that expressed HBE and HBG at higher levels than control treated cultures (Figure 3.6A, C) in contrast to the previously observed globin transcript profile (Figure 3.2). Despite this, these cells underwent a globin "switch" in that adult HBB expression was still detected in cyclopamine treatments only (Figure 3.6B). This change from previously observed downregulation in HBE and HBG is in line with mass spectrometry data showing equal protein expression of HBE and higher expression of HBG in cyclopamine treated cultures (Figure 3.5B). Based on my observations the downregulation of HBE and HBG at the transcript level occurred randomly and could not be predicted. However, the predictable expression of HBB at day 15 of differentiation could indicate that the upregulation of adult globins and downregulation of fetal globins occurs through separate mechanisms and these cells sometimes fail to engage the mechanisms for downregulation

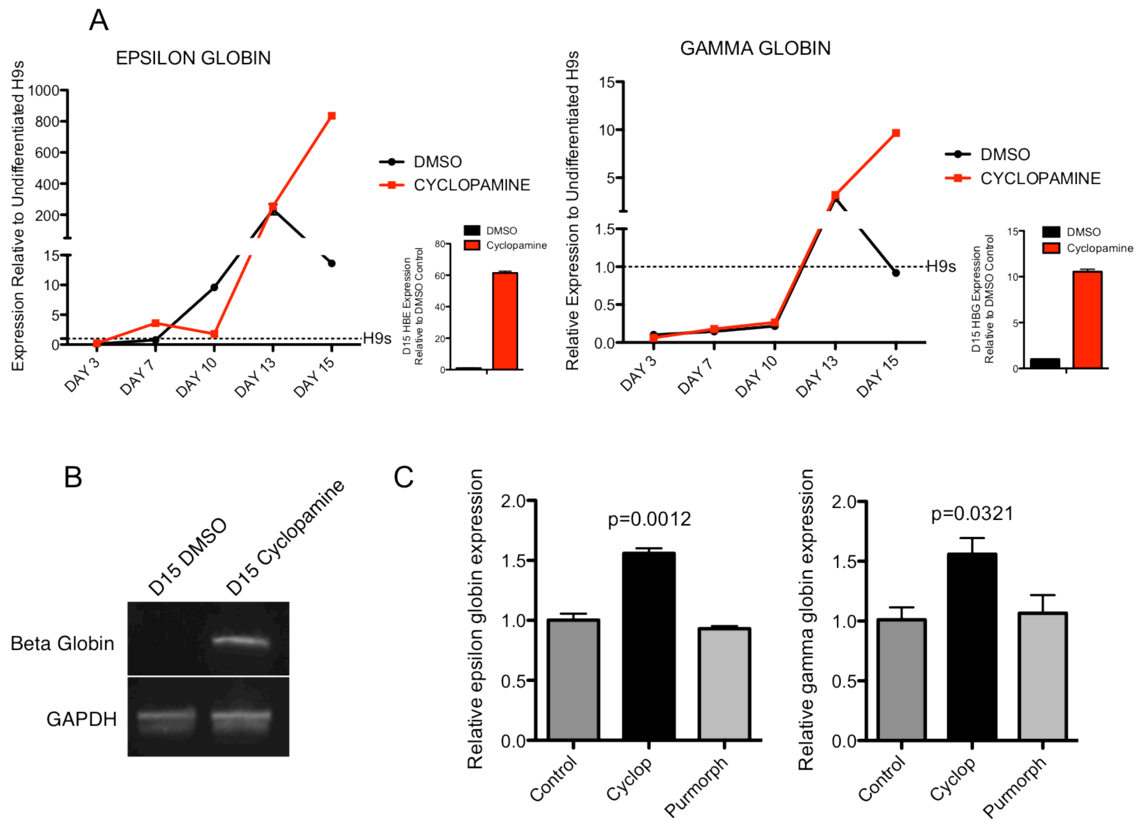

Figure 3.6 – Cyclopamine treatment produces predictable expression of beta globin but not of epsilon and gamma globins. Tracking of epsilon and gamma globins through differentiation by qRT-PCR shows gradual upregulation of epsilon and gamma globins in cyclopamine samples over controls (A), however expression of beta globin was observed at day 15 and not before (B). Upregulation of epsilon and gamma globins was observed multiple times and quantified (C).

of previously expressed globins. Alternatively, heterogeneity of the cell cultures could be showing a persistent expression of HBE and HBG in some cells while others are initiating a switch to HBB. To test if cyclopamine treatment would induce a similar maturation effect on the globin profile of immature hematopoietic cells, I treated lineage depleted human cord blood in culture with cyclopamine and measured CFU output and expression of beta globin by flow cytometry (Figure 3.7). CB was treated with cyclopamine for 48 hours in suspension and CFUs were read out. These experiments showed an increase in

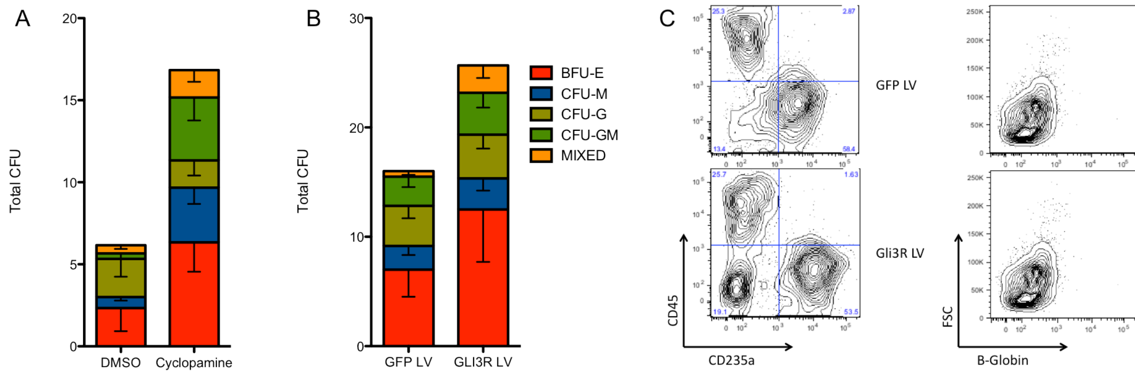

Figure 3.7 – Hedgehog inhibition in human cord blood increases CFU potential but does not induce a globin switch. Hedgehog inhibition by cyclopamine treatment (A) or Gli3R transduction (B) increases total CFU number but does not affect proportions of colony types generated. Gli3R treated cells do not undergo a globin switch to express beta globin by flow cytometry (C).

total CFU number but not in the composition of CFU colonies (Figure 3.7A). CFU number also increased over controls in Gli3R transduced cells confirming that this effect is mediated through antagonism of the hedgehog pathway (Figure 3.7B). This data is in contrast to the effect of cyclopamine on CFU output in hESCs (Figure 3.1E) and shows that inhibition of the hedgehog pathway increases proliferation of blood progenitors in somatic sources. It is therefore possible that further cyclopamine treatment of hematopoietic progenitors produced at day 15 of differentiating hESCs would respond in the same manner to somatic CFU output. Unlike its effect on hESCs however, inhibiting hedgehog signaling did not have an effect on the globin profile of CB as measured by flow cytometry on fixed erythroid colonies (Figure 3.7C). This highlights the difference of cyclopamine treatment in already generated hematopoietic progenitors (i.e. form a somatic source) vs. early commitment steps from a pluripotent state (i.e. first days of differentiating hESCs).

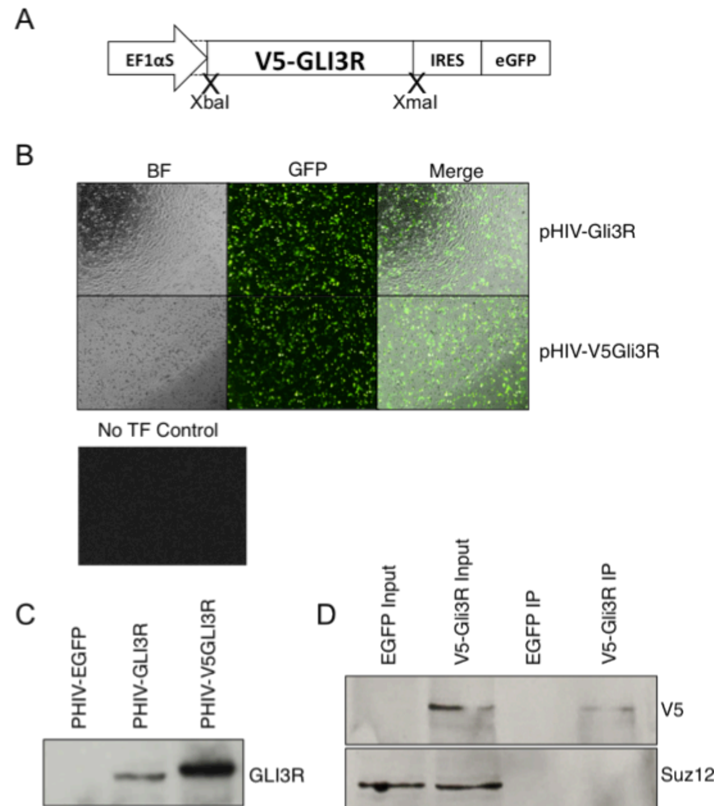

Figure 3.8 – Gli3R does not interact with Suz12. The pHIV-V5Gli3R construct contains an internal ribosomal entry site (IRES) for independent expression of GFP to allow detection (A) as seen in HeLa cells transduced with this lentivirus generated with this vector (B). The vector was confirmed to express Gli3R (C) before being used in a co-immunoprecipitation in HeLa cells to attempt to pull down PRC2 member Ezh2 (D). The Co-IP succeeded in pulling down V5-Gli3R, however Suz12 could not be detected,

3.2 Gli3R and PRC2 do not Control Epigenetic Regulation of the Beta Globin Locus

To understand the mechanisms of the globin switch we observe in hPSC-derived hematopoiesis, I interrogated the epigenetic regulation of the beta globin locus in these cells. Preliminary data from our lab has shown the potential involvement of the polycomb repressive complex 2 (PRC2) in globin switching[91], as well as an interaction between Gli3R and Enhancer of zeste homologue 2 (Ezh2). To further interrogate the mechanism of globin switching I constructed a V5 epitope tagged Gli3R lentiviral construct using a

pHIV self inactivating vector backbone[94] under the expression of an EF1a promoter (Figure 3.8A) in order to perform co-immunoprecipitation (Co-IP) to test if Gli3R interacts with Suz12, a central PRC2 member. This vector's capacity to infect HeLa cells was tested (Figure 3.8B) and confirmed to express Gli3R by western blotting (Figure 3.8C). HeLa cells were then transduced with this vector and harvested 48 hours following infection. Co-IP using an anti-V5 antibody was performed and the pull-down product was probed for Suz12 (Figure 3.8D). The CO-IP was successful (V5-Gli3R IP lane) in pulling down the target protein Gli3R, however Suz12 was not co-immunoprecipitated, indicating that this interaction likely does not occur. However, further experimentation with more permissive lysis and wash buffers could better maintain the interaction if one does occur. Yet at this point, data suggests Gli3R does not physically interact with central PRC2 member Suz12.

To test if PRC2 was indirectly affecting the globin switch that we have shown thus far to be mediated by Gli3R, I performed chromatin immunoprecipitations (ChIPs) on un-translated region (UTR) segments of the globin genes using antibodies specific to the active chromatin mark H3K4me3 and the repressive PRC2 mark H3K27me3 on hematopoietic cells generated from hESCs (Figure 3.9). ChIP data shows similar ratios of H3K4me3 and H3K27me3 marks on globin UTRs, which may indicate that these mechanisms of epigenetic regulation do not play a role in globin switching (Figure 3.9), or that these modifications are present outside the interrogated region (See Figure 3.12). For instance, the HBB UTR shows similar ratios of the two marks in DMSO and cyclopamine samples, albeit with cyclopamine having greater total amounts of both

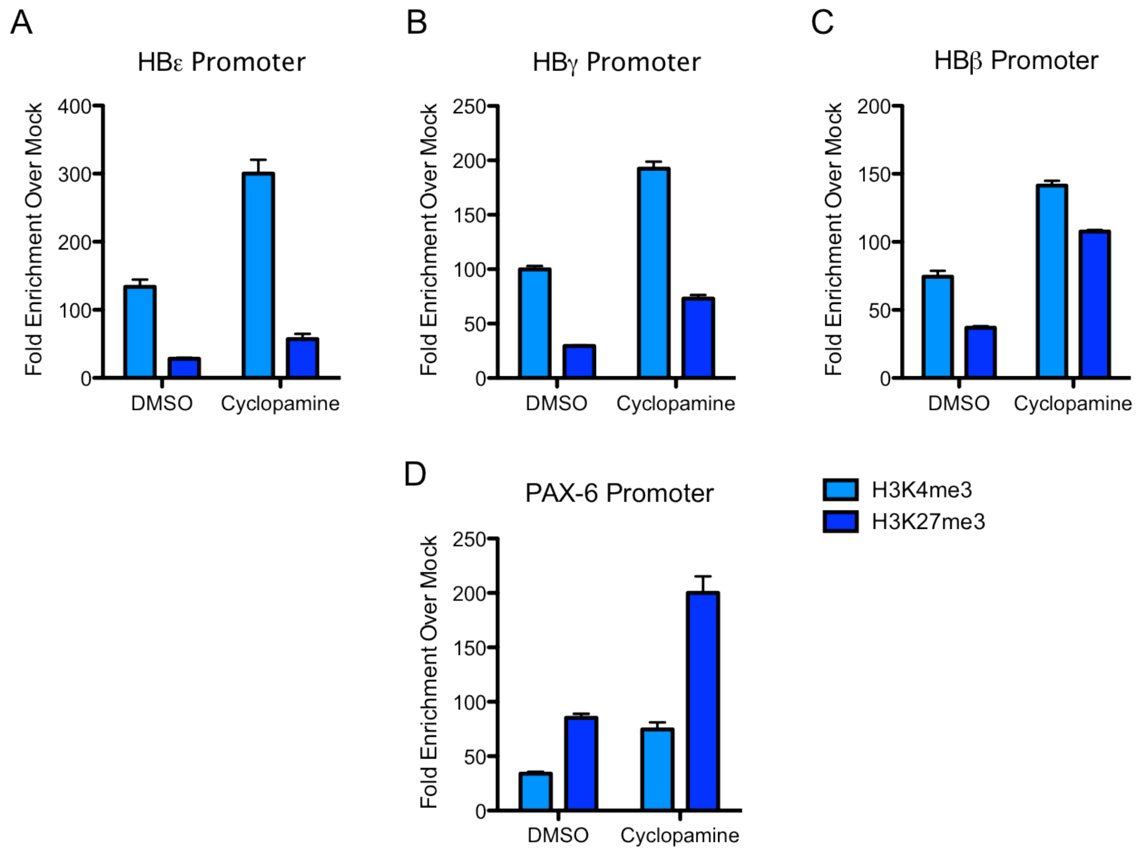

Figure 3.9 – H3K27me3 and H3K4me3 marks do not correlate with expression patterns observed from cyclopamine treatment. ChIPs for H3K27me3 and H3K4me3 on the HBE (A) HBG (B) and HBB (C) promoters show similar patters of these epigenetic marks except for cyclopamine treated cultures containing the greater total methylation of the two treatments. A neural control used, Pax6 (D), also shows the greater amounts of methylation in cyclopamine samples while maintaining similar ratios.

marks (Figure 3.9C). However, HBB is expressed in cyclopamine treated samples but not in controls. Furthermore, the methylation marks on the Pax6 promoter, an unrelated neural gene used as a negative control, also show similar ratios between the two treatments. But again in this case, cyclopamine has greater levels of both methylation marks (Figure 3.9D) indicating (a) methyl marks are not relevant to expression change in this case or (b) I have not identified the precise region of globin modification (See Figure 3.12).

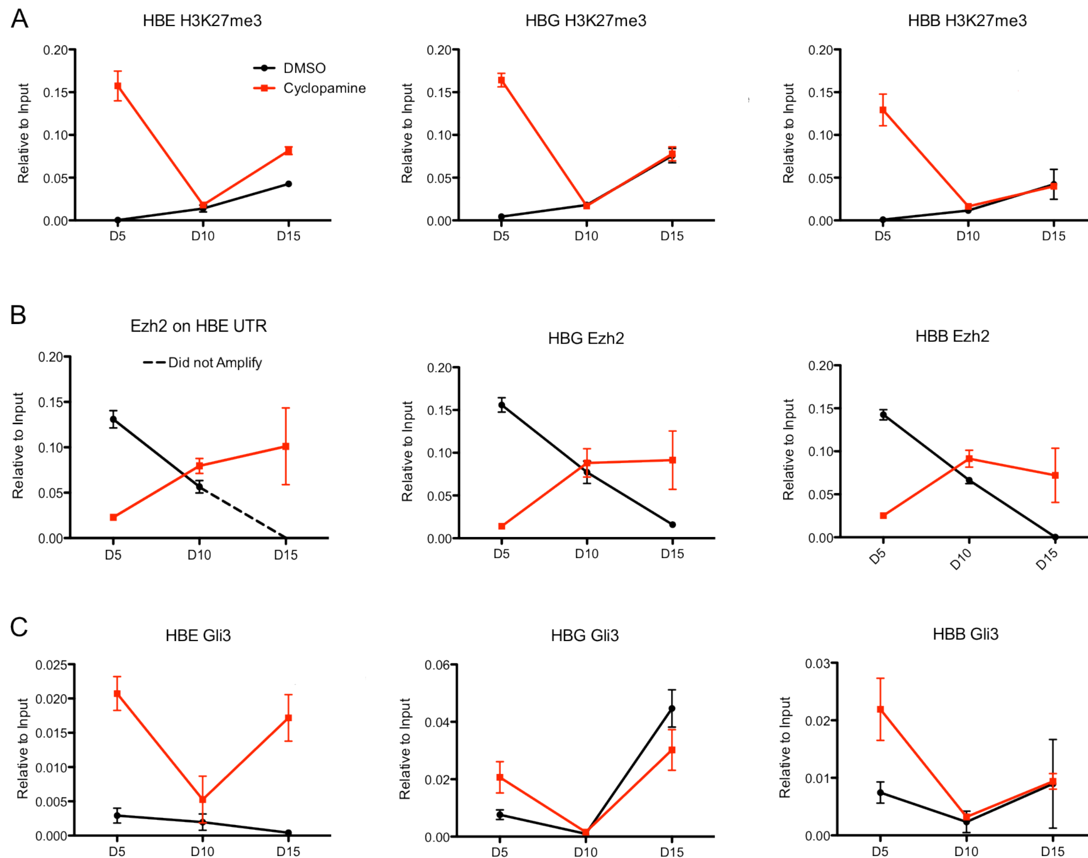

Figure 3.10 – Timecourse ChIPs for PRC2 and Gli3 involvement in globin promoter regulation does not show noteworthy patterns. ChIPs performed for H3K27me3 (A), Ezh2 (B), or Gli3 (C) performed over a timecourse through differentiation do not portray observable patterns of involvement.

Next, to test if PRC2 played a role in epigenetic regulation of the globin locus earlier in hematopoiesis, closer to the time point of cyclopamine treatment, I performed ChIP experiments at multiple time points throughout the differentiation of treated hESCs. ChIPs for H3K27me3 showed increasing methylation in control cultures following 5 days of differentiation, whereas cyclopamine treated samples showed high methylation marks relative to controls at day 5 and fell to control levels by day 10 (Figure 3.10A). A similar trend occurs with Gli3 enrichment on the HBG and HBB UTRs, but a maintained

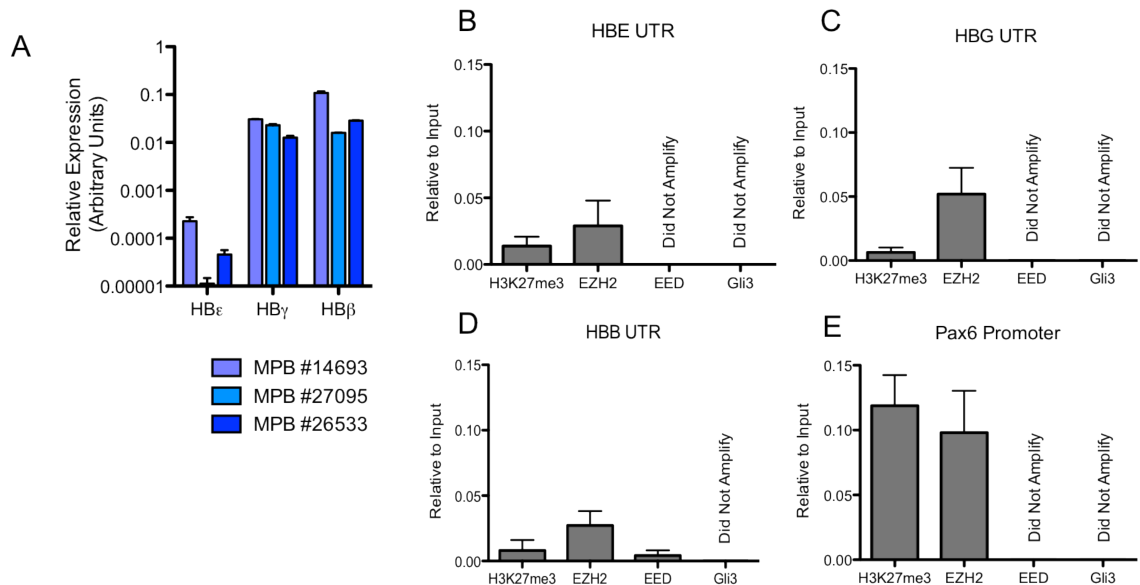

Figure 3.11 – PRC2 members and functional mark H3K27me3 do not correlate with globin expression patterns of human mobilized peripheral blood. qRT-PCR on MPB shows repression of HBE but similar expression of HBG and HBB (A). ChIP of H3K27me3, Ezh2, EED and Gli3 on the promoters of HBE (B), HBG (C), HBB (D) show similar patterns of binding and do not correlate to transcript expression levels. Pax6 control promoter shows greater enrichment of Ezh2 and functional mark H3k27me3 (E).

enrichment on the HBE UTR was seen at day 15 (Figure 3.10C). Ezh2 enrichment was maintained in cyclopamine treated cells throughout differentiation but not in control wells (Figure 3.10B). However, it is important to note that the values observed are quite low and may be within the realm of "noise" for this experimental protocol.

To further validate this data as well as test the potential involvement of Gli3R in globin regulation, I performed additional ChIPs for PRC2 members, H3K27me3, and Gli3 on the globin UTRs from human mobilized peripheral blood (hMPB) of three patients (14693, 27095, 26533). Globin expression profiling of these cells revealed low expression of HBE, but high levels of HBG and HBB transcripts (Figure 3.11A). It is noteworthy that these cells had high expression levels of HBG as they are from an adult

source. However, it is possible that the mobilization process induces a "stress" state or causes proliferative cues which have been previously documented to produce fetal globins in the adult[95]. ChIPs showed H3K27me3 marks did not correlate with the transcript expression patterns observed (Figure 3.11B-E). H3K27me3 marks were in similar amounts for all three UTR regions interrogated, where it would be expected that the HBE UTR would have greater H3K27me3 because transcript levels are greatly repressed. Similarly, levels of the methyltransferase Ezh2 do not differ greatly between the globin UTRs, and EED (a central member of PRC2) failed to amplify in almost all cases (Figure 3.11B-E). It is possible that EED did not amplify because the antibody used did not recognize a specific member of the EED family, however because the H3K27me3 marks do not correlate with the expression patterns this is unlikely. Gli3 also did not amplify in any of the promoters tested signifying that it likely does not directly play a role in globin regulation in somatic sources (Figure 3.11B-E). However, a possible caveat to this experiment is that it is based on the bulk qRT-PCR on the three MPB patients (Figure 3.11A), which was analyzed as relative to GAPDH. Therefore this PCR method did not account for primer efficiency and therefore cannot be correlated directly to transcript copy number.

It is a possibility that the measurement of H3K27me3 after ChIP was performed I attempted to amplify a non-regulated portion of the globin promoters. In an effort to ensure that this oversight was not made, I designed primers to tile the promoters of HBE, HBG and HBB beginning at the 5' UTR, and performed H3K27me3 ChIP (Figure 3.12A). The first set of primers for each globin covered the UTR, where the second and

third set extends into the promoter region of the respective globin. ChIPs were performed on MPB and confirmed that all promoter locations were similarly methylated between three MPB patients and that there wasn't a spike of methylation in any particular region of the globin promoters (Figure 3.12B-D). I therefore conclude that PRC2 does not affect epigenetic regulation of the globin locus, and Gli3R does not directly influence globin expression as a transcriptional repressor. Of course, the limitation of this assay is that coverage is very specific and limited to immediate promoters of these genes. ChIP-Seq for example would identify if there are PRC2 regulatory regions on the locus that were not interrogated, such as the locus control region of this locus.

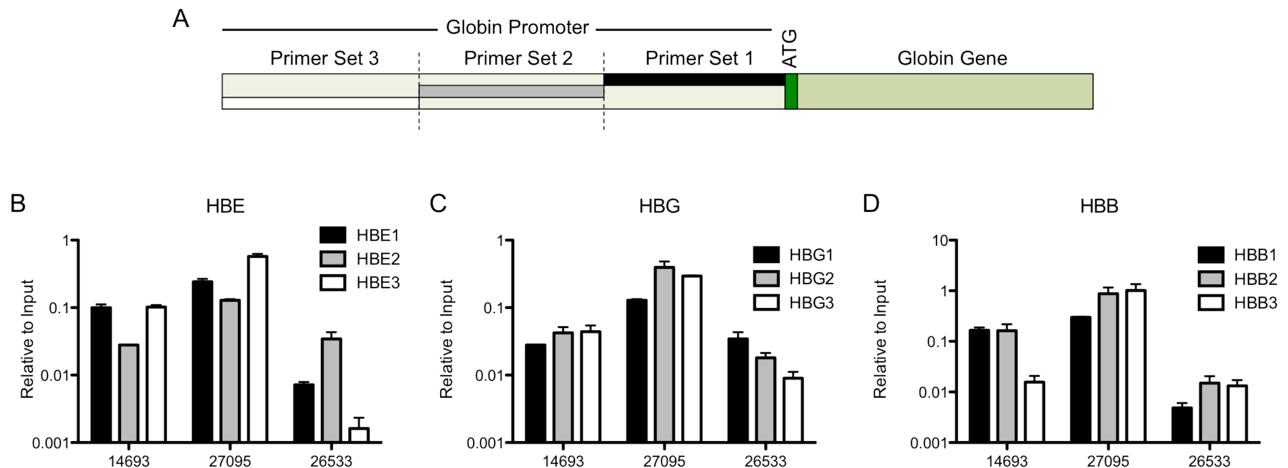

Figure 3.12 – Tiling of globin promoters did not identify specific promoter locations where PRC2 methylation occurs. Experiment design for promoter tiling (A). There was no significant difference in H3K27me3 in the 3 different amplified sequences covering the promoters of HBE (B), HBG (C) and HBB (D).

CHAPTER 4

DISCUSSION

The generation of HSCs from hPSCs remains an ongoing goal for clinical transplantation sources as well as *in vitro* modeling of hematopoiesis for drug discovery. In previous years, the development of this differentiation process had stalled to produce only minute advances in the production of the $CD34^{+}CD45^{+}$ population frequency[20,25,28,96,97]. Furthermore, due to the nature of these heterogeneous differentiation cultures, reproducibility between, or even within labs has proven difficult on some key advancements[28]. It is clear that the goal of the field is to reproducibly generate hematopoietic cells of a definitive phenotype, and not a greater number of primitive (and therefore clinically limited) hematopoietic cells. This year has seen the publication of two reports that have accomplished hematopoietic reconstitution of an irradiated, immunocomprimised mouse with cells generated from hPSCs[32,33]. Amabile *et al*. used an *in vivo* teratoma as a source of differentiation for hPSCs and isolated reconstituting hematopoietic cells, providing proof-of-principle that hPSCs can produce hematopoietic cells with greater utility than cells previously generated[33]. In contrast to the EB method of differentiation that has been described in this thesis, pluripotent cells producing teratomas *in vivo* have access to all the hormones and proteins that are in murine circulation. Therefore any cells of hematopoietic origin that may be spontaneously formed have access to molecules and supporting cell interactions that are currently missing from *in vitro* culture conditions. Teasing apart the factors involved in this *in vivo* process and then

reapplying it to EB culture is a promising avenue of experimentation. Ran *et al*, showed that overexpression of Runx1a in hPSCs produces hematopoietic cells that are capable of engrafting immunocomprimised mice[32]. These two advancements highlight that there are multiple solutions towards generating bona fide HSCs from hPSCs such as genetic manipulation or altering *in vitro* culture conditions to provide a microenvironment that better supports definitive hematopoietic differentiation.

Here I've shown that antagonism of the Hh pathway by cyclopamine treatment through differentiation of hPSCs results in not only increased hematopoietic generation, but was also the first report of producing blood cells of a definitive phenotype[91]. Upon the discovery of Hh inhibition on hematopoiesis from pluripotent sources, the project could have progressed with either functional or mechanistic experiments. Rather than perform experiments to test function alone, such as *in vivo* potential of the cells generated, I preferred to approach mechanistic understanding of the globin switch observed in hopes it would allow functional experiments on a more hypothesis based manner.

Antagonism of the hedgehog-signaling pathway in differentiating hPSCs increased the generation of CD45+ hematopoietic cells (Figure 3.1C-D), which were more mature in phenotype than in control cultures as measured by globin expression (Figure 3.5B). It is interesting that inhibition of Hh signaling would result in greater hematopoietic generation due to previous reports suggesting that Hh activity is conducive to hematopoietic progenitor maintenance and proliferation[48,98]. In the human, inhibiting the pathway resulted in decreased progenitor number after culturing human $CD34^{+}CD45^{+}$ somatic blood, while activating the pathway resulted in greater progenitor number and

greater potential to engraft immunocomprimised mice[48]. However, Dr. Ramos-Meija showed that the effects of cyclopamine on hPSC-derived hematopoiesis were most efficient with an early treatment between days 1-4 of the 15-day differentiation protocol (Figure 3.3A). This data, in combination with previous work from our lab to uncover timing of hematopoietic specification from pluripotency[99], can conclude that the cells being treated with cyclopamine at days 1-4 of differentiation are still pluripotent in nature and have not yet specified to a mesodermal lineage. Therefore antagonism of the hedgehog pathway during the first differentiation decisions of hPSCs results in later hematopoietic augmentation. In evidence of this, based on transcript levels of Hh members through differentiation, I observed that Hh signaling is reinitiated (Figure 3.3B) at the time of hematopoietic specification[99], and it is not through ongoing Hh repression that augmentation of hematopoietic production occurs. It would be interesting to test the differentiation potential of cyclopamine treated cells to other lineages such as neural or endodermal tissue to see if Hh inhibition is an important step in overcoming pluripotency for general enhanced differentiation.

Lineage depleted hematopoietic progenitors from human cord blood treated with cyclopamine for 48 hours prior to CFU formation showed increased CFU potential over controls (Figure 3.7A). This trend was also observed in cells that were transduced with Gli3R lentivirus prior to CFU formation (Figure 3.7B). This data is in line with the previously mentioned report by Bhardwaj *et al*. who observed that both inhibiting and activating the Hh pathway resulting in increased CFU formation from hematopoietic progenitors[48]. While inhibiting the hedgehog pathway results in decreased proliferation of

progenitors it also maintains them in an undifferentiated state, resulting in greater CFU potential[48]. This data shows that hedgehog inhibition acts differently when treated directly to hematopoietic cells vs. hPSCs. I observed no changes in CFU potential from hPSC derived hematopoietic cells treated with cyclopamine (Figure 3.1E).

Ultimately, the goal of this project was to generate cells of a definitive phenotype through early hedgehog antagonism. The maturity of hematopoietic cells produced through hedgehog inhibition was measured through expression of HBB, which was present in treated cultures and absent in controls (Figure 3.5A-B) indicating these cells were of definitive phenotype. This was the first report of beta globin expression from hPSC derived hematopoietic cells[91], and was tested by mass spectrometry to ensure the validity of the result due to concerns with the use of potentially non-specific antibodies. This observation indicates that these cells, at a translational level, have switched to an adult globin expression that is otherwise restricted to bone marrow hematopoiesis following birth *in situ*. Importantly, future experiments will test the capacity of the cells produced to engraft and reconstitute irradiated immunocomprimised mice. Beyond the measure of globin expression, lysed samples were probed by mass spectrometry for significant differences in protein expression in cyclopamine treated cultures (Figure 3.5C). Four proteins were found and interestingly, three of the four proteins found are actin-binding proteins. The L isoform of plastin (also known as lymphocyte cytosolic protein) is an actin binding protein that is expressed exclusively in hematopoietic tissue[100]. Annexin-2 is involved in connecting membrane proteins to the actin cytoskeleton and has been linked to HSC localization to the endosteal niche[101]. Myosin 9

is the third actin binding protein[102], although not much information in relation to hematopoiesis has been previously reported. This finding suggests that cytoskeletal structure is important in the distinction between primitive and definitive hematopoiesis. Cytoskeletal structure is involved in the function of membrane integrin proteins. In hematopoiesis, the function of integrins is critical for the interaction with the cells' microenvironment, and different integrins can be used to identify different cell populations[103]. For example, it's been shown that when L-plastin is phosphorylated in thymocytes, membrane integrins are activated causing the cells to adhere to their respective niche[104]. Because hPSCs are a heterogeneous culture of pluripotent cells and supporting stroma, it is interesting that proteins like Annexin-2 and other actin binding proteins are observed to be differentially expressed between cyclopamine treated cultures and controls. It is possible Hh inhibition is affecting the *in vitro* niche created between hematopoietic and non-hematopoietic cells that arise through hEB differentiation and these altered interactions trigger the developmental switch observed. On a similar note, it is possible that cyclopamine treatment is not affecting cells that will become hematopoietic, but affecting cells that could be differentiating to endothelial[105], osteoblastic[106] or adipocytic[107] lineages that have been reported to be supportive to the hematopoietic environment, and therefore only indirectly affecting hematopoiesis. It has been previously suggested that the developmental maturity of the supporting microenvironment is more determinant to globin expression than the developmental age of the HSCs present[108]. This was shown by injecting adult HSCs into blastocysts which produced erythroid cells that expressed HBE and HBG, while fetal HSCs injected into

adult spleens produced erythroid cells with HBB expression[108]. To test if Hh inhibition is inducing differentiation of supportive cells, interrogation of cell surface markers of these supportive populations by flow cytometry will provide insight into the changes cyclopamine is producing. These markers include CD31[109] and VE-Cadherin[110] for endothelial cells and Osteopontin[111] for osteoblasts. These receptors are used frequently in their respective fields and would not require extraordinary effort to be interrogated following differentiation culture. In addition, These specific cell types can be isolated through fluorescence assisted cell sorting (FACS) and used separately or in combinations to co-culture with differentiating hPSCs to consistently and efficiently produce hematopoietic cells with a definitive phenotype.

The role of the polycomb repressive complex was assessed due to preliminary data in the lab that suggested PRC2 was acting to repress HBE in cyclopamine treated cultures[91], and did so through binding Gli3R (unpublished data). Epigenetic modification of the beta globin locus has been a topic of interest in the globin switching field[112]. However, for some time the only report interrogating PcG involvement in globin regulation was a report showing PRC2 repression of alpha globin genes in non-erythroid cells[113]. Kim *et al*. later noted that H3K27me3 showed no observable patterns between silenced and active globin genes in the LCR or gene promoters of the K562 erythroleukemia cell line, but was highly enriched across the locus in 293FT fibroblasts[114]. PRC2 members Ezh2 and Suz12 correlated with the H3K27me3 abundance in 293FT but not in K562 cells[114]. This indicates that PRC2 is not repressing inactive globin genes. A second report was published that examined various histone modifications

on the beta globin locus and failed to detect appreciable levels of H3K27me3 in erythroid cells from various sources[115]. Similarly to these studies, I failed to observe any patterns of H3K27me3 on the globin genes in hPSC generated hematopoietic cells (Figure 3.9-3.10) or somatic mobilized peripheral blood (Figure 3.11-3.12). It is therefore unlikely that PRC2 is involved in the repression of embryonic and fetal globin genes in a hematopoietic context. PRC2 involvement was tested early in differentiation to coincide with cyclopamine treatment (Days 1-4), however ChIP experiments timed through differentiation failed to show any H3K27me3 patterns of repression (Figure 3.10). Tiling of the globin promoters following H3K27me3 ChIP ensured that a negative read-out was not due to overlooking potential regulatory sites (Figure 3.12). This data is in line with the two previous reports that failed to observe PRC2 regulation of the globin locus[114,115]. This observation is interesting because MEL cells can be induced to perform a globin switch by adding DMSO to their culture. DMSO has been previously reported to inhibit histone deacetylases (HDACs). HDAC's are often recruited by the H3K27me3 mark left by PRC2 and it would have been intuitive that PRC2 would also play a role in globin switching. However it now seems likely that HDACs are involved in globin switching through a mechanism that is independent of PRC2.

To test if Gli3R was directly interacting with PRC2 as a mechanism of recruitment I attempted to co-immunoprecipitate Suz12 with a V5 tagged Gli3R. Under permissive Co-IP conditions, V5-Gli3R failed to co-immunoprecipitate Suz12, the only non-redundant member of PRC2 (Figure 3.8). Admittedly, the experiment was performed in HeLa cells overexpressing V5-tagged Gli3R and caveats of this particular experiment

include Gli3R misfolding by interference of the V5 tag and that binding of these factors may be cell-type specific.

In conclusion, here I show that hPSCs can be used to effectively model human globin switching *in vitro* while avoiding the setbacks of transgenic mouse models and immortalized erythroleukemia cell lines. With the addition of cyclopamine, hematopoietic progenitors produced from hPSCs will undergo activation of beta globin transcription and translation but not necessarily repression of HBE and HBG (Figure 3.2, 3.5, 3.6). This data suggests that the mechanisms involved in upregulating beta globin and repressing epsilon and gamma globins may be independent, or that continued HBE and HBG expression is the result of a heterogeneous starting culture. Ultimately however, through early hedgehog antagonism, we can now begin to tease apart the mechanisms of globin switching *in vitro* and hopefully in the future test these capacity to function as true HSCs in experimental mouse models.

CHAPTER 5

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