UNIVERSITY OF CALIFORNIA, SAN DIEGO

Modeling Hematopoietic Cell Development and Chronic Myeloid Leukemia with Human Embryonic Stem Cells

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biology

by

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The dissertation of Jessica Marie Rusert is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Dedication

To my eternally devoted and beloved husband

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Publications

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ABSTRACT OF THE DISSERATION

Modeling Hematopoietic Cell Development and Chronic Myeloid Leukemia with Human Embryonic Stem Cells

by

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Professor Lawrence S.B. Goldstein, Chair

Chronic myeloid leukemia (CML) is a cancer where a hematopoietic stem cell (HSC) has been identified and suggested to give rise to this cancer by acquisition of BCR-ABL, a fusion gene that produces a constitutively active protein tyrosine kinase product p210BCR-ABL. Although the initiating event of BCR-ABL expression occurs at the HSC level, inappropriate self-renewal pathway activation in granulocyte-macrophage progenitors represents a critical event in progression to blast crisis. However, the sequence of events required for the evolution of leukemic stem cell responsible for progression to blast crisis has not been firmly elucidated. Blast crisis is the most challenging phase of CML to treat and thus, demands further understanding. Efforts to model mechanisms of disease persistence and progression as well as therapeutic resistance in human cells have been limited by the relative dearth of CML patient blood and bone marrow samples. Previous research suggests that human embryonic stem cells (hESCs) may provide a limitless and consistent source of primitive hematopoietic cells. We have used a mouse aorta gonad mesonephros stromal cell line to differentiate hESCs to hematopoietic precursor cells. In these hESC differentiating cultures we observed that the addition of vascular endothelial growth factor and basic fibroblast growth factor enhance hemogenic endothelial cell generation from the hESC line less prone to hematopoietic differentiation. Furthermore, a transcription factor necessary for hematopoietic specification, c-Myb, is not sufficiently expressed in hESC derived CD34+ cells. With c-Myb over-expression, gene expression in CD34-, CD34+CD31-, and CD34+CD31+ hESC derived populations is altered. Expressing BCR-ABL in hESC derived CD34+ cells was able to confer primary engraftment in highly immunocompromised mice, but secondary engraftment was only achieved with the addition of constitutively active β-

catenin, which is involved in self-renewal. BCR-ABL reduced the self-renewal capacity of cord blood stem and progenitor cells *in vitro*, which suggest the addition of constitutively active β-catenin is required to support the self-renewal of BCR-ABL+ cells. These findings implicate β-catenin plays a role in CML and suggest hESC can be used for the modeling of both CML and hematopoietic development.

Chapter 1. Introduction- Chronic Myeloid Leukemia

Introduction

Chronic Myeloid Leukemia (CML) is a disease characterized by granulocytosis and splenomegaly, which are in increase in granulocytes in the blood and in enlarged spleen. Patients are often asymptomatic at onset, averaging between 45-55 years old[1]. It is the result of a translocation between chromosome 9 and 22 (t(9q34;22q11)), which leads to the chromosomal abnormality named the Philadelphia chromosome[2]. This translocation produces a fusion between the Abelson (ABL) tyrosine kinase gene and the Breakpoint cluster region (BCR) gene on chromosome 9 and 22, respectively[3-5]. The BCR-ABL fusion gene, which encodes for the chimeric protein Bcr-Abl, is a constitutively active protein tyrosine kinase where the Abl kinase activity, that normally localized to the nucleus, is now located throughout the cytoplasm[2,5-9].

CML has three distinct phases: chronic-, accelerated-, and acute-phase. The acute phase is also referred to as blast crisis (BC). Chronic phase (CP) is initiated in a hematopoietic stem cell (HSC) that contains the BCR-ABL fusion gene. These cells exhibit a clonal expansion and give rise to more mature leukemia cell types that constitute the bulk of the cancer cell population called blasts[10]. Due to these acquired properties, like multipotency and self-renewal, the BCR-ABL positive HSC in CP is referred to as the leukemic stem cell (LSC)[11]. In later phases, the LSC takes on a granulocyte macrophage progenitor phenotype (GMP), but maintains these stem cell characteristics[12,13]. In CP, blast cells constitute <10% of the bone marrow. Without treatment CP can persist for years, but inevitably this phase will transition through the accelerated phase to BC[14-17]. The accelerated phase displays one or several

characteristics, usually including an increase in the percentage of blast cells present in the blood and bone marrow (BM) to10-30%. BC is characterized by the progression of the disease into a highly aggressive state where >30% of the blood and BM are blast cells, that sometimes form tumors in the lymph nodes, skin, and bone[13,18].

In patients and mouse models of CP, BCR-ABL expression is necessary for the continued expansion of the leukemic population[7,12,19-21]. Thus, treatment against the tyrosine kinase activity of Bcr-Abl with Imatinib or related tyrosine kinase inhibitors have proven highly effective in controlling the disease in 80-90% of newly diagnosed patients, although not curing it[22,23]. LSCs that produce BCR-ABL transcripts often continue to persist in the BM of patients even after cytogenetic remission has been achieved[24]. In addition, patients that achieve molecular remission, meaning BCR-ABL transcripts are no longer detectable, and discontinue anti-tyrosine kinase treatment often experience relapse[20,24-28]. This suggests these treatments are ineffective in the LSC population. Supporting this, a recent study showed convincing evidence that CP LSC do not depend on BCR-ABL tyrosine kinase activity for survival, suggesting that a BCR-ABL tyrosine kinase-independent pathway may be involved[25]. Furthermore, resistance to Imatinib type inhibitors develops in some patients due to BCR-ABL amplification or mutations, allowing the disease to progress once again. New generations of tyrosine kinase inhibitors have been and will continue to be developed in efforts to combat such occurrences. However, a better understanding of how Bcr-Abl works, and what aspects of the kinase activity are necessary and sufficient to initiate and sustain the disease, will ultimately lead to crucial insight needed in the process of achieving novel treatments. In addition, it is

important to keep in mind that once patients reach BC, current treatments are futile. Therefore, pinpointing the events that are necessary and sufficient for BC transformation are highly important[29-31].

A multitude of genetic mutations as well as additional chromosomal abnormalities have been suggested to be involved in CML progression to BC. However, which mutations are required and sufficient for this progression remain largely unknown. Blast crisis development may involve one or a combination of a number of factors or pathways that regulate them in conjunction with Bcr-Abl expression. This includes duplication of the Philadelphia chromosomes, isochromosome 17(p) which causes disruption of TP53, deletion of the p15/p16 tumor suppressor genes, changes in Stat3, GATA-2, Gli3, WNT, JunB, Fos, PRAME, β-catenin, CyclinD1, Hes1, BCL-2 family members, HoxA10, p21, or Notch signaling, or RuNX1-EV11 fusion [22,32-39]. Due to the ineffective treatment of BC at this point, and the large number of molecular components involved, it is imperative to determine which are causative versus downstream events in order to be able to promote new targets and therapeutic strategies.

CML was the first cancer found to have identifiable LSCs, which are phenotypically different during each phase, starting as an HSC during CP and becoming a GMP when the disease progresses to BC. Where initially the LSCs are HSCs with BCR-ABL as the only known mutation, the LSCs become a granulocyte macrophage progenitor GMP once the disease reaches BC[12,40]. Many additional mutations may lead to this change and probably drive the more malignant phase of CML. Identification and investigation of the LSCs in the heterogeneous population of cancer cells has extended the understanding of molecular mechanisms of this

disease. This has resulted in the possibility to study targeted treatments that may enable us to overcome the challenges related to CML.

Despite extensive research on the molecular mechanisms involved in CML, data on the emergence and progression of disease from untransformed human cells is scarce. The vast majority of research conducted on CML is performed in mice. Mouse HSC expressing Bcr-Abl confer a robust mouse model of CML, though limitations with these models exist. Most importantly, though these mouse models and CML cell lines have been in use for decades, new and effective therapies have not been developed beyond tyrosine kinase inhibitors. As treatment of human CML is ultimately the goal, it is important to study the characteristics of BCR-ABL in human cells and generate human models that allow us to study proteins that are involved in BCR-ABL transformation *in vivo*. New approaches in human cells that will help identifying novel therapies are thus needed.

We have developed a system that enables us to produce human hematopoietic precursor cells that are suitable for *in vivo* experiments on a molecular level. Furthermore, CML related oncogenes can be efficiently introduced in these precursor cells. This is an extremely useful tool in order to investigate and determine what is both sufficient and necessary for the development of CML. We show that producing a large number of hematopoietic precursor cells from human embryonic stem cells that express many hematopoietic markers is possible in culture. The differentiation of hESC to hematopoietic precursor cells has enabled us to identify several growth factors and a transcription factor that can influence the differentiation process, and allow us to use this system for both hematopoietic development and leukemic studies. Additionally, we have found that BCR-ABL alone decreases self-

renewal capacity of hESC derived hematopoietic precursor cells and cord blood HSCs, but β-catenin expression allows for BCR-ABL positive hES derived cells to sustain their self-renewal capacity. Together, these data provide the basis for modeling CML through novel techniques with human embryonic stem cells, which provides a new platform from which to dissect apart the potential molecular targets of disease.

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Chapter 2. Growth Factors and c-Myb's Influence Human Embryonic Stem Cell Differentiation to Hematopoietic Cells

Introduction

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) offer both useful tools to understand disease and development as well as a potential source for cell therapy treatments as they have the capacity to differentiate into virtually any cell lineage and have limitless self-renewal potential. Previous studies have reported hematopoietic lineage development from hESCs and iPSCs with reported *in vitro* colony forming potential and *in vivo* engraftment potential. Hematopoietic stem and progenitor cells readily form myeloid colonies in growth factors containing methylcellulose *in vitro* and will colonize the hematopoietic compartment of an immunocompromised mouse *in vivo*, often referred to as a xenograft when coming from a different species such as a human. This differentiation of hESC to cells capable of these two key functions of hematopoietic stem or progenitor cells was achieved through embryoid body (EB) formation, coculture on stromal cell lines such as OP9 or S17, or defined step-wise differentiation of adherent colonies with the addition of cytokines, growth factors, and/or small molecules[1-8]. However, a robust, reproducible differentiation technique that produces fully functional hematopoietic stem cells (HSCs) able to engraft along all hematopoietic lineages and achieve secondary transplantation has yet to be discovered. Each of these general methods has achieved varying levels of CD34+ cells, a marker found on hematopoietic precursor cells still capable of becoming endothelial or hematopoietic, hematopoietic progenitor cells which are more mature partially differentiated hematopoietic cells, HSCs which are able to differentiate to all types of blood cells, and endothelial stem cells. Although these hESC derived CD34+ cells

have shown *in vitro* functionality based on classic measures such as colony forming assays in methylcellulose, only small fractions of the cells show functionality and some methods appear to reflect embryonic/fetal stage hematopoiesis instead of the sought after definitive or adult stage of hematopoiesis[9].

Using a variety of these methods to produce hematopoietic cells from hESC, several groups have challenged the CD34+ hESC derived cells by hematopoietic reconstitution of immunocompromised mice. In the first report, embryoid body differentiation of the hESCs were used to produce CD45-PECAM-1+Flk-1+VE-cadherin+ cells. Cells displaying these markers show endothelial and hematopoietic potential *in vitro*, and can be used for direct bone marrow injection into sublethally irradiated NOD/SCID mice, which are highly immunocompromised mice having no functional B or T cells. Low levels of myeloid, erythroid, and lymphoid lineages in bone marrow (BM) was observed 8 weeks post transplant[10]. This indicates that these cells are capable of hematopoietic differentiation and engraftment, though at much lower levels than can be achieved with donor HSCs. Another method of differentiation, where hESC were cocultured with S17 stromal cells, resulted in low levels of hematopoietic reconstitution 3-6 months post intra-femoral transplantation. This was shown after harvesting femurs of the injected mice and analyzing them by FACS and PCR. Secondary transplantation was achieved in the irradiated, immunocompromised NOD/SCID mice, shown by PCR. One reason for improved success seen by the low levels of reconstitution in this case was likely due to treatment of the recipients with anti-ASGM1, a component used to deplete the Natural Killer (NK) cells present in NOD/SCID mice[11]. In a study, where cynomolgus monkey ESC lines were cocultured with OP9s before injection into the liver of fetal

monkeys, teratomas were found when the animals had not been depleted of residual SSEA4+ cells. Interestingly, after depletion of SSEA4+ cells prior to injection of OP9-differentiated mESCs, engraftment of hematopoietic progenitors into various tissues were found at birth. No long term or secondary studies were done, but cells harvested from newborn femur, cord blood, and liver were plated into methylcellulose. Colonies with hematopoietic morphology that carried GFP as a marker for donor derivation were observed[12]. hESC/OP9 coculture derived hematopoietic progenitors were further used by two different groups that attempted to achieve engraftment in immunocompromised NOD/SCID/γ_c-/- mice, which not only lack B and T cells, but also do not have functional NK cells. In one case, endothelial lineage cells preferentially engrafted, where progenitor populations were able to achieve both primary and secondary engraftment. Secondary engraftment is a measure of self renewal. Here, human cells were taken from a primary transplanted mouse, injected into a second mouse, and were found to again integrate in the endothelial tissues after neonatal, intrahepatic injection[13]. The second group found that hESC/OP9 cocultures appear to augment the survival and proliferation of EB derived hematopoietic progenitors. However, these progenitors or those derived solely from hESC/OP9 cocultures, failed to engraft after intrahepatic or intrafemoral injection into neonates or adults respectively[14]. Although some of these reports are promising, none achieved greater than 1% white blood cell hematopoietic reconstitution of any hematopoietic organ as seen by FACS, nor did they accomplish even this level of engraftment in secondary transplantations if accomplished at all.

Recently, advancement toward greater hematopoietic engraftment from differentiated hESCs has been reported. The most encouraging results were

garnered after hESC were cocultured on fetal aorta-gonad-mesonephros (AGM), fetal liver cells, or cell lines derived from these tissues. Irradiated, highly immunocompromised NOD/LtSz-Scid IL2Rγ^{null} adult mice lacking B, T, and NK cells were intrafemorally injected with whole cocultures, which included CD34+ cells derived from these cocultures. They showed primary and secondary engraftment in the peripheral blood and bone marrow at 8 and 12 weeks. About half of the recipients had human CD45+ engraftment in bone marrow ranging from 0.22-2.84% and in the peripheral blood ranging from 0.25-16.26%[2]. CD45 is a pan-hematopoietic marker excluding only platelets, red blood cells, and plasma cells. Hence, the levels of HSCs produced from hESC in this report were much lower than what has been shown with other hematopoietic sources such as cord blood or adult bone marrow. In addition, lineage engraftment was not defined and functionality of possible mature hematopoietic lineage cells was not tested by immune challenge. This report suggests that the gap between what has been achieved in reconstitution assays with cord blood (CB) cells versus that with hESC derived cells is lessening However, in CB transplantation experiments a full spectrum of blood cells can engraft to a high degree, mature, and show functional capacity, which are all still distant goals with hESC derived cells. What will be necessary to achieve results comparable with those observed with CB remains a fundamental question in stem cell and regenerative biology.

Reports that show low levels of colony formation and engraftment capacity from hESC derived hematopoietic cells exist, but no highly reproducible or efficient method has been developed. This has been for several reasons. There appears to be both lab variability and hESC line variability. Protocols utilized in one lab fall short

elsewhere based on the lack of publications with similar methods, even when the same hESC lines are being used. Furthermore, it is well established that different hESC lines have different propensities to become various cell lineages[15-19]. Lastly, when stromal cell lines are involved, it is challenging to fully control for the biological variation. Therefore, both the protocol and the hESC lines chosen or available can limit success of producing the desired cell type.

To better understand the differences between hES and iPS derived hematopoietic cells and fully functional HSCs derived from these methods, examining embryonic and fetal hematopoietic development can be useful. Fetal hematopoiesis is a complex process involving multiple sites of origin, maturation, and proliferation, where lineage specification occurs through sequential, defined steps at these locations[18,19]. Initially the embryonic, posterior primitive streak (PS) cells develop into Brachyury+ mesoderm[20,21]. A subset of these early mesoderm cells migrate to the yolk sack where they generate primitive hematopoietic cells. Slightly later, a subset of early mesoderm cells will also become definitive hematopoietic cells[22-24]. The first of these definitive HSCs bud off the ventral wall of the dorsal aorta in the AGM region[25,26]. There is now striking evidence that this occurs through a hemogenic endothelial precursor, after observing HSC emergence through tracing studies in the AGM of both the zebrafish and mouse[27-29]. Therefore, from Brachyury+ cells, CD34+CD31+Flk1+ hemogenic endothelial cells arise, which will eventually generate HSCs that are CD34+CD45^{low}CD90+Lineage-[30-33]. In addition, CD43 has been identified as a marker of hematopoietic precursors cells that appears to be expressed prior to the emergence of CD45 but after the emergence of the hemogenic endothelium[4,8]. Although these markers are pivotal to understanding and isolating the

development of definitive HSCs, the hematopoietic and endothelial genetic regulatory networks of each population have not been well characterized in human cells.

In this study we used the AGM stromal cell line UG26, with demonstrated hematopoietic stem cell generating capacity, to reproducibly generate hES derived CD34+ cells. With this coculture system, we developed a stepwise enhancement of hematopoietic development producing both hemogenic endothelium and CD43+ hematopoietic precursor cells through the addition of growth factors and other signaling molecules. In addition, through gene expression analysis, a critical transcription factor for definitive hematopoiesis, c-Myb, was identified. C-Myb has been shown to be necessary for definitive hematopoiesis and hematopoietic stem cell maintenance in multiple model organisms[34-37]. Total hESC derived CD34+ were severely lacking in c-Myb expression compared to cord blood HSC, whereas endothelial lineage genes were upregulated. C-Myb overexpression in each of these developmental populations, CD34-Flk1-, mesenchymal (CD34+CD31-Flk1-CD43-), and hemogenic endothelial (CD34+CD31+Flk1+/-CD43-) cells, is expected to show an increase in several hematopoietic genes and possibly a decrease in several endothelial genes:

Here we will show, for the first time, how c-Myb influences definitive human hematopoietic development at the genetic level. Using the Fluidigm platform for low cell number, high-through-put, gene expression detection and analysis on populations with and without overexpression of c-Myb, this will be achieved. Furthermore, in depth gene expression profiles of the various populations described above at the time of hematopoietic precursor emergence is crucial in deciphering how these populations are specified and diverge.

Materials and Methods

Construction of lentiviral vectors and lenti virus production. Vector pCDH-EF1-MCS-T2A-copGFP (Systems Biosciences, (SBI), Mountain View, CA) was used as the backbone for c-Myb constitutive expression. To generate vector pCDH-EF1-Myb-T2A-copGFP (pCDH-Myb), FLAG-tagged c-Myb was cloned from pCDNA3-Myb kindly provided by Dr. Scott Ness (University of New Mexico, Albuquerque, NM). Primers for cloning FLAG-tagged c-Myb with Nhe1 and Not1 sites: 3' CCCGGCTAGCGCCACCATGGACTACAAAGAC 5' and 3' ACGCTGGTCATGTGATAGGCGGCCGCACCC 5'. FLAG-tagged c-Myb was amplified using Phusion High-Fidelity DNA Polymerase (Thermoscientific). The product was digested with Nhe1 and Not1 and subcloned into the pCDH vector. Production and titration of lentivirus was performed in 293FT cells (Invitrogen) as previously described[38].

hESC, AM20, and UG26 cell culture. hESC lines Hues9 and HSF6 were cultured as previously described {http://stemcells.nih.gov/research/registry/ucsf.asp}. Briefly, cells were maintained by passage every 3 days with 0.05% Trypsin or collagenase respectively, and were grown on γ-irradiated mouse embryonic fibroblast (MEF) feeder layers in knock-out DMEM with 0.1mM NEAA, 2mM Glutamax, 10% knock-out serum and 10% plasmanate for Hues9 or 20% knock-out serum for HSF6, 0.1mM 2-mercaptoethanol, 1% penicillin and streptomycin, and 10ng/ml for Hues9 or 20ng/ml for HSF6 basic fibroblast growth factor. Hues16 and H1 were maintained on Matrigel with mTeSR1 (Stem Cell Technologies, Vancouver, British Columbia, Canada)

and passaged every 3-4 days by manually scraping away differentiated cells, dispase treating, and then manually dissociating the cells with a pipet tip and cell scraper. Media on all hESC cultures was changed daily.

AM20 and UG26 were grown as previously described[39]. Briefly, cells were maintained sub-confluent at 33°C in 35% alpha-MEM (Gibco), 50% Myelocult M5300 (Stem Cell Technologies), 15% FBS, 1% penicillin and streptomycin, 2mM Glutamax, 0.1mM NEAA, and 10uM beta-mercaptoethanol.

UG26/hESC Differentiation. The AM20 and UG26 coculture with hESC was performed as previously described[2]. Briefly, cells were treated with 10ug/ml mitomycin for 2.5-3 hrs and then replated on gelatin-coated 6 well plates at a density of $3x10^4$ cells/cm^2. 6-24 hrs later hESCs were plated on top at a density of $1x10^4$ cells/cm^2 in knockout DMEM, 20% FBS, 10uM beta-mercaptoethanol, 1 mM L-glutamine, 100 mM NEAA, and 1% penicillin-streptomycin and cultured for up to 21 days. ½ the media was changed every 2-3 days.

For cultures with growth factors, BMP4 and Activin A were added within 24 hrs of plating hESC onto UG26 at 50 ng/ml for 4 days (R&D Systems,). On day 5 fresh media was added with VEGF at 40 or 80 ng/ml (R&D Systems), bFGF at 50 ng/ml (Millipore), Jag1 and/or DLL1 at 250 ng/ml (R&D Systems, Minneapolis, MN). On day 11 20uM TGFβ Inhibitor SB431542 (Sigma, St. Louis, MO) was added to the cultures alongside factors already present since day 5.

Transduction of differentiation cultures. 12 well plates with H1 or Hues16 cocultured on UG26 for 7 days were spin infected with pCDH-EF1-c-Myb-T2A-copGFP virus with 5 ug/ml polybrene for 45 min at 2500 rpm at 27°C. A full media

change was performed and the cells were cultured overnight. Wells were then re-spinifected as described above and cultured to day 15. All spin infected cultures were cultured with BMP4 and Activin A for the first 40hrs and VEGF and FGF day 5-15.

Flow cytometry analysis and sorting. hES/UG26 cocultures were dissociated by 0.25% Tryple for 5 min at 37°C followed by a PBS wash. Then cocultures were further dissociated with accutase for 20 min at 37°C. Single-cell suspensions were obtained trituration with a 200ul pipetman followed by passage through a 100 um cell strainer. Cells were resuspended in differentiation media and stained for 45 min on ice with the following monoclonal conjugated antibodies: anti-human CD31-APC, CD31-PerCP-eFlour780, CD90 PerCP-eFlour710, CD45-eFlour 450 (eBiosciences, San Diego, CA), CD31-V450, CD90-FITC, CD49f-PE, CD34-APC, CD34-PE-Cy7, Flk1-PE, and anti-mouse CD49e-PE (BD Biosciences, San Diego, CA). Cells were then washed with 2.5% FBS in PBS followed by analysis and sorting on a BD FACS Aria II (BD Biosciences). Dead cells were excluded by propidium iodide(PI) staining. For qPCR gene expression analysis GFP+ cells were sorted directly into RLT buffer (Qiagen RNeasy micro kit) and frozen in greater than 3x the sorted volume upon complete lysis by pipetting up and down several times. For Fluidigm gene expression analysis 17 cells of each population from 3 distinct hESC differentiation experiments were sorted into a PCR tube with 9ul of Single Cell Lysis Buffer from the Single Cells-to-Ct Kit (Invitrogen, Carlsbad, CA). These were frozen at -20°C for further processing once all samples had been collected.

Real-time quantitative PCR (QPCR). Total RNA was prepared from cells using RNeasy micro kit (Qiagen, Germantown, MD) and on column DNase treatment was

performed according to the manufacturer's instructions. For cDNA production, RNA was reverse transcribed using Superscript III (Invitrogen) and random primers. Oligo-nucleotide primer sequences were made to amplify as many isoforms as possible producing the same size product and spanning at least one intron when possible to prevent amplification of contaminating DNA. Primers were designed using the Primer3 program and UCSC In-Silico PCR to confirm cDNA specificity. When used on hES/UG26 whole coculture RNA, human specific primers were used and verified on UG26 alone and mouse bone marrow. Primer sequences are shown in Table 2-3. QPCR was performed using FastStart Universal SYBR Green Master Mix (Roche, Indianapolis, IN) and analyzed on an Applied Biosystems 7300 Real Time PCR System using the $\Delta\Delta C_T$ method. Human ribosomal protein L27(RPL27) and glyceraldehye-3-phosphate dehydrogenase(GAPDH) were used as internal controls. All samples were run in triplicate.

Global gene expression by microarray. Two frozen, independent CB donor samples were stained with antibodies against human CD34-APC, CD38-PECy7, CD90-FITC. and Lineage (CD2, 3, 4, 8, 14, 19, 20, 56)-PerCP-Cy5.5 (BD Biosciences). Roughly 4-9,000 CD34+CD90+CD38-Lineage- cells (CB) were sorted directly into RLT from the RNeasy kit (Qiagen). Two independent day 15 Hues16/UG26 differentiated cocultures were harvested and stained as described above. From these cocultures, human CD34+mouseCD49e- cells (Dif) were sorted directly into RLT. Two different cultures of Hues16 were harvested via accutase treatment for 5 minutes and then stained with Tra1-81-AlexaFluor 647, SSEA1-FITC, SSEA4-PE for 30 minutes. From these undifferentiated hESC cultures Tra1-81+SSEA4^{HI}SSEA1- cells (Undif) were sorted directly into RLT. The number of cord

blood HSC sorted on each day of harvest (4-9,000) was then used to sort both Hues16 derived CD34+ and Hues16 hESCs. PI was added to all tubes to exclude dead cells. Test RNA was extracted from the remaining undifferentiated Hues16 and Hues16/UG26 coculture cells. Total RNA was prepared from cells using RNeasy micro kit (Qiagen) and with on column DNase treatment according to the manufacturer's instructions. RNA concentration was measured using NanoDrop (Thermoscientific, Pittsburg, PA). Equivalent amounts of RNA (40ng) were used in two rounds of Arcturus RiboAmp Plus kit 0521 (Molecular Devices, Sunnyvale, CA) for linear T7 RNA polymerase RNA amplification to produce aRNA (amplified RNA). aRNA quality was measured on an Agilent Bioanalyzer.

Illumina bead chip HT-12_v4 whole human genome microarrays were used for whole genome expression profiling. aRNA labeling, hybridization, and scanning were performed by James Sprague, SRA Supervisor at the UCSD BIOGEM genomics facility according to Illumina protocols. The resulting image files and data analysis files will be deposited on the BIOGEM SUN server. BIOGEM slide hybridization services included quantification of the slide images using Illumina's BeadArray software and export of the data for use with appropriate analysis programs (e.g. GeneSpring, GeneSight, GeneLinker Gold, D-Chip, Spotfire etc). Gene ontology and pathway analyses were carried out on the data. Additional bioinformatics analysis was carried out by Roman Sasik, Assistant Project Scientist, Dept. of Medicine, UCSD, in consultation with Gerard Hardiman, Director of the BIOGEM facility. http://microarrays.ucsd.edu/illumina/services.php. Network module analysis was performed by Giovanni Coppola, Assistant Professor, UCLA Dept. of Neurology.

Microarray Data Analysis: 1. Table of distances. Indexes 1 and 2 refer to the two biological (experimental) replicates. Distances were calculated separately for the replicates, because it shows how consistent they are when they are shown graphically. It seems that CB is equally far from both Undif and Dif. It does not mean that Undif and Dif are the same, however, and the distance of Undif and Dif is not small. They are closer to one another than either of them is to CB. One cannot rule out that this is an experimental artifact because CB received "special" treatment, namely, a higher dose of correction during normalization. The only way to be convinced that this is not an artifact is to look at significantly different go terms in this triangle and show whether they make biological sense, which is being done for publication.

Cell types compared	Undif1-CB1	Dif1-CB1	Dif1-Un1	Undif2-CB2	Dif2-CB2	Dif2-Un2
distance	105.0997	108.8346	73.0505	147.0991	129.9636	87.1479

The distance was calculated by making a union of all significantly differentially expressed genes (FDR < 0.01) in the three binary comparisons, Undif/CB, Dif/CB, and Dif/Undif. There were 2505 genes. The distance between cell types is the Euclidean distance in which a cell line is a point in a 2505-dimensional space in which coordinates are log2-transformed expression levels of the 2505 genes.

Microarray Data Analysis: 2. Heat maps of Like and Unlike Genes. The united list of genes that were significant in at least one of the three comparisons was used

and sorted by the "s" statistic. This is the product of log2(CB/Undif) and log2(Dif/Undif). The Undif was chosen as the reference for comparison as the intended goal of the experiment was to take undifferentiated human embryonic stem cells and specifically differentiate them with the goal of making them as much like CB hematopoietic stem cells as possible. We reasoned that it makes sense to look for genes whose log2(Dif/Undif) approximates the desired ratio log2(CB/Undif) - these are the "like" genes - and those whose log2(Dif/Undif) goes contrary to the desired log2(CB/Undif). The "like" genes can be considered a "success", and the "unlike" genes are the "failures" of the UG26 coculture differentiation procedure. The top 50 like genes and top 50 unlike genes were chosen and heat-mapped.

Fluidigm procedure and analysis. The Single Cell-to-Ct Kit protocol(Invitrogen) was used for DNase treatment, RNA isolation, reverse transcription, and 18 cycles of pre-amplification on previously frozen samples of 17 cells in 9ul Single Cell Lysis Solution. Samples were then Exonuclease I treated according to the Single-Cell Gene Expression using SsoFast EvaGreen Supermix “41” protocol (Fluidigm, San Francisco, CA). This protocol was followed from this step onwards. The samples and primers were loaded on the 96.96 Dynamic Array IFC and run on the BioMark HD system(Fluidigm) . A list of samples collected can be found in Table2-1. A list of genes examined and the primers used for each can be found in Table2-2.

Results

Hues16 Produces the Highest Percentage of CD34+ Cells During Coculture with UG26

Three hES cell lines, Hues9, Hues16, and HSF6 were initially tested for their differentiation capacity to the hematopoietic lineage through coculture on UG26.1B6 and AM20.1B4 mouse AGM stromal cell lines. Cells were harvested on days 12, 15, 18, and 21 based on published data of colony forming assays on these days. Single cell suspensions were stained for human CD34, CD43, CD41a, CD45, CD31, and mouse CD49e, an integrin expressed on the mouse AGM cells. Day 15 was found to have the highest percentage of human CD34+ cells in all conditions tested, and generally ~2-8% of bright CD34+ cells also expressed CD31. At most 1% of CD34+ cells were also CD45+. Hues16 plated on UG26 for 15 days produced the highest percentage of CD34+ cells (Fig2-1). HSF6 in each condition showed only a shoulder of CD34 expressing cells above negative controls and hence was not considered to be producing real CD34+ cells. CD34+ cells in a Hues16/UG26 coculture after 15 days of differentiation were found along the edges of colonies and in cell dense zones by immunofluorescence (Fig2-2).

Hematopoietic Gene Expression is Observed in Hues16/UG26 Cocultures

It has been well established that various genes are needed for the specification of the HSC during definitive hematopoiesis, such as GATA1, GATA2, SCL, Runx1, and PU.1. Some of these are also required for HSC maintenance and proliferation. GATA1 is essential for erythropoiesis, and null mice die 10.5-11.5 dpc from severe anemia[40-42]. GATA2 is a critical regulator of specification and

proliferation of the HSC in the AGM as well as modulating survival and proliferation in adult BM HSC[43,44]. SCL -/- mice die at 9.5 dpc and has been shown to be necessary for both primitive and definitive hematopoiesis[45-47]. Runx1 is required for definitive wave hematopoiesis through specification of the HSC, and mice deficient in Runx1 die at 12.5 dpc[48,49]. Although PU.1 is highly expressed in B- and myelomonocytic cells and -/- mice survive, it plays a role in HSC maintenance and is expressed at the early stages of HSC development in the AGM[50,51{Delassus, 1999 #146]. Therefore, gene expression of these genes was evaluated from 0-15 days in Hues16 differentiating on UG26. GATA1, GATA2, SCL, and Runx1 increased over this time, whereas PU.1 expression peaked at day 7(Fig2-3). Overall, this suggests hematopoietic differentiation from hESC is taking place in this coculture system.

c-Myb and Runx1c are Necessary Transcription Factors for Hematopoiesis and are Highly Differentially Expressed in CD34+ Cells Derived from hESC/UG26 Coculture as compared to Cord Blood HSC

hES derived CD34+ cells from multiple differentiation techniques have notoriously failed to mirror HSC functionality as they can not produce robust primary and secondary engraftment in highly immunocompromised mice, the gold standard for HSC identification. Furthermore, at least 50% of CB CD34+ cells will form colonies in methylcellulose, whereas only 0.1-15% of hES derived CD34 are found to form colonies regardless of the differentiation technique[2,4,8,52-55]. This suggests that key features of HSCs are missing from hES derived CD34+ cells. Similar to the published report on hESC differentiation with the AGM cell line UG26, our

Hues16/UG26 coculture differentiated cells expressed all of the hematopoietic genes examined, and renders how these CD34+ cells differ from HSCs unclear[2].

In order to tease out gene expression differences among hESC derived CD34+ cells and HSCs, a microarray was performed using human CD34+, mouseCD49e- cells sorted from two independent Hues16/UG26 cocultures, human CD34+CD90+CD38-Lin- CB HSCs sorted from two donors, and Tra1-81+SSEA4^{HI}SSEA1- Hues16 cells sorted from two independent cultures. The top 50 like genes and top 50 unlike genes were chosen and heat-mapped (Fig2-4; 2-5). CD34 was among the similarly up-regulated genes found by the microarray screen, which works as an internal positive control, as well as HOXA9, HOXB2, and LMO2, which are important for hematopoietic development. Endothelial lipase (LIPG), angiopoietin-like 4 (ANGPTL4), keratin 19 (KRT19), and collagen type IV (COL4A1) were among the identified differentially expressed genes that were up in the hESC derived CD34+ cells compared to CB HSCs. These are genes that are generally expressed in endothelial cells[56-59]. In addition, many genes that were involved in extracellular matrix and cell adhesion were more highly expressed in hESC CD34+ cells. Among the identified differentially expressed genes that were up in CB HSCs and down in hESC derived CD34+ cells, were genes involved in cell adhesion and major histocompatibility class II molecules such as CD74. In addition, c-Myb was found to be highly differentially expressed between Hues16/UG26 coculture derived CD34+ cells and CB HSC. c-Myb is a transcription factor that is indispensible for definitive hematopoiesis as well as HSC maintenance. c-Myb differential expression was confirmed by Q-PCR (Fig2-6).

Several other important hematopoietic genes described earlier were also compared by Q-PCR to in hESC derived CD34+cells and highly purified HSC. SCL was examined and found to be present only when CD34+CD31+ cells were found in the total CD34+ population. Although Runx1 was not identified by the microarray, several Runx1 isoforms have been reported to be differentially expressed during definitive hematopoiesis and differential isoform expression is not examined via microarray. Runx1b is the most abundant isoform containing 5 unique n-terminal amino acids. It is turned on by the more basic, proximal P2 promoter, which has been reported to be active in hemogenic endothelial cells that become HSCs and critical for fetal liver hematopoiesis in mice[60-62]. During definitive hematopoietic specification it appears to be expressed broadly in both mouse and human hematopoietic development[63,64]. Runx1c is transcribed from the distal, vertebrate specific P1 promoter which is particularly active in enriched fetal liver HSCs[62,65-69]. In addition, it was shown to be expressed solely during hematopoietic specification in hESC EB differentiations and in the AGM from embryonic day 10.5-11.5 of mouse embryos, suggesting it is the critical Runx1 isoform for HSC emergence[63,64]. In our hESC derived CD34+ cells Runx1b was found to be present at low levels in all hESC derived CD34+ populations examined, but Runx1c was undetectable (Fig2-7). The lack of two major transcription factors, c-Myb and Runx1c, in our hESC derived CD34+ cells confirms major differences between these cells and CB HSC and may lend to the differences in their functionality.

VEGF and FGF enhance CD34+CD31+Flk1+ cell development from hESC on UG26

The hESC derived CD34+ population is most likely heterogeneous and therefore diluting the gene expression results from those cells that are directed toward hematopoietic cells. However, we can now include additional surface markers on precursors of hematopoietic cells that were unknown or not well characterized previously. CD31 and Flk1 have been identified as important surface markers of CD34+ hemogenic endothelium. As expression of SCL was only observed in CD34+ populations where CD31 was expressed on a percentage of these cells, enhancing the CD31+CD34+ population is important for efficient hematopoietic development. Furthermore, CD43 has been shown to be a critical marker of developing hematopoietic cells differentiated from hESC and is found on fetal and adult HSCs. CD43 had not been observed in Hues16/UG26 CD34+ cells and thus finding growth factors that may lead to its expression was sought. In addition, the H1 hESC line was differentiated alongside Hues16 due to its well published propensity to differentiate toward the hematopoietic lineage[3,4,7,9,54,70-82].

During embryonic development several growth factors important for mesoderm, arterial, hemogenic endothelial, and hematopoietic specification have been identified. Bone morphogenetic protein (BMP4) is a transforming growth factor-beta (TGFβ) superfamily member that has been shown to be required for mesoderm formation in mouse genetic studies and to enhance mesoderm formation and hemogenic potential in ES differentiation[1,83-90]. Another member of this superfamily, Activin A (AA), has been suggested to encourage mesoderm and/or hemogenic endothelium in various vertebrate model systems[8,89-94]. In order to enhance the number of cells that commit to mesoderm and in turn, or in addition, the number of cells that become hemogenic endothelium, combinations of BMP4 and AA were

tested during days 0-5 of Hues16 differentiation. Great intra and inter-experimental variability was observed, but overall BMP4 alone for 4 days greatly reduced CD34+ cell percentages (Fig 2-9). This is not surprising as BMP4 has been shown to enhance trophectoderm differentiation with long-term exposure, whereas 24hr treatment enhances mesoderm formation[89,95,96]. In addition, AA alone or in conjunction with BMP4 did not consistently show improvement in hemogenic endothelium formation at day 15, though in some differentiating wells together they greatly enhanced the CD34+CD31+Flk1+ population above baseline (data not shown and Fig2-9).

Vascular endothelial growth factor (VEGF) signaling through its receptor Flk1 is required for endothelial and hematopoietic differentiation from the mesoderm[8,90,94,97-101]. Basic fibroblast growth factor (FGF) has been shown to have a positive, but not required, influence on hemogenic endothelium development[8,90,94,102,103]. Thus, VEGF in combination with FGF has been shown to produce similar levels of hemogenic endothelium as that of high levels of VEGF[8]. Transforming growth factor-beta(TGFβ) has been shown to play a role in CD43+ hematopoietic cell development from hESC, though opposing results have been reported possibly due to culture system differences[2,8]. When compared in UG26/hES differentiation cocultures, 40ng/ml of VEGF + 50ng/ml of FGF consistently enhanced the CD34+CD31+ population produced from Hues16. None of the other conditions tested provided this support (Fig2-8A). However, this was not dependably observed from H1 differentiated cells, possibly due to the variability within cultures or due to line specific differences (Fig2-8B). H1 notoriously differentiates well to blood precursors. Therefore, this hESC line may not benefit from outside signaling factors

as much as lines that differentiate less efficiently. Furthermore, TGFβ inhibitor (TGFβI) did not show reliable enhancement of CD43+ cells from H1 or Hues16, though variability was high (data not shown and Fig2-8A, B). Overall, hESC plating onto UG26 tends to be variable. If this variability could be eliminated yet cells could still be plated in small colonies, more consistent data on signaling factors may be gleaned from this system.

Due to the observation that the CD31+ population within the CD34+ population showed enhanced SCL expression, several additional hematopoietic genes were examined. CD34+CD31- and CD34+CD31+ cells were sorted from H1 and Hues16/UG26 15 day old cocultures. Indeed, CD34+CD31+ cells from both Hues16 and H1 show an increase in important hematopoietic transcription factors Runx1b, c-Myb, and GATA2 compared to the CD34+CD31- population (Fig2-9A). Furthermore, the CD34+CD31+ also show an increase in Flk1 expression, confirming that they are closer to hemogenic endothelial cells than their CD31- counterparts (Fig2-9B).

Definitive hematopoiesis cannot occur in the AGM without Notch1 signaling to turn on Runx1 through the Notch intracellular – RBPjκ complex[104-107]. Delta like-1 (DLL1) is one of 5 mammalian Notch ligands and is required for arterial specification during vasculogenesis, which must occur prior to hematopoietic specification in the AGM[108]. In addition, it is expressed in the subaortic patches of the para-aortic splanchnopleure/AGM region suggesting its role in hematopoiesis[109]. Jagged-1 (Jag1) is another mammalian Notch ligand and is expressed in the endothelium of the AGM[109]. In its absence, AGM hematopoiesis is impaired yet arterial development remains unchanged suggesting it may be the key Notch ligand for specification[110]. As

Runx1 is crucial for HSC specification in the AGM and the two isoforms examined were either under-expressed or undetectable in hESC derived CD34+ even after VEGF and FGF treatment of cocultures, DLL1 and Jag1 were added to one set of differentiation cultures. Only slight differences were observed in CD34+CD31+ percentages and CD43 was not induced or enhanced (data not shown). CD34+CD31- and CD34+CD31+Flk1+ cells were sorted for gene expression comparison of Runx1a, Runx1b, and Runx1c. In Hues16 differentiated cells Runx1b gene expression was induced 5-12 fold in CD34+CD31-Flk1- cells in all conditions tested. This includes the addition of the TGFβ inhibitor where no Notch1 ligand was added, which may be due to high well-to-well variability or possibly induction of Runx1b expression after TGFβ inhibition(Fig2-10A). Furthermore, Runx1b went up 2 fold in CD34+CD31+Flk1+ cells when DLL1 was added alone or in conjunction with Jag1 (Fig2-10A). In H1 differentiations Jag1 alone induced 3 and 2 fold higher expression levels of Runx1c in CD31-Flk1- and CD31+Flk1+ populations respectively, but expression level differences were minor and therefore probably due to well to well variation rather than effects from the Notch1 ligands(Fig2-10B). These results were not encouraging enough to pursue Runx1 induction from either of these Notch1 ligands. By extrapolating data from two different experiments, the levels of each isoform still falls at least 8 fold below that of HSCs tested side by side with Hues16 derived CD34+ cells(Fig2-7 and Fig2-9). Furthermore, the results were highly variable between the two hESC lines tested suggesting the effects seen were not strictly due to the addition of these ligands, but a result of the cultures they were added to. Therefore finding the proper conditions to turn on Runx1, especially

Runx1c, in the desired CD34+CD31+Flk1+ population of hESC cocultured on UG26 appears to be more complex and was not further pursued.

Overexpression of c-Myb may alter hematopoietic and endothelial gene expression in different hESC derived CD34+ and CD34- populations.

We have found that CD34+ cells from hESC differentiations express low levels of c-Myb, an important hematopoietic transcription factor that is needed for definitive hematopoietic development. VEGF and FGF enhanced hemogenic endothelial cells, CD34+CD31+Flk1+, within this CD34+ population from Hues16 which appears to lead to induction or enhancement of c-Myb expression (Fig2-9A). Therefore, this hESC/UG26 coculture system offers a unique opportunity to study how over-expression of c-Myb can influence gene expression in different hematopoietic developmentally relevant populations. Days 7 and 8 hESC/UG26 differentiation cultures were transduced with c-Myb and GFP expressing lentivirus. On day 15, GFP+ and GFP- cells of each of the following populations were sorted for gene expression profiling of 96 genes: humanCD34-mouseCD49e-, humanCD34+CD31-, humanCD34+CD31+Flk1+/- (Fig2-12). Transduction efficiencies of whole cultures were very low with a bias against CD34+CD31+ cells(data not shown). Thus, gene expression analysis was carried out on only 17 cells of each population of interest. The CD34+CD31+ cells from both hESC lines were generally $Flk1^{dim/+}$, but due to the low efficiency of transduction, Flk1 was not included in the GFP+ cell sort (Fig2-11). However, CD34+CD31+Flk1+GFP- and CD34+CD31+Flk1-GFP- cells were collected for comparison. In addition, H1

consistently produced CD34+CD31+CD43+ cells whereas Hues16 only occasionally did (Fig2-11). This population from H1 was therefore also sorted to understand how CD43 further distinguishes the CD34+CD31+ cells. For a genetic expression comparison to endothelial stem and more mature cells, 17 cells each of CD34+CD31+ and CD34-CD34+ primary human umbilical vein endothelial cells (HUVEC) and primary human aortic endothelial cells (HAEC) were also sorted. Furthermore, three sets of 17 CD34+CD90+CD38-Lineage- CB HSCs were sorted from a mixed donor (3-5 donors) CB sample to compare the genetic profiles of these hESC derived populations to that of a long-term HSCs. For a baseline cell type to compare to markers of stemness, SSEA4^{Hi}Tra1-81+, were used to sort 17 H1 and Hues16 cells each(Table2-1).

The results of this gene expression analysis will not only show us what c-Myb regulates either directly or indirectly in hematopoietic and endothelial precursor populations, but will also provide a genetic snapshot of the genes examined in each population sorted in the absence of c-Myb overexpression. The majority of genes chosen are expressed in mature and developing hematopoietic and endothelial populations (Table2-2). Currently the gene expression analysis using Fluidigm's dynamic 96.96 array chip is in progress and data will be available shortly.

Discussion

In this study we show that although efficient hematopoietic differentiation to a mature hematopoietic stem cell population remains unsuccessful, hESC cocultured

on the AGM stromal cell line, UG26, can be used to probe distinguishing genes and factors involved in hematopoietic development. Here, gene expression profiles of specific early human hematopoietic precursor cells will be well characterized. Although data on only a handful of genes are presented here currently, data is forthcoming comparing 96 hematopoietic and endothelial developmental, stem, and maintenance genes. This data will cover 4 different populations of hESC derived cells from H1 and Hues16/UG26 cocultures sorted from 3 independent experiments: CD34-, CD34+CD31-CD43-, CD34+CD31+CD43-Flk1+, and CD34+CD31+CD43+Flk1+/-. This data will provide a full spectrum of the gene expression differences between cells that are supportive or capable of hematopoietic precursor development. Furthermore, the identification that c-Myb expression is lacking in the general Hues16 derived CD34+ cell population led to the use of this system to study how c-Myb regulates other important hematopoietic genes needed for the specification, maturation, and maintenance of HSCs. This data is expected to shed novel insight into the c-Myb regulatory network of developing HSCs.

C-Myb is an important hematopoietic transcription factor expressed during hematopoietic specification and necessary for HSC maintenance[34-37]. However, most of the studies on what genes it regulates have been performed in fibroblasts, epithelial cells, cancer cell lines, and mature blood cells. Through these studies over 80 targets have been identified[111-118]. In addition, there have been just 2 reports specifically looking at how c-Myb regulates hematopoietic specification using mouse embryonic stem cells (mESCs), but only a handful of well known hematopoietic genes were evaluated between the 2 studies: GATA1, GATA2, SCL, LMO2, HOXB4, MLL, Etv6/Tel, BCL2, and Runx1[119,120]. Therefore, defining the genetic pathways regulated

by c-Myb expression at the time of hematopoietic specification remains a fundamental goal of developmental and stem cell biology. In addition, hematopoietic development is known to vary between mouse and human, such as the differences in the phenotypic markers used to identify a mouse HSC versus a human HSC. Therefore, performing these studies in human cells will provide a better understanding of human hematopoietic specification, which is imperative to achieving potential hematopoietic cell therapies and disease modeling with hESCs.

Previously, several similar gene expression studies have been carried out on various hematopoietic and endothelial populations derived from hESCs. During embryoid body (EB) differentiation, *Flk-1, VE-Cadherin, RUNX1/AML1, GATA-2, GATA-4, TAL1/SCL, Smad-3, Flt1, NF-E2, β-catenin, HoxB4*, and *Lmo-2* were found to emerge in whole EBs on day 6, which corresponded to the emergence of hemogenic endothelial phenotypic cells (CD34+CD31+VE-Cadherin+Flk1+). These genes were maintained out to day 16 when CD45+ cells began to arise suggesting they are not responsible for definitive hematopoietic stem cell specification. A specific group of genes was identified at days 12-14 of differentiation that corresponds with a peak in colony forming capacity of EB cells. Many of these genes have been shown previously to be involved in hematopoietic self-renewal i.e PIK3R1, RGS18, ABCB1/MDR-1, IRS1, SENP6/SUMO-1, Wnt5a, etc[121]. This suggests self-renewal genes may be responsible for hematopoietic specification in the right context of hematopoietic transcription factor expression, though specific phenotypic populations derived from EB differentiations were not analyzed. Therefore, these genes may be involved in the emergence of other populations of cells within the pool examined. Another group compared fetal blood (FL) and hESC EB derived

CD34+CD38-CD45+ cells by microarray where only 2.4% of genes differentially expressed from undifferentiated hESC were common between these populations. This suggests the hESC derived CD34+CD38-CD45+ have a very different transcriptome profile than FL population. Particularly, key members of the Notch and Wnt signaling pathways necessary for self-renewal were down-regulated in the hESC derived cells by comparison[122]. However, this profile for FB HSCs is not highly stringent. Thus, this analysis included more mature cell types without using CD90, CD49f, or CD49RA to identify the true long-term, multipotent HSCs[123,124,125]. This may slightly explain the lack of overlap in their gene expression profiles as the hESC derived population is possibly more immature and the FL population more mature overall. However, similar to the first study, this group also found genes involved in self-renewal to be under-expressed in hESC derived hematopoietic stem-cell like populations suggesting this may be a common theme among these hESC derived cells. When gene expression libraries of lineage-CD34+CD43+CD45+ and lineage-CD34+CD43+CD45- populations derived from hESC differentiated on the stromal cell line, OP9, were compared, myeloid differentiation, cell cycle, and biosynthetic pathway genes were differentially expressed. Furthermore, when compared to human fetal liver (FL) and CB CD34+CD38-lineage- cells the FL libraries were more closely related to the hESC derived Lineage-CD34+CD43+CD45- derived libraries than those from CB. Again, this may be due to lack of more stringent HSC markers for the FL and CB samples as more mature blood cells will be included in the CB sample and possibly endothelial cells will be included in the fetal liver sample[31]. This may also suggest this group is fairly close to achieving FL-like HSCs from hESC through OP9 cocultures. However, hESC/OP9 cocultures have been reported to be

much less successful by other groups in a few studies, and does not produce functional HSCs that can engraft in an animal, where the FL HSCs do[14,126-128]. Furthermore, the lack of more reports from other investigators using this method, though the first publication came out 6 years ago and the same group has put out several more successful studies using this method, suggests other have also failed to replicate their findings[4,7,75-77,81]. This lack of reproducibility suggests this is not a reliable method to obtain HSC-like cells and findings from this group are difficult to apply to the field as a whole. Another study found high levels of ID protein expression to potentially cause the inability of hESC derived CD34+ cells to produce B cells, as a true functional HSC should be capable of differentiating to all blood cell lineages[128]. High levels of ID genes may therefore play a role in overall HSC specification. However, the hESC CD34+ cells were compared to total CD34+ cells from CB, thus including a largely heterogeneous population of different types of hematopoietic progenitors cells with variable differentiation capacities and only a small percentage of HSCs[123]. Hence, this result is difficult to interpret with respect to an HSC. Another study analyzing only 4 genes in different hESC derived populations, reported CD34+CD31+Flk1+ cells were found to express c-Myb, SCL, GATA2, and LMO2, where CD34+CD31-Flk1- were lacking in c-Myb and CD34-CD31-FLk1- lacked both c-Myb and SCL[55]. This data supports our findings thus far, though a more in dept analysis of these populations is needed to further identify genes that may be involved in HSC specification. Lastly, the B-cell development transcription factor LNK, was identified as differentially expressed between hESC derived CD34+ and several subsets of CB CD34+ cells, where it was found to be expressed even prior to the establishment of hematopoietic cells from differentiating

hESCs. The knockdown of LNK increased the output of CD34+CD45+ from hESC by 4 fold, though functionality of the cells was not tested[129]. Identifying genes such as these that may release a block in generating fully functional HSCs from hESC is our goal through these gene expression experiments. Although all of these reports have made contributions to the field, they have not identified factors that can yet account for the functional differences between hESC derived CD34+ cells and HSCs.

Upon expression of c-Myb in the Hues16 derived CD34+CD31- cell population, which was found to lack Myb expression entirely, it is expected that several known or proposed c-Myb target genes will be activated. These genes may be turned on directly or indirectly as the genetic expression analysis was performed on differentiating cells 8 days after they were transduced. LMO2 is required for the definitive wave of hematopoiesis and is regulated by Myb during erythropoiesis[111]. Furthermore, LMO2 alongside GATA1, SCL, HOXB4, MLL, Etv6/Tel, BCL2, and Runx1, all important genes for hematopoietic development, were found to be upregulated upon c-Myb expression in mESC derived endothelial cells. Thus, several or all of these genes may be enhanced in the hESC/UG26 derived CD34+CD31- population as well as the other populations examined when c-Myb is expressed. BMI-1, which is involved in hematopoietic stem cell maintenance and proliferation, was found to be a c-Myb target in p190 BCR-ABL dependent B-cell leukemia in mice[130]. Thus, it may be upregulated after Myb expression in the hESC derived cell populations analyzed as well. Furthermore, expression of BCL2, GATA3, c-Kit, c-Myc, GATA1, Fli1, Meis1, PBX1, TGFβ1, CD31, and CD34, all of which are expressed in HSCs, may also be enhanced as their levels have become elevated after c-Myb expression in a variety of systems[111-114,116,131-137]. Finally, CCNA1(cyclin

A1), which is involved in the cell cycle, and CTNNAL1 (alpha-catulin), which may be a fetal HSC marker, have been identified in several screens in multiple cell types for c-Myb regulators[111,113,114]. Therefore, their expression will likely be induced or enhanced in hES/UG26 derived Myb expressing cells. Beyond these reports however, little is known about the regulatory networks c-Myb controls to promote HSC specification. Analyzing the different hematopoietic and endothelial related populations developing from differentiating hESCs with and without Myb expression, will contribute to this understanding and possibly identify novel targets.

Although the addition of BMP4, Activin A, FGF, VEGF, and TGFβI to hESC/UG26 cocultures showed great variability, the results were only based on 2-3 independent experiments with at least 4 wells total being represented. hESC plating between wells was noticeably different in several cases and may contribute to this variability. However, the addition of FGF and VEGF showed reproducible enhancement of the CD34+CD31+Flk1+ population developed from Hues16. VEGF is known to be critical for hemogenic endothelial development and FGF has been shown to enhance this, though is not required. Therefore, this result is not surprising. As expected based on the number of hematopoietic publications using the hESC cell line H1, this line differentiated more efficiently to the hemogenic endothelial state in our experiments without the need for additional factors[3,4,7,9,54,70-82]. Thus, there are inherent differences between these two hESC lines that lead them to be more or less prone to hematopoietic development. This is further supported from the initial differentiation experiments presented here comparing HSF6, Hues9 and Hues16. Furthermore, differences among hESC lines have been reported regularly in the literature[15-19]. Thus, the frequent use of H1 for hematopoietic differentiation

experiments and the differentiation differences shown here between all of the lines tested for CD34+ cell development, suggest that creating a lineage specific differentiation protocol applicable to all hESC lines may not be possible. Instead, a line or multi-line specific protocol is much more likely to be replicable between labs. This is an extremely important observation when currently many different protocols for making hematopoietic precursor cells have been reported, yet a consistent protocol has not been adopted in the field nor even among several labs. Furthermore, no method using any hESC line has shown efficient development and isolation of HSCs, signifying that a major piece to the puzzle of hematopoietic development is still yet to be discovered.

Chapter 2 is currently being prepared for submission for publication. Rusert, Jessica M; Court-Recart, Angela; Plaisted, Warren; Jamieson, Christina AM; Jamieson, Catriona HM; Goldstein, Lawrence SB. “c-Myb Influences Hematopoietic Gene Expression During Hematopoietic Differentiation of Human Embryonic Stem Cells.” I am the primary author and researcher.

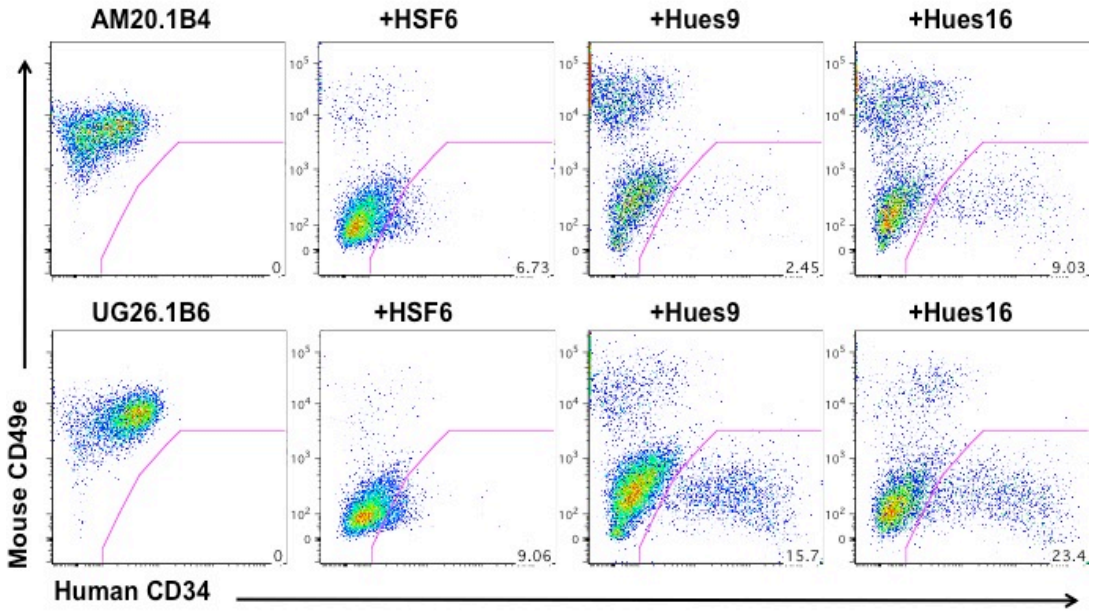


Figure 2-1. 3 hESC lines differentiated on AGM stromal cell lines for 15 days. HSF6, Hues9, and Hues16 hES cell lines were cocultured with AM20.1B4 or UG26.1B6 for 15 days, which was found to be the peak of CD34 expression. Mouse CD49e is an integrin expresed on the mouse stromal cell lines and CD34 is a characteristic marker of HSC, though is thought to be expressed before HSC specification.

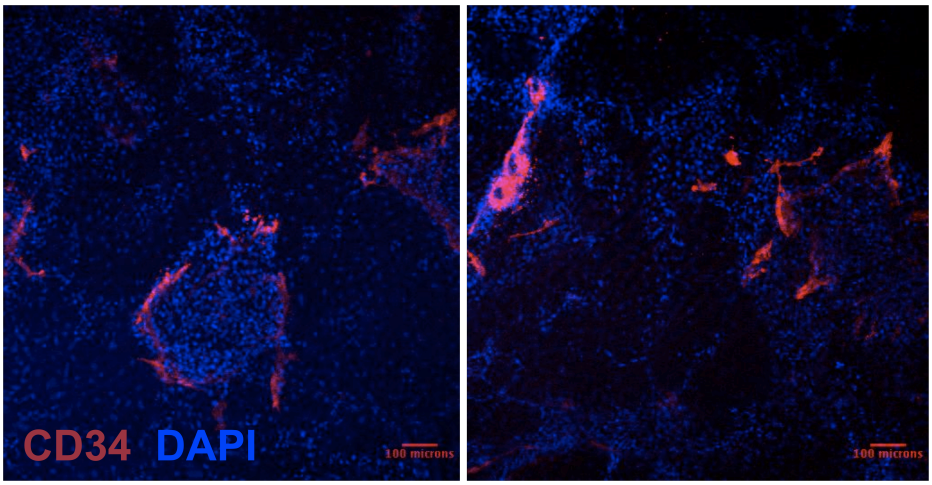


Figure 2-2. Immunofluorescence of Hues16 15 days after plating on the AGM cell line UG26. Human CD34 expression in red is seen around the edges of colonies and in cell dense areas. Dapi, which stains nuclei, is shown in blue.

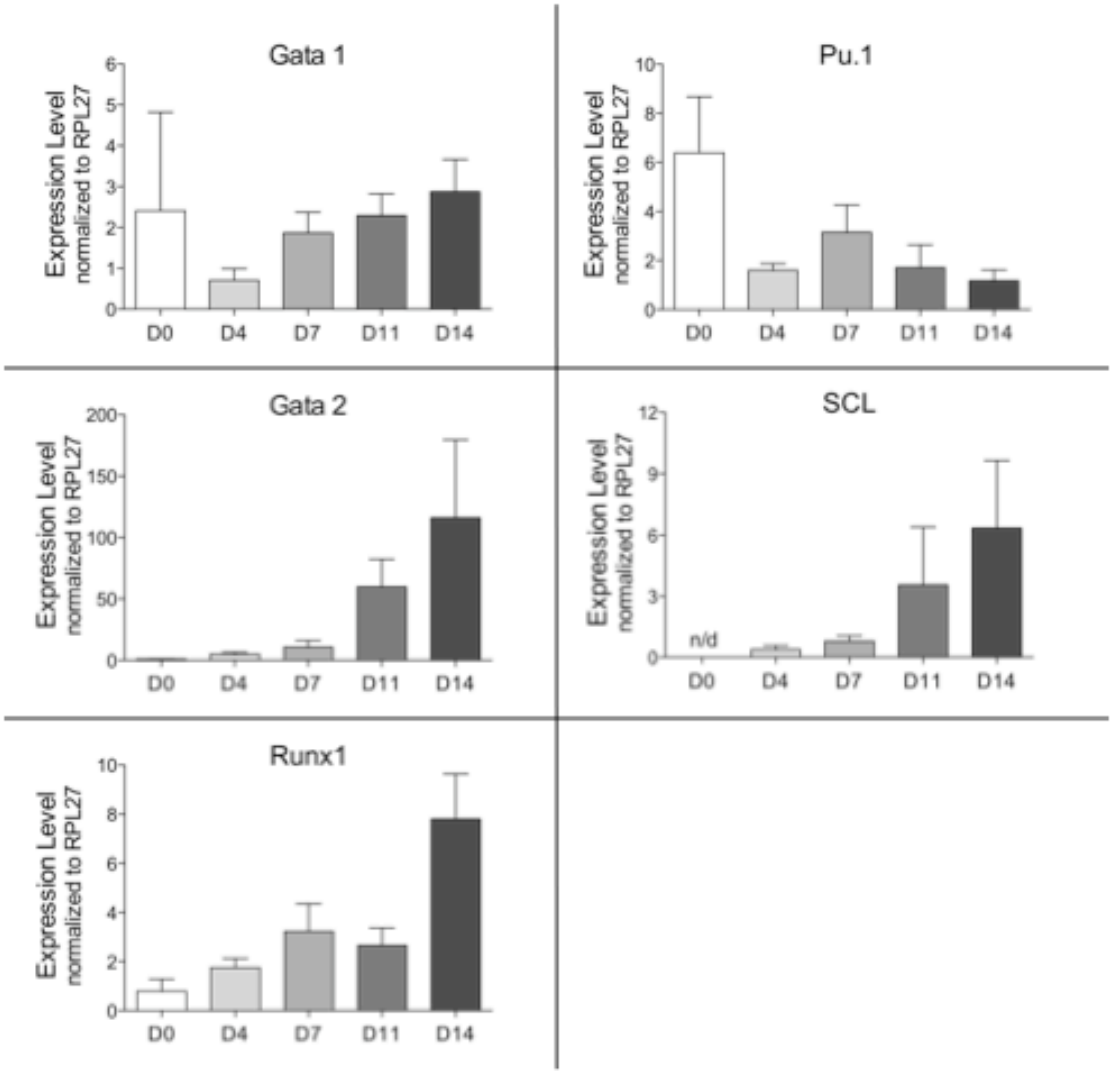


Figure 2-3. **Expression profiles of Hues16/UG26 cocultures over 14 days**. Important transcription factors generally increase over a 14 day period of Hues16 differentiation on the stromal cell line UG26. High levels of GATA1 and PU.1 were observed in 1 sample of Hues16 at day 0 which may indicate the culture contained differentiation while still on MEFs (n=3).

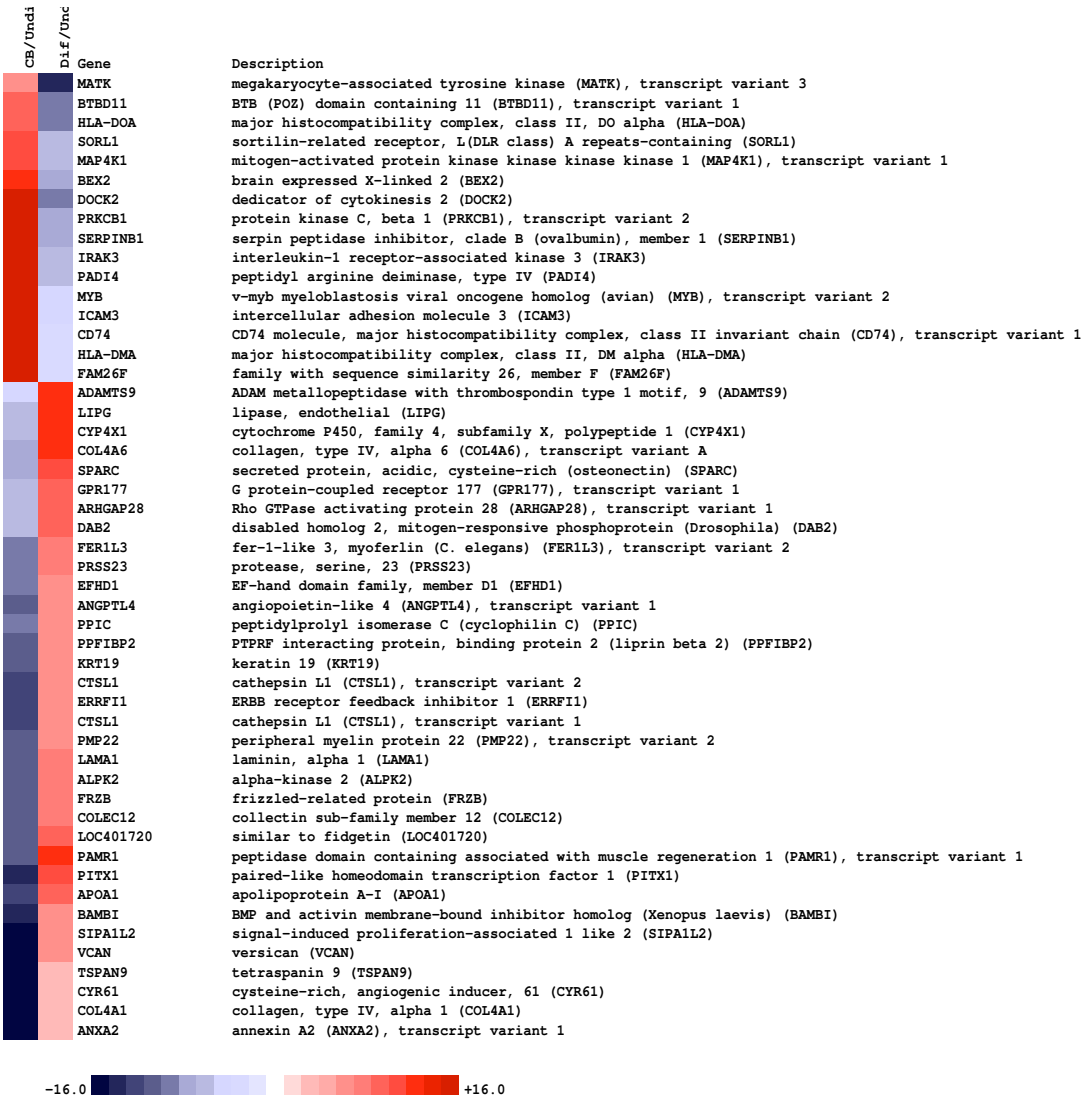


Figure 2-4. Genes found to be differentially expressed by microarray analysis of Hues16 derived CD34+ cells and CB HSC compared to undifferentiated Hues16.

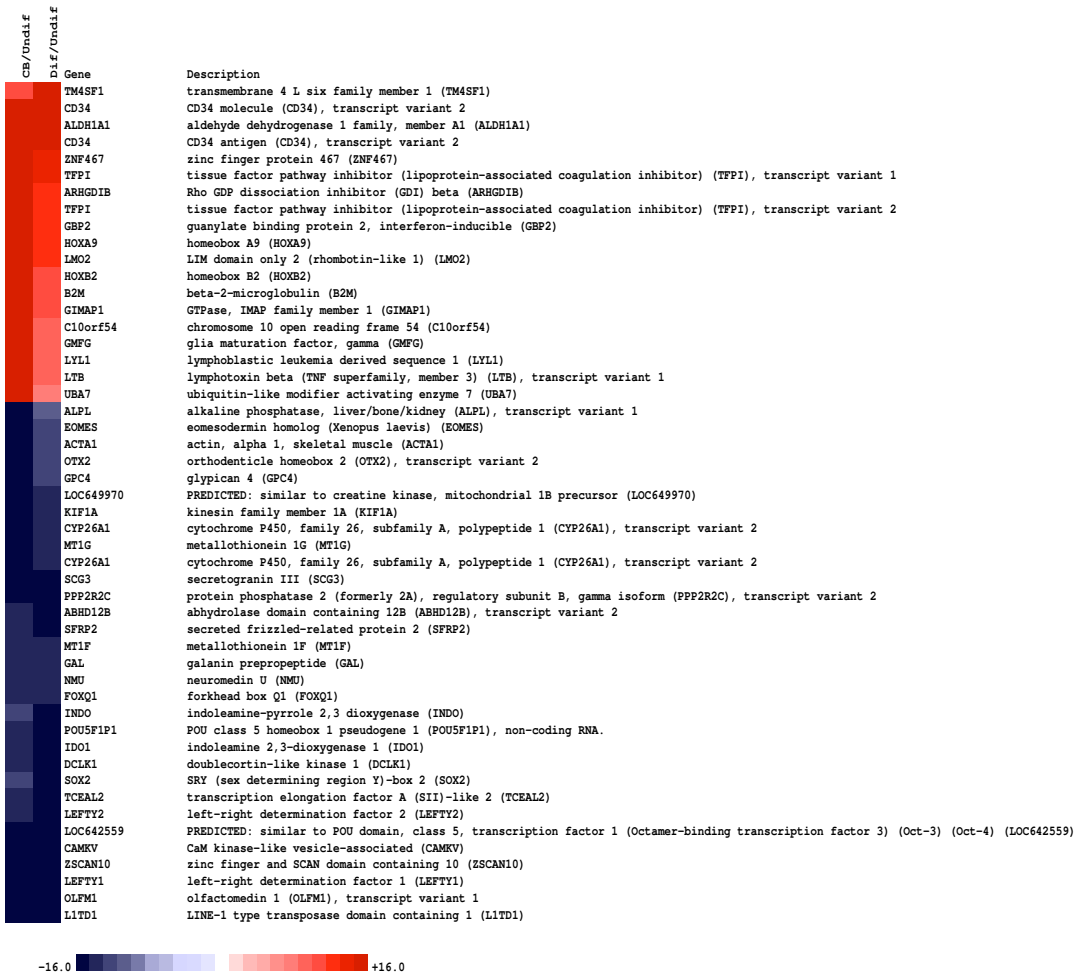


Figure 2-5. Genes found to be similarly expressed by microarray analysis of Hues16 derived CD34+ cells and CB HSC compared to undifferentiated Hues16.

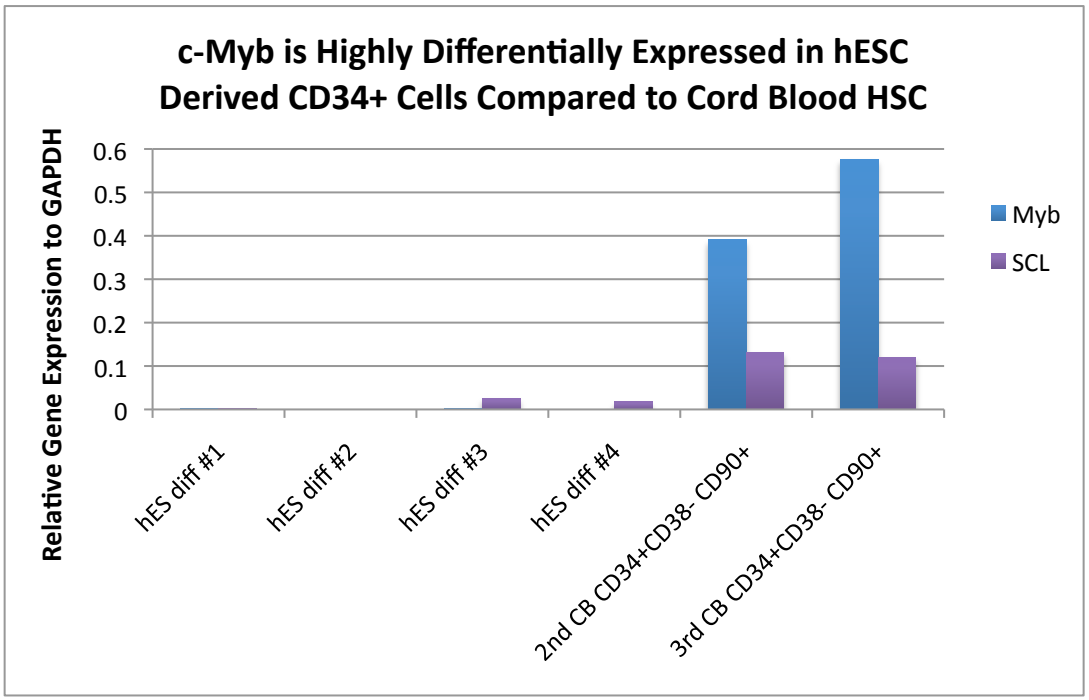


Figure 2-6. **QPCR examination of c-Myb and SCL in Hues16/UG26 derived CD34+ cells and CB HSC**. 4 different sets of Hues16/UG26 coculture differentiations showed extremely low levels of c-Myb compared to 2 individual donors of CB HSCs (CD34+CD38-CD90+Lineage-). Hues16 differentiation sets #3 and 4 show an increase in SCL expression, which was found to correspond to elevated levels of CD31 surface marker expression within the CD34+ population. C-Myb, however, appeared not to change with the increase in CD31 expression.

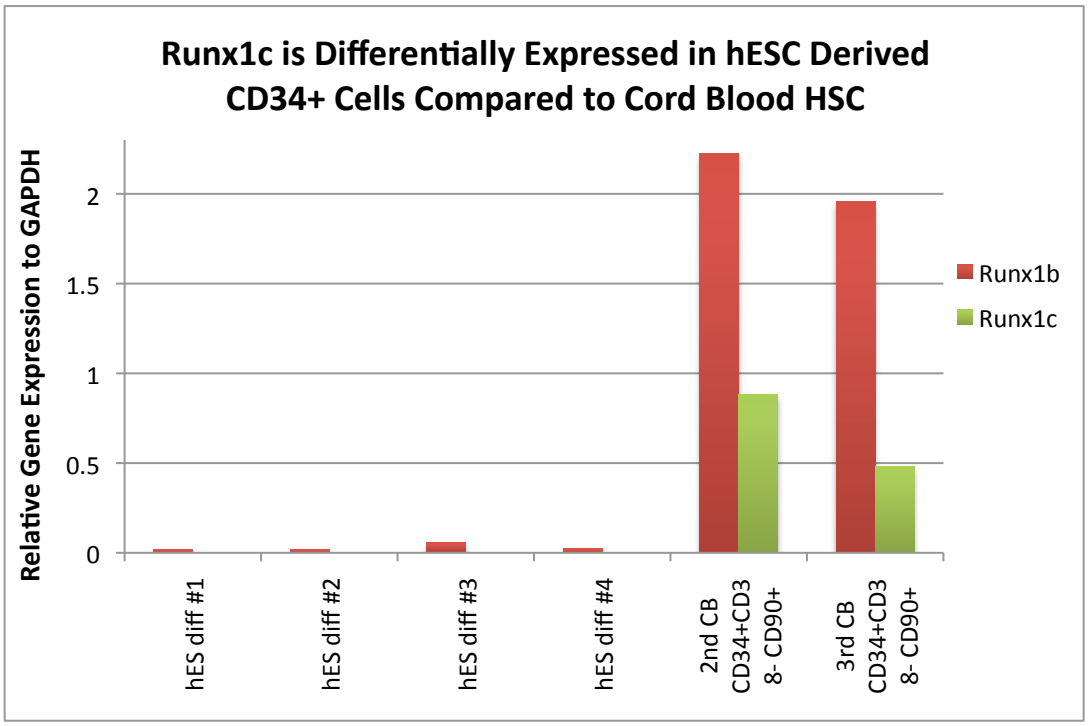


Figure 2-7. **QPCR examination of two important Runx1 isoforms in Hues16/UG26 derived CD34+ cells and CB HSC**. The Runx1b isoform show extremely low levels of expression in Hues16 derived CD34+ cells compared to 2 donors of CB HSCs (CD34+CD38-CD90+Lineage-), however is detectable in all populations of CD34+ cells examined from 4 different sets of differentiation. The Runx1c isoform was practically undetectable in all Hues16 derived CD34+ samples where it was readily detectable from CB HSCs.

A)

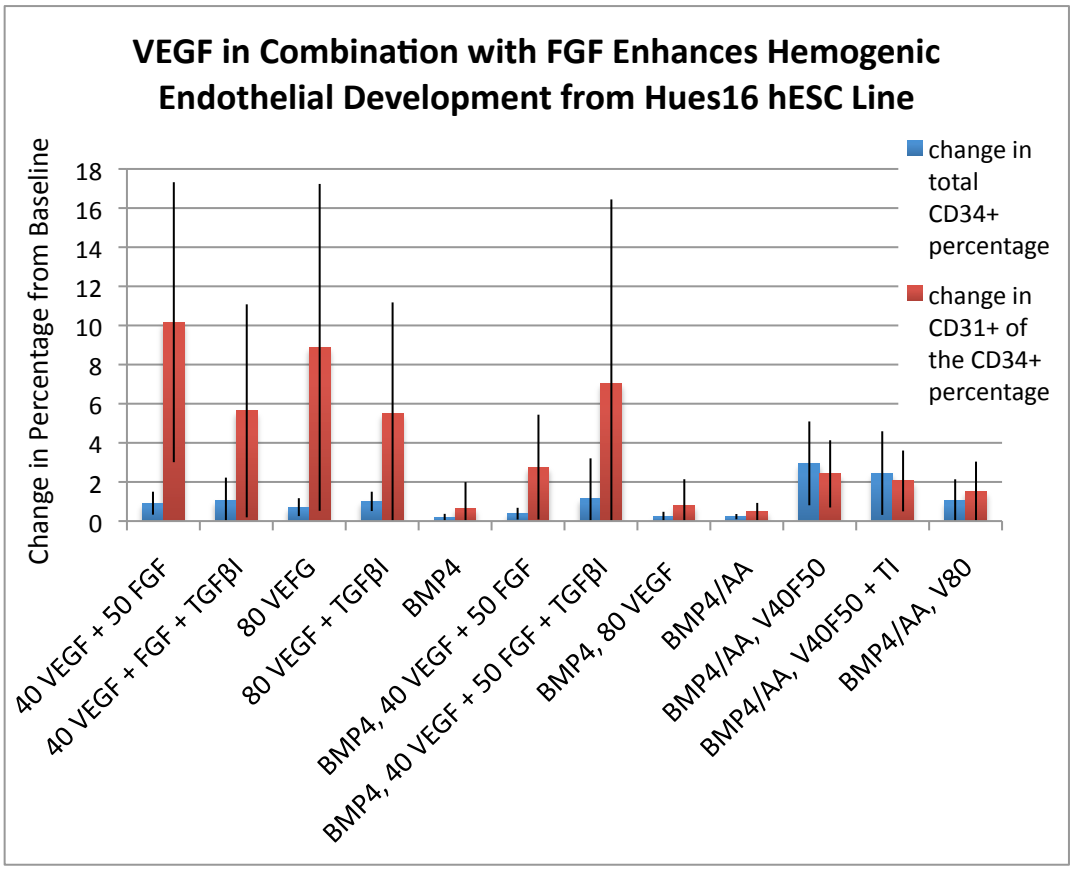


Figure 2-8. **VEGF only encourages hemogenic endothelium differentiation from Hues16, not H1. A)** VEGF with FGF consistently enhances CD34+CD31+ differentiation from Hues16 by at least 3 fold. No other condition tested achieved this (n=4-7wells from 2-3 sets of differentiation) **B)** Neither VEGF alone nor in conjunction with FGF or TGFβ inhibitor enhanced CD34+CD31+ differentiation from H1, though VEGF slightly increased the total CD34+ population when added with FGF and decreased the CD34+ population when the concentration was doubled but no FGF was added.

B)

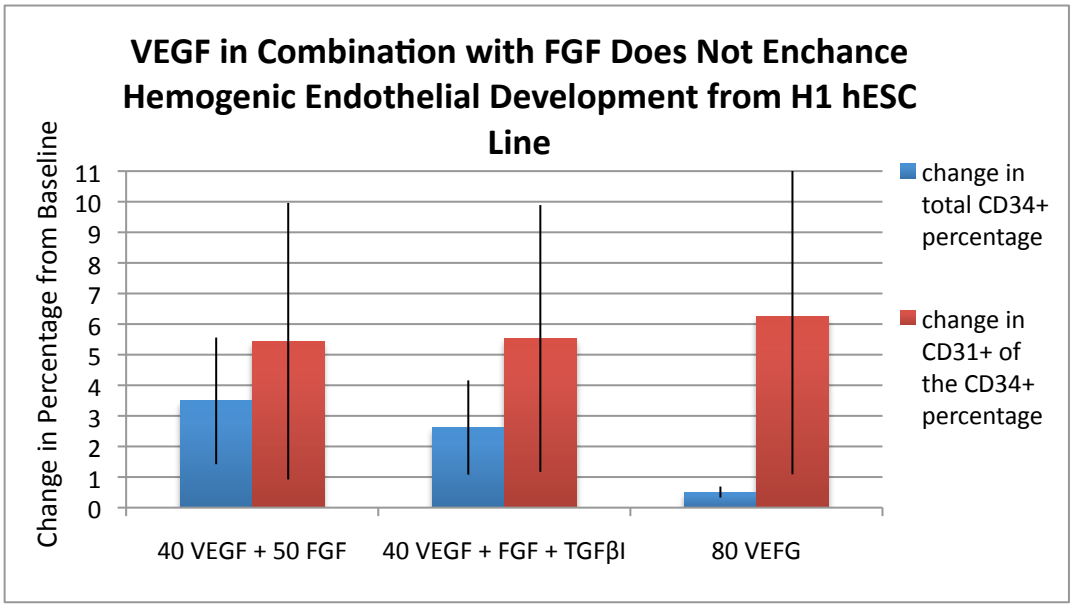
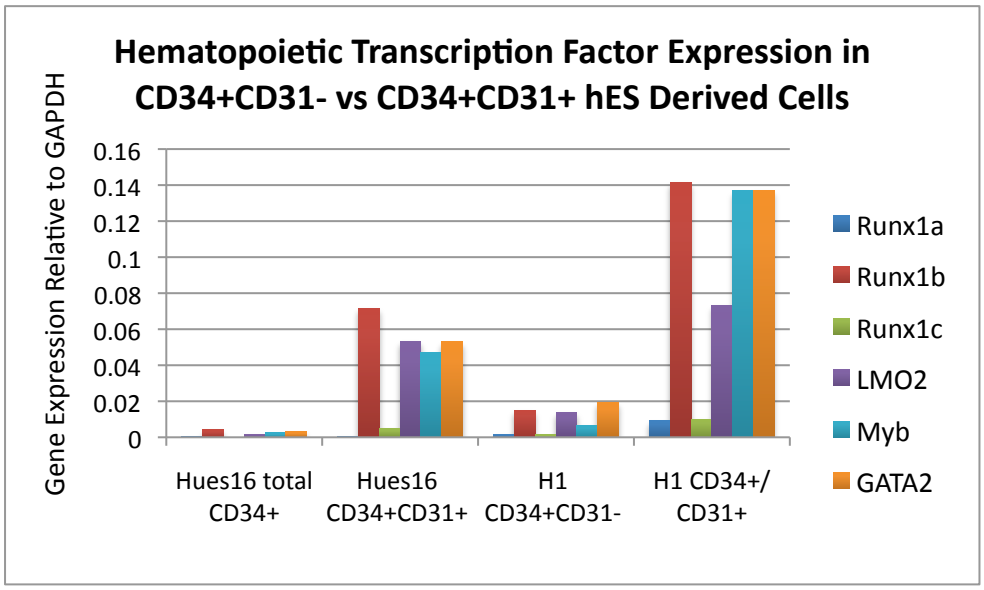


Figure 2-8 continued. **VEGF only encourages hemogenic endothelium differentiation from Hues16, not H1. A)** VEGF with FGF consistently enhances CD34+CD31+ differentiation from Hues16 by at least 3 fold. No other condition tested achieved this (n=4-7wells from 2-3 sets of differentiation) **B)** Neither VEGF alone nor in conjunction with FGF or TGFβ inhibitor enhanced CD34+CD31+ differentiation from H1, though VEGF slightly increased the total CD34+ population when added with FGF and decreased the CD34+ population when the concentration was doubled but no FGF was added.

A)



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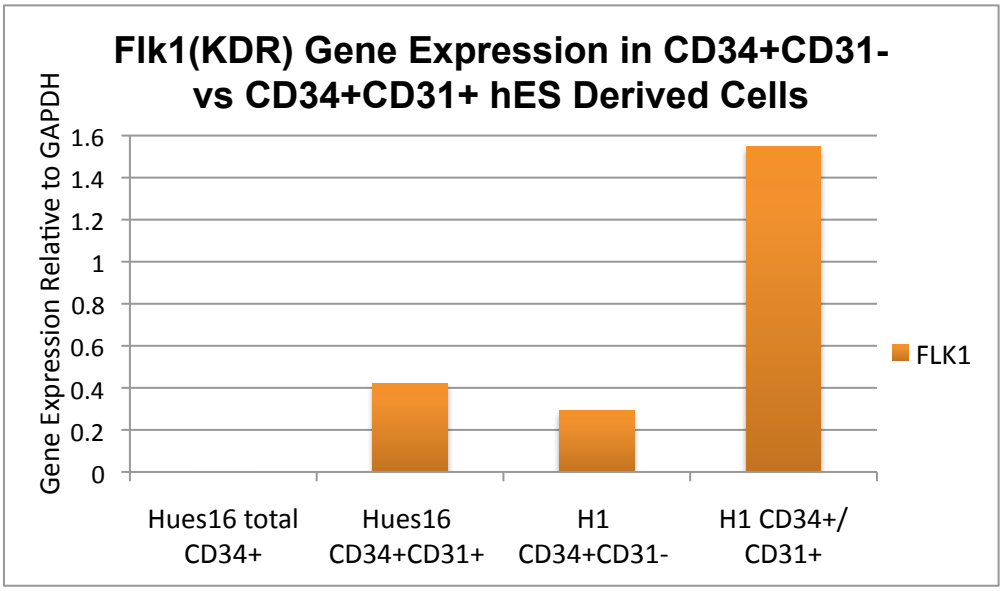

Figure 2-9. **hESC derived CD34+CD31+ cells express higher levels of important hemogenic genes. A)** The transcription factors Runx1b, LMO2, c-Myb, and GATA2 are more highly expressed in CD34+CD31+ hESC derived cells compared to their CD34+CD31- counterparts. **B)** Flk1 gene expression is increased in hESC derived CD34+CD31+ cells.

A)

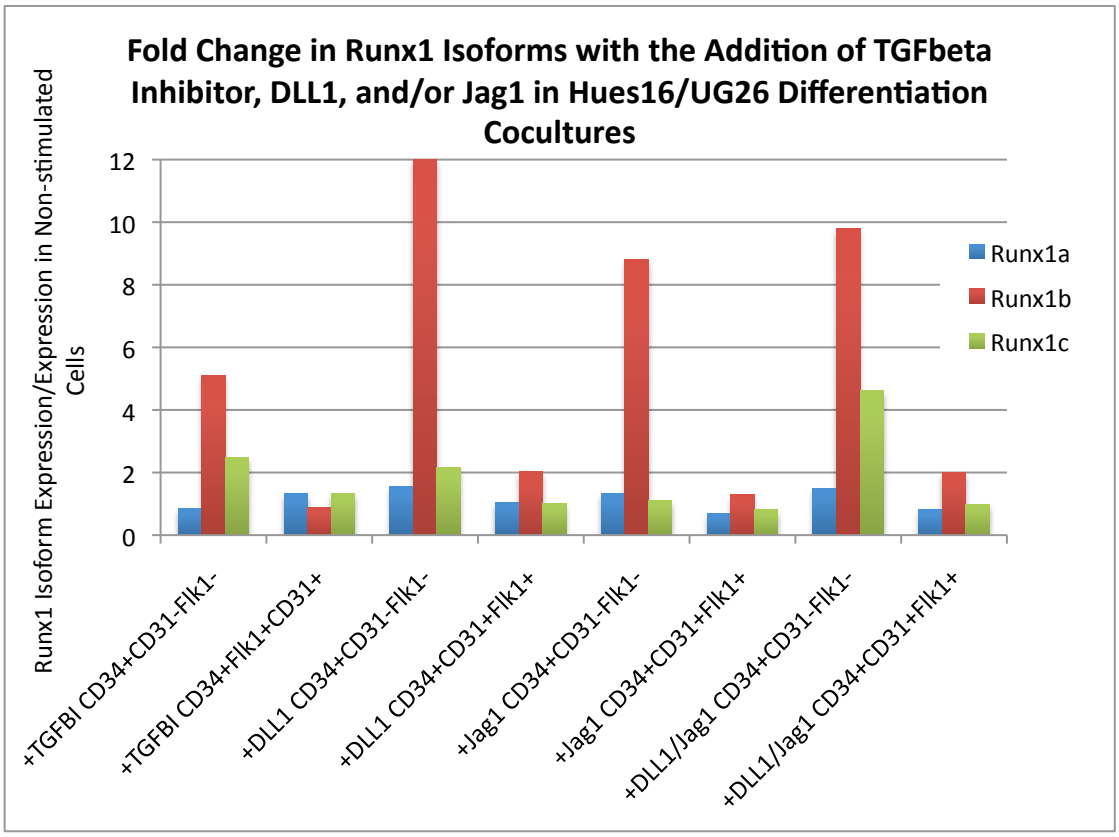

Figure 2-10. **DLL1 and Jag1 fail to sufficiently enhance Runx1 isoforms in hemogenic endothelium differentiated from hES/UG26 differentiation cultures.** **A)** Runx1a, b, and c isoform expression after differentiation of Hues16 on UG26 in the presence and Notch1 ligands DLL1 and/or Jag1 compared to expression in cultures that had no Notch1 ligands. **B)** Runx1a, b, and c isoform expression after differentiation of H1 on UG26 in the presence and Notch1 ligands DLL1 and/or Jag1 compared to expression in cultures that had no Notch1 ligands.

B)

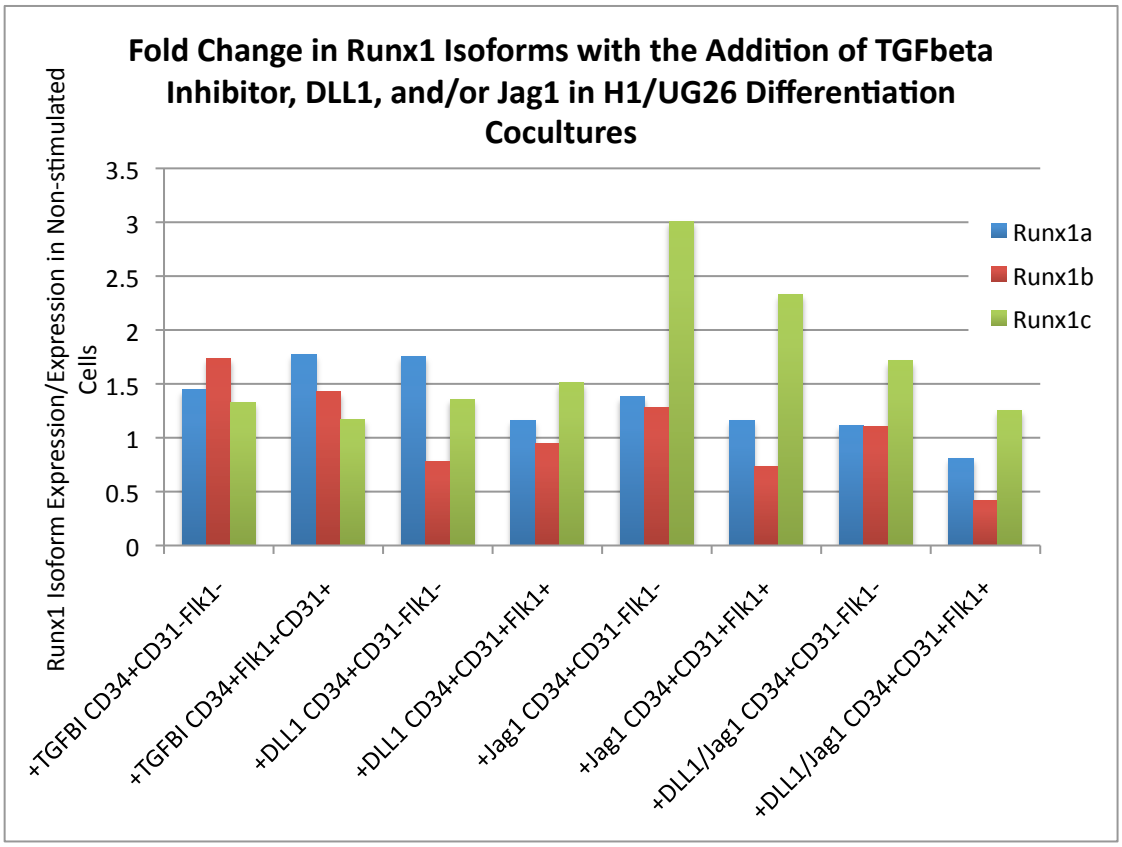

Figure 2-10 continued. **DLL1 and Jag1 fail to sufficiently enhance Runx1 isoforms in hemogenic endothelium differentiated from hES/UG26 differentiation cultures. A)** Runx1a, b, and c isoform expression after differentiation of Hues16 on UG26 in the presence and Notch1 ligands DLL1 and/or Jag1 compared to expression in cultures that had no Notch1 ligands. **B)** Runx1a, b, and c isoform expression after differentiation of H1 on UG26 in the presence and Notch1 ligands DLL1 and/or Jag1 compared to expression in cultures that had no Notch1 ligands.

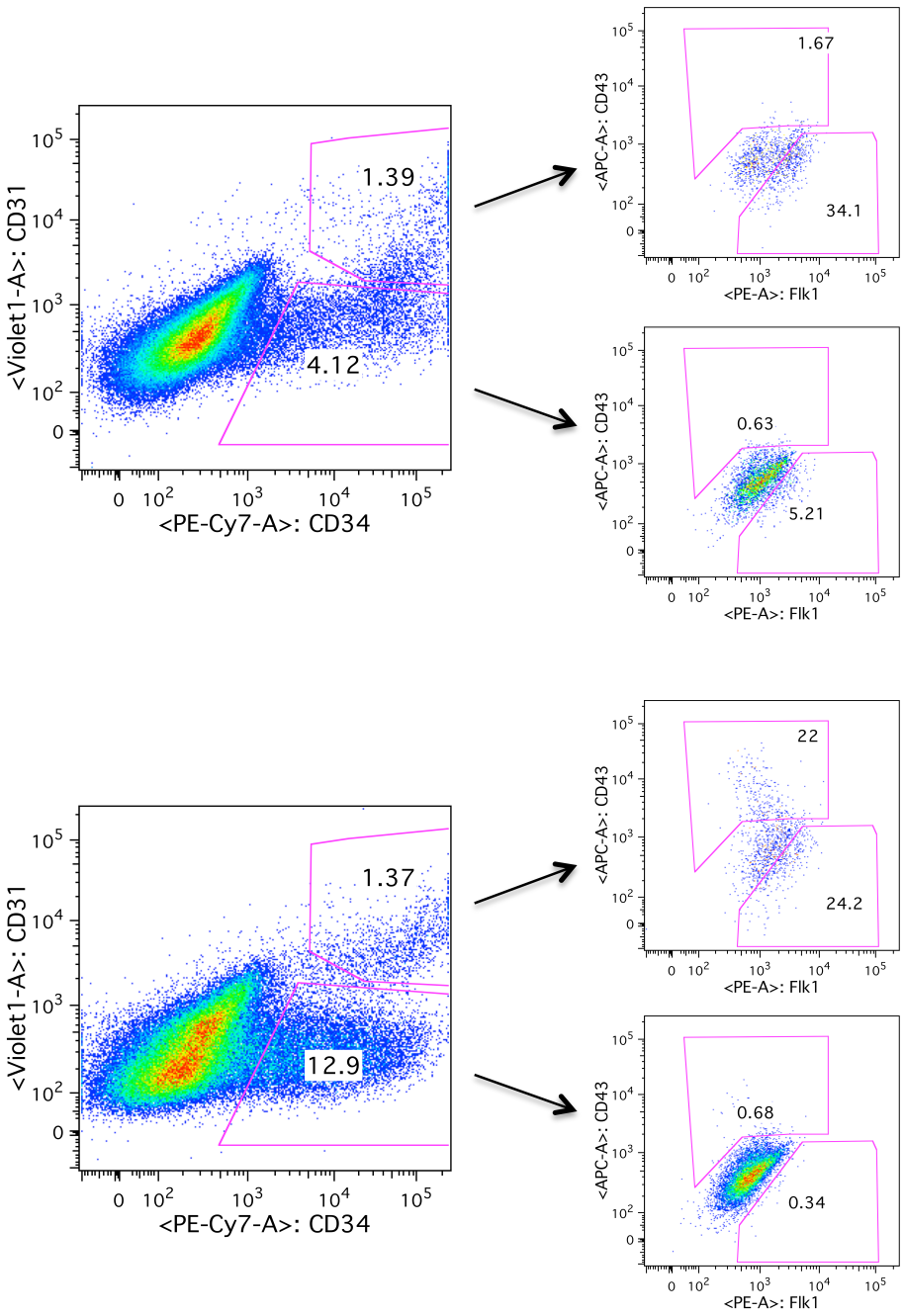

Figure 2-11. Characteristic sorting plots for hESC differentiated cell populations that were used in the Fluidigm gene expression dynamic array. Examples of Hues16 (top) and H1 (bottom) CD34, CD31, Flk1, and CD43 expression profiles 15 days of differentiation on UG26. These populations were sorted to compare the hematopoietic and endothelial gene expression profiles of each phenotypically distinct population.

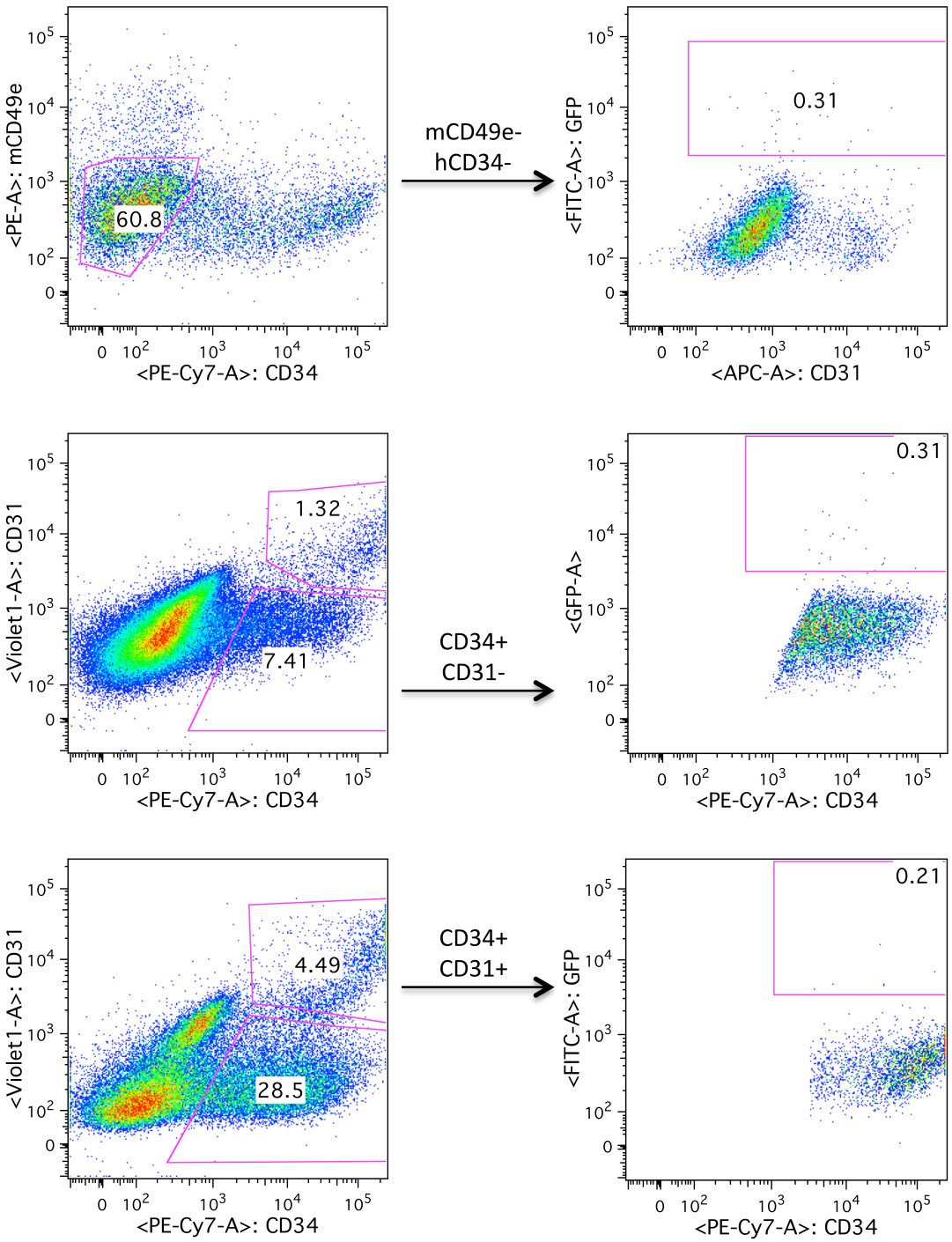

Figure 2-12. GFP expression in CD34+ and CD34- populations of hESC differentiated cells. Examples of GFP+ cells that were sorted for Fluidigm gene expression analysis. From top to bottom: mouseCD49e- humanCD34- GFP+; human CD34+CD31-GFP+; and human CD34+CD31+GFP+.

Table 2-1. **Samples sorted for Fluidigm dynamic array gene expression analysis**. 17 cells of each of the below populations were sorted and used for gene expression analysis of 96 genes.

		Myb/GFP-	Myb/GFP+
differentiated Hues16	CD34-mouseCD49e-	3 bilogical replicates	3 bilogical replicates
	CD34+CD31-	3 bilogical replicates	3 bilogical replicates
	CD34+CD31+Flk1+/-	3 bilogical replicates	3 bilogical replicates
differentiated H1	CD34-mouseCD49e-	3 bilogical replicates	3 bilogical replicates
	CD34+CD31-	3 bilogical replicates	3 bilogical replicates
	CD34+CD31+Flk1+CD43-	3 bilogical replicates	3 bilogical replicates
	CD34+CD31+CD43+	3 bilogical replicates	-----
mixed donor (3-5 individuals) CB HSC	CD34+CD38-CD90+Lin-	3x	
HUVEC	CD34+CD31+	1x	
	CD34-CD31+	1x	
HAEC	CD34+CD31+	1x	
	CD34-CD31+	1x	
H1	Tra1-81+SSEA4^{HI}	1x	
Hues16	Tra1-81+SSEA4^{HI}	1x	

Table 2-2. **Sequences for Fluidigm primers**. 5' to 3' sequences of successful primers for Fluidigm based nano-fluidic QPCR.

Gene ID	Forward primer	Reverse primer	Product size
Oct4	AGAAGGATGTGGTCCGAGTG	GCCTCAAAATCCTCTCGTTG	91
Sox2	CAAGATGCACAACTCGGAGA	GTTCATCGACGAGGCTAAGC	95
c-Myb	CAGCCCACTGTTAACAACGAC	CATGTAACGCTACAGGGTATG	91
LMO2	AGGAACCAGTGGATGAGGTG	CGATGGCCTTCAGGAAGTAG	99
HOXA9	CAATGCTGAGAATGAGAGCG	AGTGGAGCGCGCATGAAG	85
HOXB3	CACCCCACCTACACAGACCT	GTTAATTTGGGCGCTTCTTG	77
HOXA3	TCCCCATCCGTTCGTATTTA	TGAACAAAAGGCCCAAGAAC	91
HOXB4	AGAGCCCGTCGTCTACCC	GTGTAGGCGGTCCGAGAG	99
GATA1	AGATGAATGGGCAGAACAGG	GGCAGTTGGTGCACTGAGTA	98
GATA2	GGCTCGTTCCTGTTCAGAAG	GGCATTGCACAGGTAGTGG	100
GATA3	CAATGCCTGTGGGCTCTAC	TTTCTGGTCTGGATGCCTTC	81
Runx1a (AML1a)	AACCCTCAGCCTCAGAGTCA	GGAGAGGGATGGACAGAAGA	90
Runx1a/b - (AML1b)	ACTTCCTCTGCTCCGTGCT	CTAGGGCCACCACCTTGAA	78
Runx1c - (AML1c)	GTAGGGCTAGAGGGGTGAGG	TCCTGAAAATGCACCCTCTT	108
HDAC1	TGTCGGAGTACAGCAAGCAG	AGAACTCAAACAGGCCATCG	91
CBFbeta	ACAGCGACAAACACCTAGCC	GACTCCATTCAGAATCATGGG	91
Hhex	TCAGAATCGACGCGCTAAAT	TCTGATCACAGGAACTGTCCA	98
IKAROS	TGGTTGATTGGGGTTTGATT	AGTTCTGGATGCTCCCAATG	78
SCL (TAL1)	AGGAGAGCGAGCAGATCAAG	CTTGGTCTCCAGCAGCTTGT	98
PU.1	GAGGTGTCTGACGGCGAG	AGGAACTGGTACAGGCGGA	98
MEIS1	ACATTCCTGGACACCCTCAC	TTGATGCTGACATTGGCATT	81
BMI-1	TGTGTGCTTTGTGGAGGGTA	GCTGGTCTCCAGGTAACGAA	99
SOX17	TGAATGTGTCCCAAAACAGC	AAAACACACCCAGGACAACA	76
MLL	TCCAGGAAGCTCGATCAAAT	GGAACACAACTGCATCATGG	99
NF-Y	GTGGTGAAGGTGGACGATTT	CGGATGATCTGTGTCATTGC	100
LEF-1	AGCACAAACCTCTCAGGAGC	GGGTCCCTTGTTGTAGAGGC	94
TCF-1	GGCCTTGTTCTGTCACCAAT	CATGTGGGTCACTAGCTGGA	76
NOTCH1	GTTGTGCTCCTGAAGAACGG	GTCTCGTAGCTGCCCTCCC	95
NOTCH2	CAATAATGTGGAGGCAACTCT	CTCCCGGGCAGCAAGAAA	100
NOTCH4	CCCAGGAATCTGAGATGGAA	CCACAGCAAACTGCTGACAT	85
CSL/RBPjk	CAAACAGTCAAACTTGTGTGC	CATCTGCATCCAATAATGCG	97
Hes-1	CCAAAGACAGCATCTGAGCA	TCAGCTGGCTCAGACTTTCA	91
EphrinB2	CAATCCCAACAGCCTCAAAG	CACCGTGTTAAAGCTGGTGT	100
Mushashi	CTGCCTACGGACCAATGGC	GGAGTCGAACCTGGAGGG	100
Evi1	AGTGCCCTGGAGATGAGTTG	TGATCTAGAGCAGAAAGTCCA	83
CDX4	TATGCATGGATGCGCAAG	TTCCAGCTCCAATCTTTGATG	99
MOZ/MYST3	AAAGATGAAGAAGAAGATGAA	GAGGAGAATTCTTCACATCAC	100
Gfi1	CCACCAGAAGTCAGACATGAA	GTTGGAGCTCTGGCTGAATG	100
Fli1	CCGTATCAGATCCTGGGC	AGAGCAGCTCCAGGAGGAAT	91
ERG	TCTTGGACCAACAAGTAGCC	ACAGGAGCTCCAGGAGGAAC	80
Ets1	GTCAACCCAGCCTATCCAGA	GAACTCCGATGGTGGAACAC	93
Ets2	TGGAGACGGATGGGAGTTTA	TTCTCGTAGTTCATCTTGGGC	93
Elf1	CCAGTACCATGCAGGATGAA	CAACCACTGGAACTTGGGTT	84
TEL (ETV6)	TAGTGGATCCCAACGGACTG	TTGTAGTAGTGGCGCAGGG	100
PBX1	CCAGTGAGGAAGCCAAAGAG	TCTTCTTGTACCGGATTCGC	99
Lyl1	GGAGACCAAGCCACTGTGAG	CGGCTGTTGGTGAACACG	82
Ldb1	AGCTAGCACCTTCGCCCT	AGCCTCTCGTCCTCGTCC	99
Mib1 (Mindbomb)	TGCTCTGACAAGAAAGCAGC	CTCGACACTGCACACACTTT	100

Table 2-2 continued. **Sequences for Fluidigm primers**. 5' to 3' sequences of successful primers for Fluidigm based nano-fluidic QPCR.

Gene ID	Forward primer	Reverse primer	Product size
beta-catenin	ACTTGATATTGGTGCCCAGG	CTGGCCATATCCACCAGAGT	88
BCL2	GCACCTGCACACCTGGAT	GAGAAATCAAACAGAGGCCG	90
c-kit	TTGTTCAGCTAATTGAGAAGCA	GTCTACCACGGGCTTCTGTC	98
CTNNAL1	GGACGGAACATGTCCAGTATG	TTGATGAATTAAATCCTGGGAA	90
TGF-BETA1	AGCATCTGCAAAGCTCCC	AAGTCAATGTACAGCTGCCG	100
CCNA1	AAGGAGTGTGCGTCAGGACT	TTTCAAGAATGGATCTGCTTCA	80
Rasgrp3	TGAACTTGACCAGGATGAAGG	TTATTGCCTTCAGTTTCCGC	84
Egfl7 (VE-statin)	TACACTCTGTGTGCCCAAGG	CAGCCTCTGCACTTCTTCCT	91
WASP	CGTGATGCAGAAGAGAAGCA	AGCCACTCAGTCATCCCATT	100
HCAM (CD44)	TAAGGACACCCCAAATTCCA	ACTGCAATGCAAACTGCAAG	85
STK-1	CAAATCTCAGGGCTTCATGG	CCTCCTCTTCTTCCAGCCTT	85
VCAM-1	AAAAGCGGAGACAGGAGACA	TACTCCCGCATCCTTCAACT	85
VE-CADHERIN	TAACCTGACTGTGGAGGCCA	ATGTGGACTTGCACAATGGA	78
Tie-1	AGATTGCGCTACAGCTAGGC	CCGCGTAAGTGAAGTTCTCA	84
Tie-2	GCTACAGACTGGAGAAGCCC	TGATGGCCTCTCATAAGGCT	92
CoupTFII	CCGAGTACAGCTGCCTCAAG	GCTTTCCACATGGGCTACAT	80
EphrinB4	TGTGATGTGGGAGGTGATGT	GCAGCCGGTAGTCCTGTTC	95
Flt4	GTGTGGTCTCCAACAAGGTG	ATGGCTTGGATTCGATGGT	93
Endogolin (ENG)	TCATGCGCTTGAACATCATC	ATGAGGAAGGCACCAAAGGT	100
Endothelin-1	AAATCAGAAGAAGTTCAGAGG	TTCAGCTTGGGATCATGAAA	94
VWF (von willebrand factor)	GCACCATTCAGCTAAGAGGA	CCTCTCTCATTGACCTTGCAG	100
Endomucin	CCAAGAACTGACAGCTTGAGG	TTCCACGCTTGGTGCATAG	85
Lectin	CACTGGTTAGCAACCCCAAT	TAGGCCTGACTGGAGCACTT	87
Etv2 (ER71)	CTTTGAAGCGGTACCAGAGC	GTTAGTTTTGGGGCATCGAG	95
Hey1	TGGATCACCTGAAAATGCTG	CGAAATCCCAAACTCCGATA	91
TAB2	TGGATTTGTAGGTCCTGTGC	CCACTGAGCTCCCTCATCAT	100
TM9SF2	TGGTTATTACCTGTTCTGAAGC	ACTCGTAAGGAATGAACGCC	95
Dvl3	CAAGATCACCTTCTCCGAGC	CCATCGTGATCGTGGAGAG	87
Dvl1	GAGCTTGAGTCCAGCAGCTT	CGGATGAGTCTGGATGAGGT	98
ID1	GGGATTCCACTCGTGTGTTT	CTGAGAAGCACCAAACGTGA	74
c-Myc	AAAGGCCCCCAAGGTAGTTA	AGCTTTTGCTCCTCTGCTTG	75
E2-2	GGAGACGCATCGAATCACAT	GCTGTTAAGGAAGTGGTCTCT	89
E2A	CTCGTCCAGCCCTTCTACC	GTAGCTGGGCGATAAGGCAC	100
alpha-smooth muscle actin (a-SM-actin)	AATGCAGAAGGAGATCACGG	TCCAGACAGAGTATTTGCGCT	86
dHand	CCGACGTGAAAGAGGAGAAG	TGGTTTTCTTGTCGTTGCTG	81
MetAP2	GGACAAGAATGCGAATACCC	TTCACTTGCCTGATCTAATGCT	96
ADAMTS9	CCAGATGGATATCACAAGGGA	ACAGTAACCACCGCATTTCC	92
LIPG	GACCCTGAGAACACCAGCAT	CAGTGGGACTGGTTTCGTTT	94
COL4A6	ACATCACCATCCCGCAGT	GACTGGCCTCCACCCTCG	100
KRT19	CCGCGACTACAGCCACTACT	TGTCGATCTGCAGGACAATC	98
MATK	TTTGACATTGGGAGCACAGA	TATTCTTCACGGCCACCTTT	89
SORL1	AGCCTGCCATCCTGCTGTA	ACATCCGTAGATCTGGCAGC	79
SERPINB1	CGGATGACATTGAGGACGAG	CAGGTTTAGTCCACTCATGCA	87
FLK1	TTCTTGGCTGTGCAAAAGTG	CCGCCGTGCCTACTAGAATA	93
CD31	TGAGGGTGAAGGTGATAGCC	TTGCAGCACAATGTCCTCTC	92
GAPDH -1	GAGTCAACGGATTTGGTCGT	AATGAAGGGGTCATTGATGG	95
RPL22	GAAGGAGGAGTGACTGGGAA	GTGGCATGAGGCAGCTATTTC	74
MDH1	TGGTGTTCCTGATGATCTGC	TCACGTGAGAAATCATTAATAG	99
RPS29-1	AATATGTGCCGCCAGTGTTT	CCCGGATAATCCTCTGAAGG	91

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Chapter 3. BCR-ABL is Necessary but Not Sufficient for Human Leukemia Development from hES Derived Cells

Introduction

Model systems of human disease have been vital to the advancement of scientific understanding and the treatment of previously fatal conditions. Organisms such as the fruit fly, zebrafish, and mouse have genes in common with humans and therefore have been used as model organisms. Our current understanding of hematopoietic and leukemic development is based on decades of research using organisms such as these, where mouse models have arguably been one of the most useful tools for approaching questions related to disease development and treatment screening. However, as with all model systems, limitations that make the lack of human (cell-based) models apparent are numerous. The most striking is that although many mouse models of diseases have been developed, few have led to cures[1-4]. This may be due to how the disease is modeled in the organism or the biological differences between the human and the mouse. Advancement in treatment measures with these mouse models have been made however, such as the inhibition of the tyrosine kinase, p210 Bcr-Abl, in chronic myeloid leukemia (CML).

There are two types of CML mouse models (involving only mouse cells) that are commonly used. The first and most widely used entails a BCR-ABL retroviral bone marrow transduction followed by transplantation of these cells into a new mouse. After optimization of this mouse model by various groups, all of the transplanted mice developed a myeloproliferative disease similar to human CML[5-7]. This is seen by an increased number of mature granulocytes expressing BCR-ABL in the peripheral blood, splenomegaly, and leukemic stem and progenitor cells take over the bone marrow (BM)[8]. All of these symptoms are similar to those seen in patients, and therefore this has been a useful model for testing new therapeutics and

dissecting the genetic networks involved in BCR-ABL leukemogenesis[8-10]. The second CML mouse model is the BCR-ABL transgenic model. In this model constitutive BCR-ABL expression resulted in acute lymphoblastic leukemia (ALL) shortly after birth, whereas using a CMV tetracycline-off driven system, whereby BCR-ABL expression is induced only after removal of tetracycline, a rapid lymphoblastic leukemia occurred[11,12]. Neither of these models drove a CML-like disease possibly due to when and where BCR-ABL was expressed. Using a tet-off system under the control of the murine stem cell leukemia gene 3' enhancer to drive BCR-ABL however, an inducible CML-like disease was created[13]. This system models human disease more closely probably due to the specificity of cells it is expressed in and has already been used to study unique properties of leukemic stem and progenitor cells from different microenvironments[14,15]. However, with both of these CML mouse models, disease development is much more rapid than what is observed in patients as full blown acute-like disease from BCR-ABL expression alone occurs within weeks in the mouse models, not years which is the case in patients. This limits the ability to study the transition from CP to BC CML in these models, which is an important point in disease as no known treatments are effective once CML progresses to the BC phase.

These mouse models have offered an opportunity to learn how BCR-ABL affects the development, proliferation, localization, maturation, and survival of the cells it is expressed in. Furthermore, an all mouse cell model allows for the affected cells to respond to endogenous cytokines and same species protein-protein interactions, possibly making a disease phenotype easier to establish. However, as with most mouse models, these do not recapitulate all disease pathologies, require

additional or different mutations than those seen in patients in order to achieve similar pathologies, and most often do not predict treatments that will be effective in patients[1-4]. This suggests that alternative hematopoietic disease models are needed.

Leukemic patient samples can be used for disease studies through genetic analysis, the production of cell lines, short-term in vitro studies, and xenograft models. Genetic analysis of patient samples have proven highly effective in proving genetic aberrations are causative in diseases like chronic myeloid leukemia where chromosomal translocation causes constitutive expression of BCR-ABL, but the complexity of many cancer genomes confound effective treatment predictions[16]. Cell lines derived from cancers have proven useful in high-through-put screening and human signaling pathway analysis. However, these cells have become adapted to life in a dish rather than their natural microenvironment, which often cause selective survival of certain cells over others. This can also result in additional mutations to be acquired which in turn may provide growth and survival advantages to the surviving cells, as well as altered engraftment potential, maturation, and response to drugs[17,18]. Still, for short-term *in vitro* and xenograft models, patient samples are extremely useful in order to gain insight into sub-groups of disease, treatment efficacy, malignancy, and metastasis. A third and widely used CML mouse model is the human xenograft non-obese dynamic/severe combined immunodeficient (NOD/SCID) or recombinase-activating gene(RAG) 2-/-γchain-/- mouse model[19-22]. This is the most physiologically relevant model as it uses human patient samples that have been transplanted into one of the above immunocompromised mice and these mice go on to display many of disease pathologies seen in patients. However, genetic variability between donors, the difficulty in establishing CP patient sample xenograft models,

and the relative lack of patient samples render limited progress on treatment targets and establishing wide-spread *in vivo* and *in vitro* disease models with CML patient cells.

Finally the use of genetically manipulated human hematopoietic cells to model leukemias have been another useful avenue. Through the introduction of an oncogene or knock-down of a tumor suppressor in the initial affected cell type a developmental approach to leukemia can be taken. A myeloproliferative-like disease was indeed established at low levels in mice transplanted with CB that was retro-virally transduced with BCR-ABL, however an increase in erythroid and megakaryocytic cells was observed over granulocytic expansion, which is seen in patients[23]. In addition to this pathophysiological difference in BCR-ABL transformation, the limited supply of HSCs from individual donors, expense of acquiring cells, and the inability to maintain, expand, and efficiently genetically manipulate HSCs in culture has thwarted high-through-put use of this type of disease modeling. Because of these limitations there is a great need for a better source of human cells to study aspects of human hematopoiesis and blood diseases and to create better xenograft models.

In this study, we describe a novel mouse model of CML using human embryonic stem cells (hESCs). hESCs offer a unique solution to the problem as they are essentially limitless in number, are pluripotent, and can be genetically manipulated with greater ease than their more mature counterparts. These qualities allow us to investigate how different genes affect the development of cell fate and disease in a normal human, genetically homogeneous background. Furthermore, there is all together a lack of studies on the development of CML in human cells, and

therefore is crucial to establish reliable systems that allows us to conduct these kinds of studies. Here, we use a lentiviral system to efficiently transduce different sources of human hematopoietic precursor, stem, and progenitor cells with CML relevant oncogenes. This enables us, in a reproducible and reliably way, to test which genes are involved and sufficient to drive characteristics of CML.

Materials and Methods

Construction of lentiviral vectors and lenti virus production. Vector pCDH-EF1-MCS-T2A-copGFP (Systems Biosciences (SBI), Mountain View, CA) was used as the backbone for BCR-ABL and (constitutive,) mutant β-Catenin expression. To generate vector pCDH-EF1-BCR-ABL-T2A-copGFP (BCR-ABL), BCR-ABL was cloned from a retroviral vector kindly provided by Dr. Jean Wang (University of California, San Diego, La Jolla, CA). Cloning BCR-ABL into the MCS was done in 2 parts using primers with EcoR1 and Kpn1 sites: Forward 3' CGGAATTCGCCACCATGGTGGACCCGGTGGG 5' and Reverse 3' GGGGTACCAGGAGTGTTTCTCC 5', and then Kpn1 and Not1 sites: Forward 3' CCGGTACCATGGGCCTGTGTCC 5' and Reverse 3' TTTTCCTTTTGCGGCCGCCCTCTGCACTATGTC 5'.
BCR-ABL pieces were amplified using Phusion High-Fidelity DNA Polymerase (Thermoscientific, Pittsburg, PA). The products were digested with EcoR1 and Kpn1 or Kpn1 and Not1 respectively, then subcloned into the pCDH vector.

Mutant mouse β-Catenin vectors were subcloned as described above using EcoR1 and Not1 sites in primers as follows: Δ-N131-β-Catenin Forward 3'

CGGAATTCGCCACCATGGTTGAAACATGC 5' and Reverse 3' TTTTCCTTTTGCGGCCGCTTACAGGTCAGTATC 5'; S33A-S37A-T41A-S45A-β-Catenin Forward 3' CGGAATTCGCCACCATGGCTACTCAAGCTGAC 5' and Reverse 3' TTTTCCTTTTGCGGCCGCTTACAGGTCAGTATC 5'. Subcloning was performed from mutant S33A-S37A-T41A-S45A-β-Catenin cDNA kindly provided by Dr. Karl Willert (University of California, San Diego, La Jolla, CA).

GSK3β shRNA vector was constructed by Ifat Geron of Catriona Jamieson's lab (University of California, San Diego, La Jolla, CA). BCR-ABL, S33A-S37A-T41A-S45A-β-Catenin (mβCat), and GSK3β shRNA lentivirus production was carried out essentially as described[24].

LEF/TCF reporter assay. 293FT cells (Invitrogen, Carlsbad, CA) were transfected with Δ-N131-β-Catenin, S33A-S37A-T41A-S45A-β-Catenin , and LEF/TCF reporter constructs using the TCF/LEF Reporter Kit (SAbiosciences, Valencia, CA) according to the manufacturer's instructions. 40 hrs post transfection luciferase activity was measured using the EnVision Multilable Plate Reader (Perkin Elmer, Waltham, MA).

Western Blot. Cell lysates were prepared 24 hours after transfection of plasmid DNA and 48 hours after transduction of lentivirus on MEFs or 293FT cells (Invitrogen). Cells were washed in PBS before resuspension in lysis buffer containing protease inhibitor cocktail (Roche, Indianapolis, IN). 1ug/ml anti-Abl antibody clone 8E9 (Pharmingen, San Jose, CA), anti-β-Catenin (clone 8E7, Millipore, Billerica, MA), or anti-GSK3β (#9315, Cell Signaling Technology, Danvers, MA) was incubated with the membrane overnight followed by peroxidase-conjugated goat anti-mouse IgG antibody.

hESC, AM20, and UG26 cell culture. Hues16 was maintained on Matrigel with mTeSR1 (Stem Cell Technologies, Vancouver, British Columbia, Canada) and passaged every 3-4 days via manually scraping away any differentiated cells, dispase treating, and then manual dissociation with a pipet tip and cell scraper. Media was changed daily.

AM20 and UG26 were grown as previously described[25]. Briefly, cells were maintained sub-confluent at 33°C in 35% alpha-MEM (Gibco, Life Technologies, Grand Island, NY), 50% Myelocult M5300 (Stem Cell Technologies), 15% FBS, 1% penicillin and streptomycin, 2 mM Glutamax, 1 mM NEAA, and 10 uM beta-mercaptoethanol on gelatin coated plates.

UG26/hESC Differentiation. Hues16/UG26 coculture was performed as previously described[26]. Briefly, UG26 cells were treated with 10 ug/ml mitomycin C for 2.5-3 hrs, washed 3 times with PBS, harvested via trypsin, and then replated on gelatin-coated 6 well plates at a density of $3x10^4$ cells/cm^2. 6-24 hrs later hESCs were harvested via dispase, washed 3 times, and plated on top of the UG26 cells at a density of $1x10^4$ cells/cm^2 in knockout DMEM, 20% fetal bovine serum, 10 μM beta-mercaptoethanol, 1 mM L-glutamine, 100 mM non-essential amino acids, and 1% penicillin-streptomycin and cultured for 15 days. One half of the media was changed every 2-3 days.

Transduction of Hues16/UG26 derived CD34+ cells. hESC/UG26 cocultures were dissociated via 0.25% Tryple for 5 minutes at 37°C followed by a PBS wash. Then cocultures were further dissociated with accutase for 10-20 minutes at 37°C. Single cell suspensions were then obtained after vigorous trituration and passage through a

100 µm cell strainer. Cells were resuspended at a concentration of $5x10^4$ per 100 ul StemPro (Stem Cell Technologies) plus 300 ng/ml Flt3, 100 ng/ml IL-6, 400 ng/ml SCF, and 62.5 ng/ml TPO in ultra-low binding plates (Corning, Corning, NY). GFP-Luciferase, BCR-ABL, mutant-β-Catenin, and/or GSK3β shRNA lenti virus was added at an MOI of 200 in 5 ug/ml Polybrene. Transduction was carried out overnight at 37°C.

Isolation and Transduction of Chronic Phase(CP) CML Patient CD34+ cells and Cord Blood CD34+ Cells.

CP CML PBMC patient samples previously ficolled and frozen were thawed, CD34+ selected using MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and plated at a concentration of $5x10^4$ per 100 ul StemPro (Stem Cell Technologies) plus 300 ng/ml Flt3, 100 ng/ml IL-6, 400 ng/ml SCF, and 62.5 ng/ml TPO (R&D Systems, Minneapolis, MN). GFP-Luciferase, mutant-β-Catenin, and/or GSK3β shRNA lenti virus was added at an MOI of 200 in 5 ug/ml Polybrene and cells were cultured over night. Frozen, single donor cord blood CD34+ cells (Stem Cell Technologies) were thawed, transduced, and cultured identically.

CFU assays. After a 24 hour transduction, roughly 1000-2000 CB CD34+ cells were plated in 10 ml of Methocult H4435 Enriched (Stem Cell Technologies) in 12 well plates. Cells were cultured for 12-14 days before colonies were scored. Colonies were replated into 1 well each of a 96 well plate in 100 ul Methocult for another 14 days.

Nanofluidic phospho-proteomic immunoassay (NPI). 4 nl of 10 mg/ml transduced CD34+ CB cell lysates were diluted to 0.2 mg/ml in 200 nl HNG (20 mM HEPES pH

7.5, 25 mM NaCl, 0.1%, 10% glycerol) with Phosphatase Inhibitor Cocktail 1 diluted 1:100 (Sigma, St. Louis, MO) and Protease Inhibitor diluted 1:100 (Calbiochem, EMD Millipore, Rockland, MA) per capillary tube. 200 nl sample mix containing internal pl standards was added. The firefly system first performed a charge-based separation (isoelectric focusing) in a 5 cm long, 100 micron inner diameter capillary. Predicted pls were calculated with Scansite. Each sample was run on a panel of different pH gradients (pH 3-10 and pH 2-11) to optimize the resolution of different peak patterns. After separation the photo-activated in-capillary immobilization, CRKL was detected using anti-CRKL (Cell Signaling Technology). Anti-B2-microglobulin (Millipore) was used to normalize the amount of protein loaded. The peaks were quantified by manually selecting the start and end of each peak and a flat baseline for calculating the area under the curve (AUC). The NPI data was normalized to B2-microglobulin by dividing the measured peak area for the protein of interest by the measured peak area for B2-microglobulin. Experiments were performed with the Nanopro 1000 (Cell Biosciences, Santa Clara, CA) and all samples were run in triplicate or more.

Rag2-/-γchain-/- xenografts. Transduced hES and CP CML patient CD34+ cells were washed 2x in 10 ml PBS 24 hrs post transduction. Cells were resuspended at $5x10^6$/ml in StemPro (Stem Cell Technologies). $1x10^5$ cells were injected intrahepatically into P0-P2 neonates. Primary transplanted mice were sacrificed between 8 and 12 weeks of age. Liver, spleen, and bone marrow from tibias, femurs and humeri were harvested for fluorescence activated cell sorting (FACS), RNA, and immunofluorescence analysis. Single cell suspensions were made of all tissues followed by red blood cell lysis (RBC lysis buffer, eBioscience, San Diego, CA) with a portion of spleen and liver being saved for sectioning. For secondary transplants,

after red blood cell lysis, roughly ¼ of the spleen and bone marrow of primary animals were combined and injected intrahepatically into two P0-P2 neonates each. These mice were sacrificed at 15 weeks for FACS, RNA, and immunofluorescence analysis.

Flow cytometry of xenograft tissue. Single cell suspensions of each tissue were blocked with human and mouse Fc block (Miltenyi Biotech) for 30 min on ice. The cells were then stained with anti-mouse CD45-PerCP-Cy5.5, anti-human CD34-APC, CD45-APC-H7, CD90-PE, and CD38-PE-Cy7 (BD Biosciences, San Diego, CA) on ice for 45 minutes. FACS analysis was run on the FACS Aria I (BD Biosciences).

Immunofluorescence of tissue section. Roughly ¼ of spleen and liver were suspended in OCT media and frozen in iso-butane. 20 um slices of the spleen and 50 um slices of the liver were sectioned and mounted on glass slides. Slides were stored at -80°C. For staining, slides were removed from freezer and moisture was allowed to evaporate. Tissues were circled with nail polish and fixed in 4% PFA for 15 min before washing 3x with PBS. The samples were then blocked for 2 hrs at room temperature in 2% Tween 20 with 1:50 MAX Block blocking reagent (Active Motif), 1:50 human Fc block, 1:50 mouse Fc block (Miltenyi Biotec), and 40ul/ml M.O.M. Blocking Reagent (Vector Labs, Burlingame, CA). After three washes of 10 min, mouse anti-human CD34-AlexaFluor 647 (1:10, Biolegend, San Diego, CA) and APEX kit (Invitrogen) pre-conjugated mouse anti-human CD45-AlexaFluor 555 (1:20, BD biosciences) in 5% BSA, and 0.2% Tween 20 in PBS was added and incubated for 1 h. Tissues were washed 3 times then incubated with goat anti-mouse IgG-AlexaFluor 488 (1:1000, Invitrogen) in 5% BSA/0.2% Tween 20 in PBS for 30 minutes. After washing with PBS 3 times tissues were covered with VectaShield Hard Set Mounting Media containing Dapi (Vector Labs) and a coverslip was placed

on top. These were dried in the dark overnight at room temperature. Imaging was carried out on an Olympus FV1000 Confocal Microscope (Olympus America, Center Valley, PA).

Real-time quantitative PCR (QPCR). Total RNA was prepared from a small aliquot of single cell suspensions of each tissue after treatment with red blood cell lysis buffer (eBioscience) using RNeasy mini kit (Qiagen, Germantown, MD). On column DNase treatment was performed according to the manufacturer's instructions. For cDNA production, RNA was reverse transcribed using Superscript III (Invitrogen) and random primers. Quantitative real time-PCR (Q-PCR) was performed in triplicate on an iCycler using SYBR GreenER Super Mix (Invitrogen). Human ribosomal protein L27(RPL27) was used to identify all human cells present in xenograft tissues to distinguish normal from leukemic, BCR-ABL positive cells. All positive samples were confirmed by a second run.

For analysis of replated colonies, each well of the 96 well plate containing a replated colony was added to 500ul of RLT buffer and RNA was isolated with RNeasy Micro Kit (Qiagen). On column DNase treatment, reverse transcription, and Q-PCR was performed as above with all samples being run in triplicate. mRNA levels for BCR-ABL transcripts were normalized to HPRT and compared using the delta-dealt CT method. The following primers were used:

BCR-ABL Forward: ctccagactgtccacagcat

BCR-ABL Reverse: ccctgaggctcaaagtcaga

RPL27 Forward: atcgccaagagatcaaagataa[27]

RPL27 Reverse: tctgaagacatccttattgacg[27]

HPRT Forward: cgtcttgctcgagatgtgatg

HPRT Reverse: tttatagccccccttgagcac

Results

Lentiviral BCR-ABL construct produces Bcr-Abl protein and alters CB CFU results.

BCR-ABL cDNA is 6.093kb and produces a protein of 210kD making it a large oncogene for cloning and over expression. After subcloning from a retroviral backbone, the EF1α promoter driven BCR-ABL lentiviral vector was tested for functionality. It expressed GFP upon transfection similar to that of the backbone vector, pCDH (Fig3-1A). In addition, it expressed full length BCR-ABL protein after transfection of the plasmid DNA and transduction of the lentivirus produced from it (Fig3-1B). Furthermore, BCR-ABL transcripts are highly overexpressed and p-CRKL levels, a direct target of BCR-ABL phosphorylation, are increased after transduction in CB CD34+ cells (Fig3-1C,D)[28]. To date, this is the second lenti viral vector produced that expresses the fusion gene BCR-ABL[29]. With this novel BCR-ABL expression system pseudotyped with the VSVG envelope, human CB CD34+ cells that do not readily divide in culture and are classically difficult to infect, could be efficiently infected.

Mouse models of CML using retroviral or transgenic expression of BCR-ABL in mouse hematopoietic stem and progenitor cells have not fully recapitulated human

CML. For example, an aggressive CML-like myeloproliferative disease was found within 4 weeks of transplant by Li et al. whereas patients undergo a lag time of up to 5 years before experiencing symptoms[7]. Uchida et al. observed B-cell, not myeloid, acute leukemia that developed within 3 months of transplantation in roughly 50% of the recipients[29]. Studies such as these suggest BCR-ABL is enhancing self-renewal and/or survival or providing a growth advantage of the mouse hematopoietic stem and progenitor cells. To test this in human cells *in vitro* CB CD34+ cells were infected with backbone or BCR-ABL lentivirus. BCR-ABL expression was found to enhance erythroid differentiation from both donors (Fig3-2), which has been observed previously using a retroviral system of expression. It was suggested that this may be due to the high overall expression of BCR-ABL in infected cells compared to the endogenous levels expressed in CML patients[30]. In addition, instead of the growth advantages seen in most transduction/transplantation or transgenic mouse models, BCR-ABL reduced the self-renewal capacity of human CB stem and progenitor cells in CFU assays by roughly 50% (Fig3-2). This suggests that in human cells, BCR-ABL is not a highly transformative oncogene but may only confer cancer when present in a cell that already has long-term self-renewal capacity. This is supported by the fact that the CP LSC is a HSC, not a progenitor cell, as progenitor cells expressing BCR-ABL cannot transmit disease whereas BCR-ABL+ HSCs can[14,31].

Evaluation of mutant β-Catenin and GSK3β vectors

Enhanced β-catenin expression and activity have been observed in CML LSC[31]. Furthermore, loss of β-catenin expression greatly reduces BCR-ABL

transformation to a myeloproliferative state[32]. Whether elevated β-catenin signaling in CML LSC is a transformative or down-stream event, however, is not known. Therefore, 2 constitutively active mutant β-catenin lentiviral expression vectors were created by subcloning Δ-N131-β-catenin and S33A-S37A-T41A-S45A-β-catenin into the pCDH-EF1 backbone. Each of these mutants prevent degradation of the protein caused by phosphorylation at the S33, S37, T41, and S45 residues. The size of the proteins produced by these constructs was confirmed by western blot (Fig3-3A). Upon comparison of these two mutants, the S33A-S37A-T41A-S45A-β-catenin was found to have a greater transcriptional activation capacity as seen by the LEF/TCF reporter assay (Fig3-3B). This is a classic measure of activated β-catenin levels as β-catenin complexes with LEF-1 and TCF transcription factors, which then bind and activate canonical Wnt target genes. The S33A-S37A-T41A-S45A-β-catenin (now referred to as mβCat) expression vector thus allowed us to test if the enhanced self-renewal activity provided by the addition of constitutively active β-catenin in BCR-ABL positive cells is sufficient to reverse BCR-ABL loss of self-renewal as seen in the colony formation assays.

GSK3β is involved in several important developmental, cell cycle, homeostasis, and metabolic signaling pathways and is a serine/threonine kinase. In the canonical WNT signaling pathway, GSK3β phosphorylates β-catenin which is then degraded. In effect, β-catenin can no longer turn on its transcriptional targets. GSK3β functions in a similar way in the hedgehog pathway, where Gli family members are prevented from turning on transcriptional targets[33,34]. Therefore, one can argue that by knocking down GSK3β, elevated β-Catenin and Gli dependent WNT and hedgehog signaling can occur. We hypothesized that this may produce a

stronger transformative phenotype due to GSK3β's role in two pathways known to be involved in oncogenesis. Testing our GSK3β lentiviral showed knock-down of His-tagged GSK3β that was transfected alongside. However, endogenous GSK3β was not affected in the analyzed time-frame (Fig3-4). This is a relatively stable protein with a long half-life of around 48 hours, which presents a challenge for knocking down expression by transient transfection[35]. Therefore the lack of endogenous GSK3β knock-down was not concerning as transfected GSK3β was sufficiently decreased.

BCR-ABL alone is not sufficient to drive a CML-like myeloproliferative disease in hESC derived hematopoietic precursor cells

hESC derived CD34+ cells differentiated for 15 days on the UG26 AGM stromal cell line were infected with GFP-luciferase (GLF), BCR-ABL, mβCat, and/or GSK3β shRNA lentiviruses and intrahepatically injected into P0-P2 RAG2-/-chain-/- pups in three separate experiments. QRT-PCR and FACS analysis were performed on liver, spleen and bone marrow (BM) cells 8-12 weeks post primary transplantation and 15 weeks post secondary transplantation (of ¼ primary harvested BM and spleen cells). A low level, 0.2-7.13%, of human CD45/CD34+ cell engraftment was detected by FACS in a small percentage of animals in each primary transplant set though no trend between groups was observed (Fig3-5). This demonstrates that hESC derived CD34+ cells are capable of engrafting along the hematopoietic lineage and homing to hematopoietic relevant organs, though at much lower levels than CB CD34+ cells.

QRT-PCR analysis of all primary and secondary animals revealed differences between BCR-ABL positive and negative cells. Overall, human cell engraftment was

measured by human ribosomal protein L27 (RPL27) transcripts, and RPL27 and BCR-ABL detection were found to be mutually exclusive other than in one sample, a tumor. This suggests that the detection limit for BCR-ABL in transduced cells is much lower than that of endogenous RPL27. This is supported by QPCR of BCR-ABL transduced CB CD34+ cells showing BCR-ABL is highly over-expressed after lenti-viral infection compared to endogenously expressed RPL27, for which it is normalized to(Fig3-1C). Furthermore, although lentiviral infection is used to express BCR-ABL, infection efficiencies ranged from roughly 10-30%. Thus, 90-70% of the transplanted cells were BCR-ABL negative even after transduction. Therefore, it can be inferred that RPL27+ tissues contain only BCR-ABL negative cells in every sample but the tumor, which was comprised of mostly human cells generating easily detectable levels of RPL27. However, the mouse organs we analyzed primarily consisted mouse cells with low levels of human cell engraftment based on our FACS data (Fig3-5).

Between 22-37.5% of recipients in each primary transplant group, other than the GLF + mβCat group, contained engrafted human cells by detection of RPL27 in BM and/or liver samples. Similarly, between 22-37.5% of BCR-ABL infected groups contained BCR-ABL+ cells found in liver and/or spleen tissues. This suggests no survival differences between BCR-ABL positive and negative cells in primary transplanted mice, but may point to a homing difference. However, although 9-18% of BCR-ABL negative cells were found in 3/5 of secondary transplanted mouse groups in liver and/or BM, BCR-ABL positive cells were found only in spleens of secondary recipients where mβCat was expressed. This suggests that neither BCR-ABL alone nor in conjunction with knock-down of GSK3β confers self-renewal

advantages. To push this further, BCR-ABL may even decrease self-renewal in transplanted cells as fewer mice overall were found with BCR-ABL positive cells upon secondary transplantation than those with BCR-ABL negative cells. BCR-ABL thus appears to reduce self-renewal potential in the hESC derived cells, which has been reported by others in different systems and was observed in our CB colony forming assays[14]. However, in the presence of elevated levels of activated β-catenin self-renewal was possibly either restored or enhanced as 18% of these recipients had secondary engraftment. HSCs have high levels of activated β-catenin which promotes self-renewal and thus enforcing this state in the presence of BCR-ABL may be necessary to support self-renewal in hESC derived CD34+ cells[31].

It might be expected that constitutively active β-catenin may enhance self-renewal and engraftment of CD34+ hESCs on it's own because elevated levels are present in HSCs. However, normal self-renewal still occurs in HSCs lacking β-catenin and although the phenotypic population of HSCs can be expanded upon Wnt-β-catenin signaling, these cells were shown to have little stem cell capacity *in vivo*[36-38]. Following this, we did not observe increased engraftment of mβCat expressing hESC derived CD34+ cells but instead possibly saw decreased engraftment as none of the mice in this group contained detectable RPL27 transcripts. However, only one hESC differentiation was infected with mβCat alone after CD34 selection and injected into 6 pups. Possibly this number of mice was too few to pick-up low engraftment potential or the differentiation was not optimal and therefore had less potential to engraft. Overall, this suggests that dramatic positive differences in engraftment of hES derived CD34+ cells would not be observed between cells expressing elevated levels of βCat or not. Further investigation is needed to fully clarify if constitutive β-

Catenin plays a significant role in the efficiency of hESC derived CD34+ cell engraftment.

BCR-ABL in conjunction with constitutively active β-catenin or GSK3β knock down are not sufficient to support engraftment of chronic phase CML patient samples

Engraftment of CP CML patient samples is notoriously difficult with successful reports needing to use an excessive number of cells per mouse, from 2-5x10^6, even though these cells carry the endogenous oncogene BCR-ABL[39]. With a dearth of CP patient samples that have not been treated with tyrosine kinase inhibitors, and the low number of CD34+ cells that can be recover from these samples even when they are obtained, this mouse model for CP CML has not been used by many groups. In order to study the transition of CP to the accelerated and BC phases of the disease, we transplanted 10^5 CD34+ CP CML cells per mouse. The mice were expressing GLF with mβCat or GSK3β shRNA to test if these mutations provide BCR-ABL positive cells with a growth, survival, or self-renewal advantage. Although 25-33.3% of mice in the primary transplant groups showed human cell engraftment at the time of sacrifice, only 5% of the mice in the GSK3β knock-down group, and none from the GLF alone or mβCat group were found to have BCR-ABL+ cells engrafted. Furthermore, 5.8-16.7% of mice in the secondary transplanted groups had human cells engrafted, yet no BCR-ABL+ cells were able to achieve secondary transplantation. Therefore, although BCR-ABL negative CD34+ cells from CP-CML patient samples were able to confer both primary and secondary engraftment upon

transplantation at fairly similar rates between each group, neither mβCat nor GSK3β knock down provided enough of a growth, survival, or self-renewal advantage to achieve similar results in BCR-ABL positive cells. This may suggest that more than one additional mutation is required for further transformation and transition to accelerated and BC phases of disease.

hESC derived and CP CML patient sample CD34+ transplanted cells can be visualized in liver and spleen and engraft along the hematopoietic lineage

To confirm the presence of human hematopoietic cells in both hESC and CP CML CD34+ cell transplanted mice, both liver and spleen tissue slices were examined by immunofluorescence microscopy. Interestingly, we discovered that hESC derived CD34+ cells, that were CD45 negative upon transplantation, became CD45 positive as a result of the new environment they were transplanted into in both tissues analyzed (Fig3-6A,B). In one report, hESC derived CD34+ cells were found to engraft along the endothelial lineage[40]. This clearly shows that hematopoietic development is indeed taking place in these cells CD45 is exclusively expressed on hematopoietic cells. In addition, CD45+ cells were found in the livers of several CD34+ CP CML transplanted mice confirming RPL27 QRT-PCR results of hematopoietic engraftment as well (Fig3-6B).

Discussion

In this study we describe a method for modeling CML in several human cell types both *in vitro* and *in vivo*. We were able to do this by efficiently expressing BCR-ABL in cells that are normally hard to infect using a lenti viral vector expression strategy. CB HSCs were used as the *in vitro* modeling cell type, because it proved to be efficiently transduced by this lentiviral system and they readily form mature hematopoietic colonies in methylcellulose. With this system there was no need for forced cell division in order for BCR-ABL integration to take place, as is necessary with the retroviral systems used previously[23]. This prevents loss of the HSC's stemness and capacity to engraft, which occurs with extended culturing and forced cycling. Therefore this is a more robust measure of how stem cell qualities are maintained versus differentiation in the presence of BCR-ABL. Within this system, BCR-ABL skewed differentiation of CB stem and progenitor CD34+ cells to the red blood cell lineage in colony forming assays. Furthermore, BCR-ABL caused loss of self-renewal potential in colony replating assays of CB CD34+ cells. This result differs from what is seen in patients, where an expansion of the granulocyte lineage occurs when BCR-ABL is expressed in the HSC. Based on those observations, one would expect to see an expansion of granulocytes rather than a loss of self-renewal in BCR-ABL transduced CB cells. However, this discrepancy has been addressed after retroviral insertion in CB CD34+ cells[23]. They suggest that this disparity is due to the extreme levels of BCR-ABL overexpression compared to the endogenous levels seen in patients. To test this, the use of induced pluripotent stem cells (iPSC) from CP CML patient samples may be fruitful as they contain endogenous levels of BCR-ABL. In addition, BCR-ABL has been shown to cause HSC differentiation at the expense of

self-renewal in an inducible transgenic mouse model[14]. This correlates with our findings that BCR-ABL positive CB colonies loose self-renewal capacity.

BCR-ABL induced loss of self-renewal was further observed upon BCR-ABL positive hES derived and CP CML CD34+ cell transplantation experiments. BCR-ABL positive cells were unable to confer secondary transplantation, which is a measure of self-renewal potential. However, BCR-ABL negative cells from both sources were able to engraft in both primary and secondary recipients of both liver and BM. For the first time, in this study the effects of constitutive expression β-catenin or knock down of GSK3β were evaluated in BCR-ABL positive human cells. Upon expression of mβCat, BCR-ABL positive hESC derived cells were able to achieve secondary engraftment in the spleen. This enhanced engraftment was not, however, observed in CP CML patient sample transplanted mice. Interestingly, this is the first report of a hESC derived model system for CML, and it indicates that hES derived hematopoietic precursor cells expressing BCR-ABL may have inherent survival and self-renewal advantages over CP CML CD34+ patient samples. This suggests that elevated levels of activated β-catenin may be necessary and sufficient to maintain self-renewal in BCR-ABL expressing hESC derived cells, but not in CD34+ cells from CP patients with endogenous BCR-ABL.

Activated β-catenin is known to be elevated in normal HSCs and the leukemic stem cells in CML: HSCs in CP and granulocyte-macrophage progenitors in BC[31]. Furthermore, in mouse transduction/transplantation models of CML, lack of β-catenin almost doubled the time of disease onset, decreased the rate of morbidity, led to ALL in most cases rather than CML, and was necessary for successful secondary transplantation and for the survival of the leukemic stem cell following Imatinib

treatment[9,32]. Based on these observations, an interesting follow-up would be the evaluation of how BCR-ABL positive CD34+ human CB would respond to knock-down and elevated levels of activated β-catenin both *in vitro* and *in vivo*. The lack of β-catenin would be expected to diminish the transformation capacity of BCR-ABL in CB transduced cells further supporting the evidence that β-catenin is necessary for BCR-ABL induced CML. However, constitutively active β-catenin in the presence of BCR-ABL expression in CB CD34+ cells may enhance transformation by either increasing the incidence of disease and/or shortening the length of onset.

A subtle but possibly important observation is that hESC derived BCR-ABL positive and negative cells localized to different areas in both primary and secondary transplantations. Both cell types were found in the liver by Q-PCR. As the liver was the site of injection, this observation was not surprising. However, beyond the injection site, the BCR-ABL negative cells were exclusively found in the BM, the BCR-ABL positive cells were exclusively found in the spleen suggesting a homing difference due to BCR-ABL expression. Splenomegaly is not only an important diagnostic indicator of CML, but also an important prognostic determinant[41-44]. Spleen size in addition to basophil count are the most important clinical predictive indicators for complete cytogenic remission assessment after 18 months of treatment with Imatinib[45]. It remains unknown if correlation of spleen size and clinical outcome is simply due to leukemic cell load or a difference in the biology of the leukemic cells that reside there. Supporting the theory that there may be a difference in the leukemic spleen cells themselves there is anecdotal evidence of CML patients achieving prolonged complete cytogenic remission after splenectomy[46,47]. Furthermore, in an inducible transgenic CML mouse model BCR-ABL positive BM

cells were found to home preferentially to the spleen[48]. More importantly, spleen derived BCR-ABL positive cells were more potent than BM derived BCR-ABL positive cells and this did not appear to be due to support from the non-leukemic microenvironment[15]. Our results therefore suggest that the hESC derived CD34+ cells are inherently more supportive of BCR-ABL transformation after the addition of mβCat and that they might more easily mirror important disease pathologies upon xenograft than CP CML patient cells.

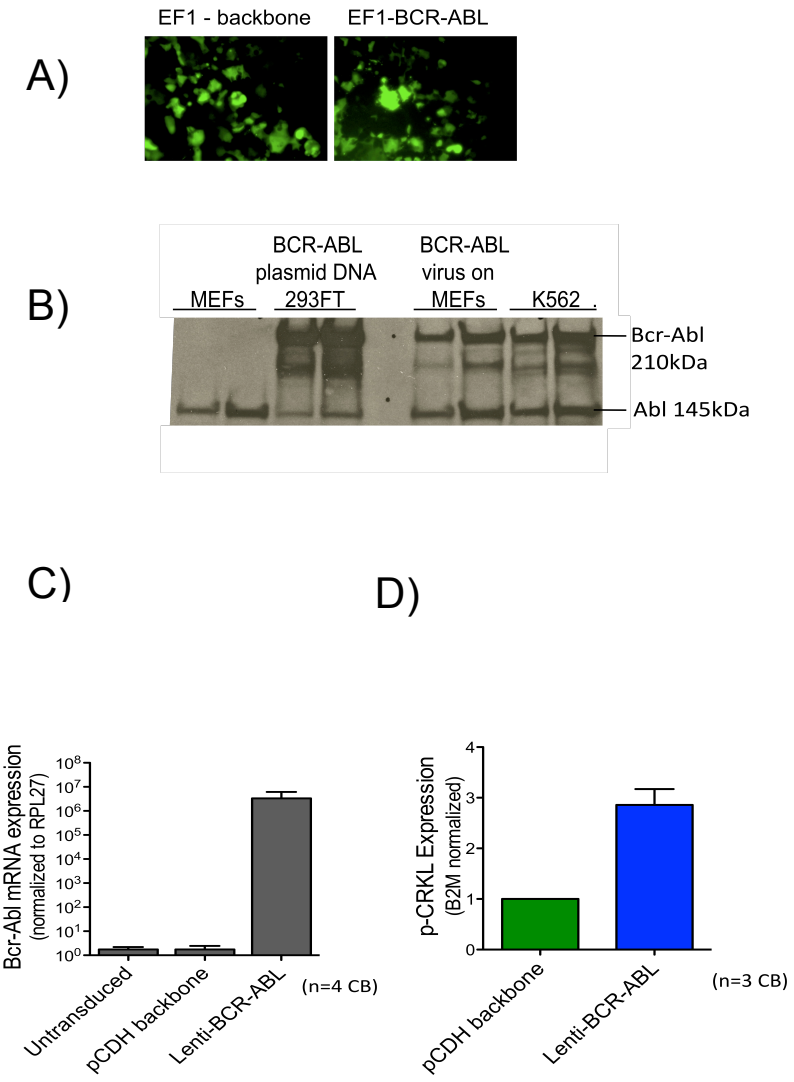
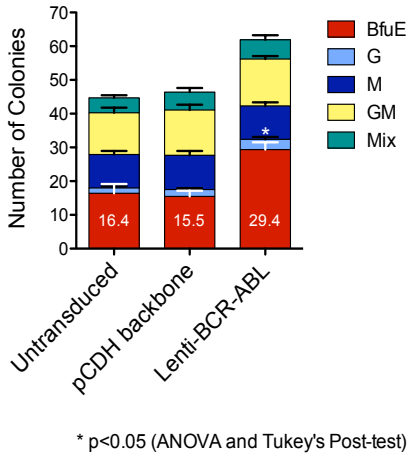


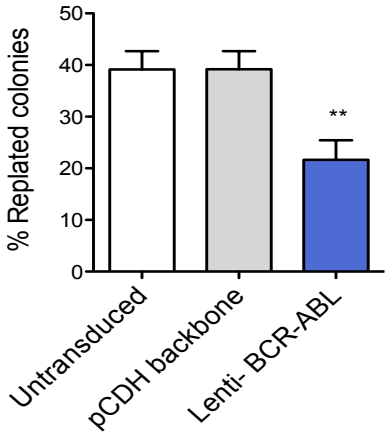
Figure 3-1. Confirmation of BCR-ABL expression construct. A) Transfection with lipofectamine of pCDH-EF1-MCS-T2A-copGFP versus pCDH-EF1-BCR-ABL-T2A-copGFP on 293FT cells shows both parent vector and BCR-ABL containing vector express GFP. **B)** Western blot of BCR-ABL expression after transfection of plasmid DNA and transduction of lentivirus with BCR-ABL expression vectors compared to K562, a CML cell line with endogenous BCR-ABL, probed with anti-Abl. **C)** BCR-ABL transcript levels are highly overexpressed in BCR-ABL transduced CB cells compared to untransduced and parent vector transduced CB CD34+ cells. **D)** p-CRKL expression levels in backbone and BCR-ABL transduced CB CD34+cells. Abbreviations: MEF- mouse embryonic fibroblasts; CB – cord blood

A)



* p<0.05 (ANOVA and Tukey's Post-test)

B)



* p<0.05 (ANOVA and Tukey's Post-test)

Figure 3-2. BCR-ABL increases the percentage of BfuE colonies and reduces self renewal capacity in cord blood CD34+ cells. A) Comparison of untransduced, pCDH backbone transduced, and BCR-ABL transduced CB CD34+ colony formation and **B)** replated colony formation in methylcellulose. n=2 (2 cord blood donors were used, 5 of each colony type was used in replating assay for each). Abbreviations: BfuE- burst forming units erythroid, G – granulocytes, M – macrophage, GM – granulocyte/macrophage, Mix – red blood cell and white blood cell mixed colony

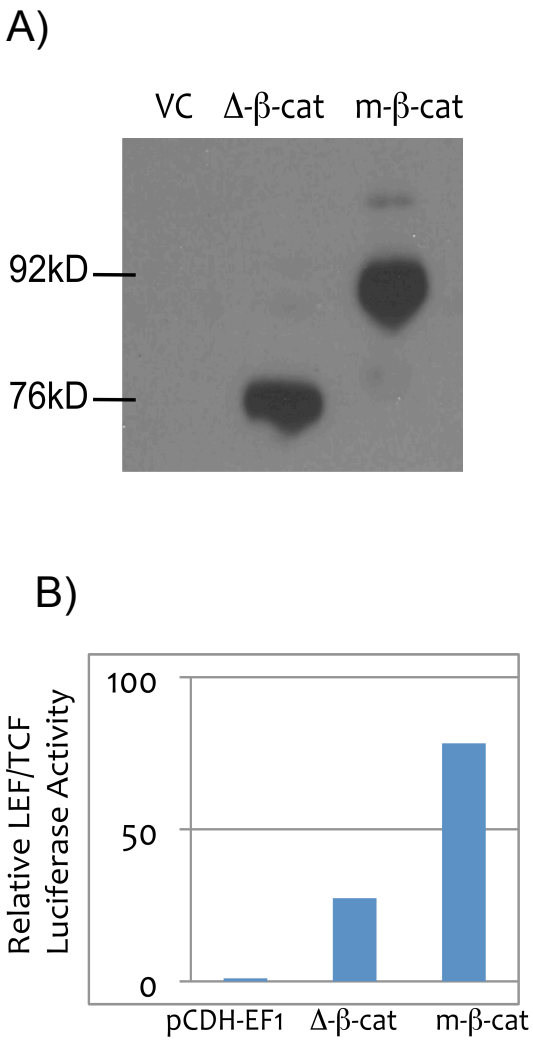


Figure 3-3. Confirmation and Comparison of mutated β-Catenin constructs. A) Western blot of 293 cells transfected with pCDH-EF1-Δ-βCatenin-T2A-copGFP versus pCDH-EF1-mβCatenin-T2A-copGFP. **B)** LEF/TCF reporter assay on 293 cells transfected with each construct shows mutant β-Catenin has more transcriptional activation activity. Abbreviations: VC - parent vector control, pCDH-EF1 – also the parent vector control

A)

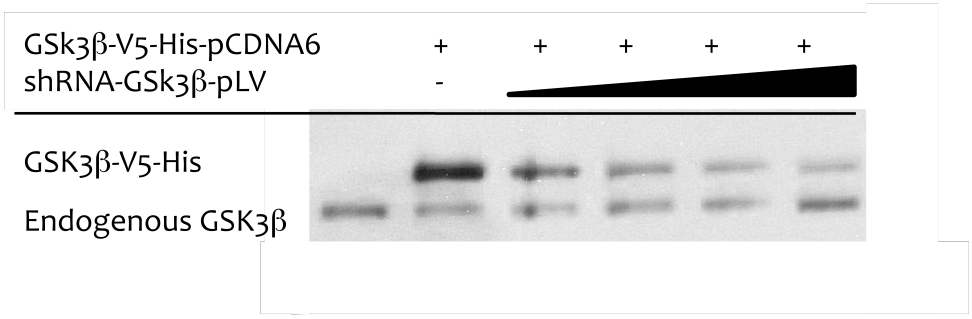


Figure 3-4. Confirmation of GSK3β shRNA construct. A) Western blot of 293 cells transfected with His-tagged GSK3β construct plus increasing amounts of shRNA-GSK3β-pLV construct. Endogenous levels do not change though His-tagged GSK3β levels decrease with increase in shRNA.

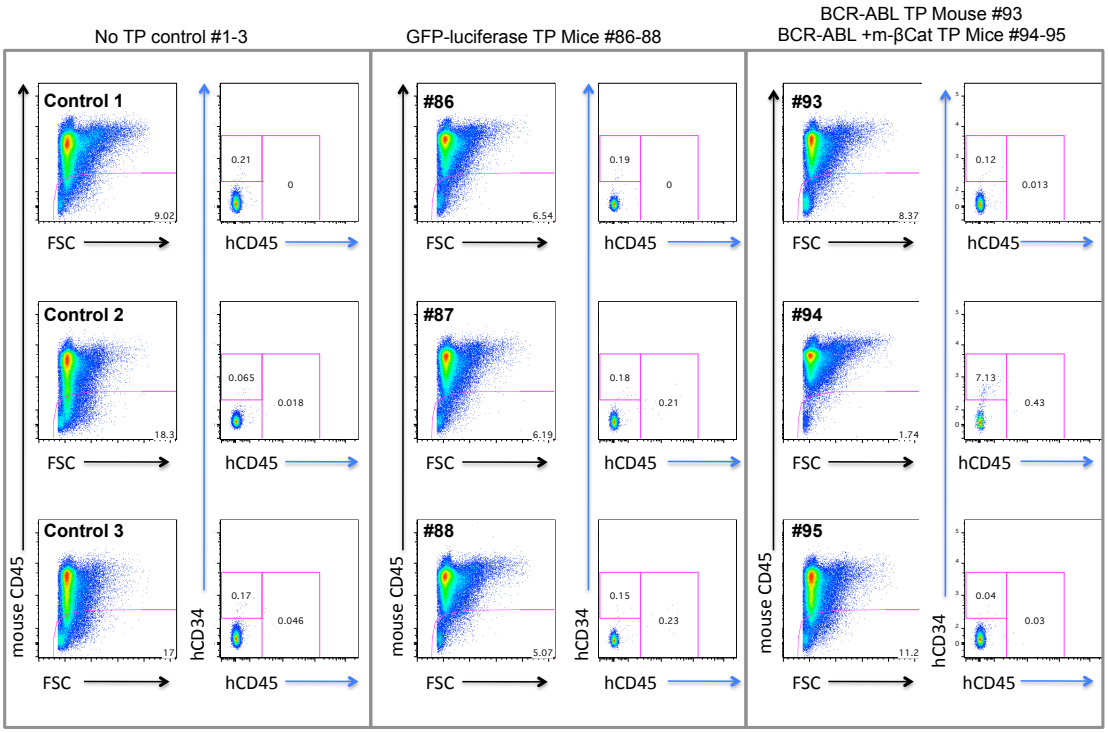


Figure 3-5. **A low percentage of human cells can be found in mouse bone marrow by FACS analysis in several primary transplanted mice. A)** FACS analysis of BM from 3 no TP control mice and 6 TP mice, 3 that received cells transduced with GLF virus, 1 that received cells transduced with GFL and BCR-ABL virus, and 2 that received cells transduced with GLF, BCR-ABL, and m-βCatenin virus. Abbreviations: GLF - green fluorescent protein-luciferase fusion gene, TP – transplant, BM – bone marrow, FACS – fluorescent activated cell sorting.

Table 3-1. mβCatenin overexpression may be necessary to support BCR-ABL positive hematopoietic cell self renewal. Overall primary and secondary TP results of hES derived CD34+ cells. Showing the number of mice transplanted with cells expressing each gene set that were found to contain human cells of any kind (RPL27) or BCR-ABL positive cells in tissues harvested between 8-12 weeks in primary TP and at 15 weeks in secondary TP. Abbreviations: GLF - green fluorescent protein-luciferase fusion gene, RPL27 – ribosomal protein L27, TP – transplant, BM – bone marrow.

	1º TP				2º TP			
Transduced gene(s)	**BCR-ABL (+) nº of mice** (nested PCR)	**Tissue(s) (+)**	**RPL27 (+) nº of mice** (Q-PCR)	**Tissue(s) (+)**	**BCR-ABL (+) nº of mice** (nested PCR)	**Tissue(s) (+)**	**RPL27 (+) nº of mice** (Q-PCR)	**Tissue(s) (+)**
GLF	0/11	n.a.	3/11	Liver, BM	0/15	n.a.	2/15	Liver, BM
GLF + BCR-ABL	3/8	Liver, Spleen	3/8	Liver, BM	0/11	n.a.	1/11	BM
GLF + BCR-ABL + m-βCatenin	2/8	Liver, Spleen	3/8	Liver, BM, Tumor	2/11	Spleen	2/11	Liver
GLF + BCR-ABL + GSK3β shRNA	2/9	Liver, Tumor	2/9	Liver, Tumor	0/10	n.a.	0/10	n.a.
GLF + m-βCatenin	0/6	n.a.	0/6	n.a.	0/12	n.a.	0/12	n.a.

Table 3-2. Mutant β-Catenin nor GSK3β knock down were able to confer self renewal of CP CML patient sample CD34+BCR-ABL+ cells. Overall primary and secondary TP results of CP CML CD34+ cells. Showing the number of mice transplanted with cells expressing each gene set that were found to contain human cells of any kind (RPL27) or BCR-ABL positive cells in tissues harvested between 8-12 weeks in primary TP and at 15 weeks in secondary TP. Abbreviations: GLF - green fluorescent protein-luciferase fusion gene, RPL27 – ribosomal protein L27, TP – transplant, BM – bone marrow.

	1º TP				2º TP			
Transduced gene(s)	**BCR-ABL (+) nº of mice** (nested PCR)	**Tissue(s) (+)**	**RPL27 (+) nº of mice** (Q-PCR)	**Tissue(s) (+)**	**BCR-ABL (+) nº of mice** (nested PCR)	**Tissue(s) (+)**	**RPL27 (+) nº of mice** (Q-PCR)	**Tissue(s) (+)**
GLF	0/18	n.a.	5/18	Liver, Spleen	0/17	n.a.	2/17	Liver
GLF + m-βCatenin	0/18	n.a.	6/18	Liver, BM, Spleen	0/17	n.a.	1/17	Liver
GLF + GSK3β shRNA	1/20	BM	5/20	Liver, BM	0/24	n.a.	4/24	Liver, BM

A)

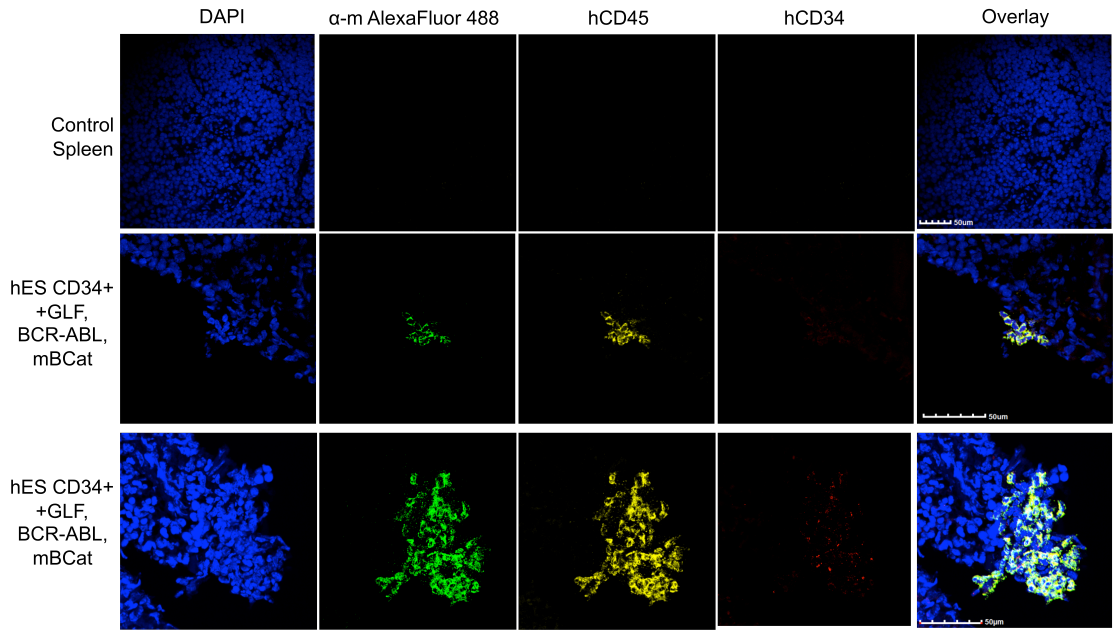


Figure 3-6. CP CML and hES CD34+ transplanted cells become CD45+ and can be found in mouse liver and spleen sections by immunofluorescence. A) Spleen sections from mice that received hES CD34+ cells expressing the genes shown contain human CD34 and CD45+ cells. **B)** Liver sections from mice that received hES or CP CML patient CD34+ cells expressing the genes shown contain human CD45+ cells.

B)

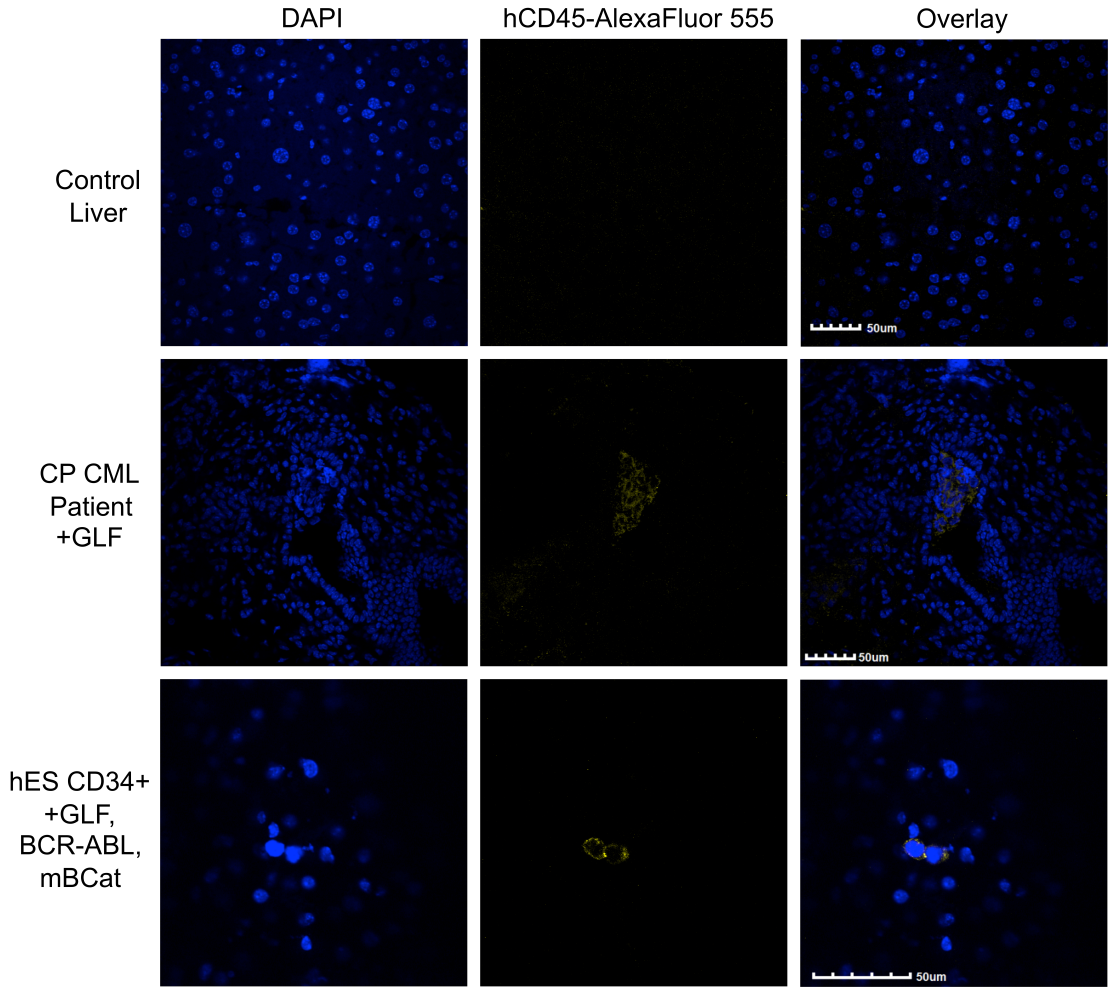


Figure 3-6 continued. CP CML and hES CD34+ transplanted cells become CD45+ and can be found in mouse liver and spleen sections by immunofluorescence. A) Spleen sections from mice that received hES CD34+ cells expressing the genes shown contain human CD34 and CD45+ cells. **B)** Liver sections from mice that received hES or CP CML patient CD34+ cells expressing the genes shown contain human CD45+ cells.

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Chapter 4. Conclusions

Discussion

Research on chronic myeloid leukemia (CML), and cancer in general, was revolutionized by the discovery of the causative reciprocal chromosomal translocation t(9;22) (q34;q11) creating the fusion gene BCR-ABL[1-3]. This fusion gene produces a p210 Bcr-Abl protein product with constitutive tyrosine kinase activity[4-6]. This led to the first highly successful targeted therapy approach to treat cancer using an Abl kinase inhibitor such as imatinitb[7,8]. Approximately 80-90% of CML patients treated with such an inhibitor achieve complete cytogenic remission, which means cells expressing BCR-ABL can no longer be detected[9,10]. However, for those patients that progress to blast crisis (BC) phase of the disease, either through acquisition of imatinib resistance or new mutations, treatments are futile. The literature has a barrage of proposed mutations that may be causative in this transition from chronic phase (CP) to BC, though none have yet to be clearly identified for this role[10-18]. Contributing to this problem is the lack of human cell based models. Most of the evidence that suggests these various mutations play a role in BC development are based on studies in mice. Mouse bone marrow (BM) that expresses BCR-ABL either through retro-viral infection or from a specific transgenic insertion have been shown to develop a replicable CML-like disease within weeks to months[19-21,22-24,25,26]. These models are wonderful tools to study how BCR-ABL causes transformation, the cell types capable and involved in this process, how tyrosine kinase inhibitors affect the disease in these animals, and how BCR-ABL circumvents these inhibitors. However, like many mouse models, the discovery of treatment options for BC patients have waned, possibly due to the lack of efficacy once they reach a patient. Many

treatments are highly effective in mouse models but fail to produce the desired outcomes in humans[27-30]. Therefore, better human cell based models are desperately needed to identify causative mutations and to test and produce effective treatments for CML BC patients.

Although patient samples offer unique qualities such as endogenous mutations, there is a shortage of material which make them challenging to use even for low through-put analysis and treatment screenings. Furthermore, genetic variability between patient samples often confounds research findings and the intra- and inter- heterogeneity of samples makes causative mutations difficult to identify. In addition, only a finite number of normal patient hematopoietic stem cells (HSCs) can be garnered from an individual donor. As HSCs can be neither cultured long-term nor expanded without loosing their stem cell properties, widespread modeling of certain hematopoietic diseases from human HSCs remains challenging. In order to address this need for a genetically homogeneous and tractable human cell based model, we aimed to 1: establish a renewable source of human hematopoietic cells suitable for the study of CML, and 2: to study how BCR-ABL alone or in conjunction with other mutations affects engraftment and self-renewal in these and other hematopoietic cell sources.

To generate a source of human hematopoietic cells to use as a tool to study CML, we adapted a previously published protocol that uses mouse aorta gonad mesonephros (AGM) stromal cell lines in a cocultures system with human embryonic stem cells (hESC) to differentiate hESC to hematopoietic precursor cells. This protocol has to date achieved the highest level of functional hematopoietic stem cells, which was demonstrated by secondary engraftment in a mouse. They were able to

detect the cells by FACS, though levels were extremely low in most cases and engraftment was only achieved in about half of the animals[31]. The fact that this remains to be the best demonstrated technique to produce functional, engrafting HSCs, yet produces engraftment far inferior to that when using cord blood (CB), establishes the need for better differentiation methods[32,33]. Therefore, although we based our hESC differentiation techniques on this method to induce hematopoietic differentiation, we aimed to improve it through the addition of growth factors and to further characterize the cell types it produces.

Initially 3 different hESC lines were tested for their capacity to produce CD34+ cells. CD34 is a reliable a marker of HSCs, hematopoietic progenitor cells, and hemogenic endothelial cells that give rise to HSCs, and is frequently used for this purpose[33-39]. Hues16 routinely produced the highest percentage of CD34+ cells after 15 days in the coculture system. This time-frame also correlated with an increase in several important hematopoietic transcription factors including GATA1, GATA2, Runx1, and SCL, and therefore both this hESC line and the time frame were used in all future experiments. Additionally, the most highly published hESC line in hematopoietic differentiation reports, H1, was included in our hematopoietic development studies as a frame of reference to the majority of the literature. Markers besides CD34 were included in future studies to differentiate what are proposed to be the hemogenic endothelial cells (CD34+CD31+Flk1+), hematopoietic-committed precursor cells (CD34+CD31+Flk1+CD43+), and the non-hemogenic endothelial and/or mesenchymal cells (CD34+CD31-). This system was used to test additional factors that may promote hemogenic endothelial and committed hematopoietic precursor cells. Furthermore, we identified an important hematopoietic

transcription factor, c-Myb, that was insufficiently expressed in Hues16 derived CD34+ cells. Thus, overexpression of c-Myb was examined for its affect on hematopoietic and endothelial gene expression in these distinct hESC derived populations.

Reports suggest BMP4, Activin A, FGF, VEGF and TGFβ play a role in definitive hematopoiesis[40-48,47-53,31,48,49,53-58]. Although variable results were obtained when these factors or combinations of these factors were added to our hESC/UG26 differentiating cocultures, we established that VEGF in combination with FGF enhanced the hemogenic endothelial development from Hues16 by at least 3 fold. This was not observed with H1, though these cells readily differentiate to the hematopoietic lineage compared to most other hESC lines[59,60]. Thus, H1 may be less particular of the differentiation conditions and/or require less guidance from outside factors as they may be inherently primed for hematopoietic development. These results suggest that this system can be used to further elucidate important environmental cues necessary for the efficient differentiation of hESCs to hematopoietic stem cells. However, hESC line variability and possibly the plating variability between wells due to i.e. small clumps of cells, need further investigation before a standard, reproducible protocol can be established.

The results from the microarray analysis that was performed on Hues16 derived CD34+ cells from UG26 coculture differentiations and compared to highly purified CD34+CD90+CD38-Lineage- CB HSCs, found that c-Myb is one of the most differentially expressed genes between these cell types. C-Myb is a major transcription factor necessary for the definitive wave of hematopoiesis and for HSC self-renewal and maintenance[61-64]. After the addition of VEGF and FGF to

Hues16/UG26 cocultures to enhance hemogenic endothelial production, QPCR analysis further displayed how c-Myb is differentially expressed between the hESC derived CD34+CD31- and the CD34+CD31+Flk1+ hemogenic endothelial cells. Both Hues16 and H1 CD34+CD31- cells were found to have very low levels of c-Myb expression, whereas their CD34+CD31+Flk1+ counterparts had around 20 fold enhanced expression. Therefore, we sought to exploit how c-Myb is differentially expressed in these distinct hESC derived CD34+ populations. By overexpressing it in populations with very low levels of expression we hope to delve into c-Myb's role in hematopoietic specification. Furthermore, by overexpressing c-Myb in populations that already express it we hope to see which genes it may be regulating in those phenotypic populations due to their unique genetic environments. Upon overexpression of c-Myb we expect to find several reported target genes to be turned on or enhanced as well as novel direct or indirect targets within each of the sorted populations. Very few studies have been carried out on c-Myb's specific gene regulatory contribution to the definitive wave of hematopoiesis through and therefore novel findings are expected.

Alongside the evaluation of c-Myb's influence on the hematopoietic and endothelial gene expression profiles of the various hESC derived populations, the data collected on non-Myb expressing hESC derived CD34-, CD34+CD31-, CD34+CD31+Flk1+, and CD34+CD31+CD43+ populations will be a great contribution to the hematopoietic and endothelial developmental biology field. Several previous reports have examined the transcriptome differences between mature hematopoietic stem and progenitor cells from fetal liver, fetal blood, and cord blood with that of hESC derived CD34+ populations. Results from these studies have

suggested genes involved in self-renewal pathways and lymphocyte development may possibly be involved in the functional differences between these populations[65,66,67,68,69,70]. Through our gene expression analysis of the above mentioned hESC derived populations, we aim to identifying genes that may promote or hinder the generation of fully functional HSCs from hESC. Although all of these reports have made contributions to the field, they have not identified factors that can yet account for the functional differences between hESC derived CD34+ cells and HSCs. Gene identified through our screen and genes described in these reports should be tested for their ability to alter hESC derived output of CD34+ hematopoietic populations and their HSC functional capacity.

The second goal of this work was to study how BCR-ABL alone or in conjunction with other mutations affects engraftment and self-renewal in our hESC derived CD34+ cells and other hematopoietic cell sources. We used a lentivirus carrying BCR-ABL to efficiently expression the p210 Bcr-Abl protein in CB and hESC derived CD34+ cells. In colony forming assays, BCR-ABL skewed differentiation of CB CD34+ cells to the erythroid lineage. In addition, it reduced the self-renewal capacity of these cells in colony replating assays. Although BCR-ABL causes an expansion of the granulocyte compartment in CML, this increase in red blood cell differentiation was observed previously and attributed to the high levels of overexpression caused by transduction compared to the endogenous levels seen in CML patient samples[71]. However, the reduction in self-renewal is a novel finding in human CB samples and suggests BCR-ABL is not as potent of an oncogene in human hematopoietic stem and progenitor cells as it is in mouse, as mouse BM cells expressing BCR-ABL readily proliferated within weeks to months after transplantation

and can confer serial transplantation that also display robust expansion of white blood cells (WBCs)[19-21,22-24,25,26]. Indeed retroviral expression of BCR-ABL alone was reported to skew transplanted CD34+ CB samples to a myeloid or B-lymphoid fate, but an expansion of BCR-ABL+ white blood cells (WBCs) was only observed in 7.4% of the mice at 5 months[71]. This time frame better mirrors human disease than many mouse models, as CP patients experience a debatable latency of up to 10 years after the acquisition of the initial BCR-ABL mutation[72]. It further supports how important human (cell-based) models are for developing treatments when such a clear distinction exists between human HSC and mouse HSC response to BCR-ABL expression.

To examine BCR-ABL's role in the engraftment capacity of human cells, we transduced hESC derived CD34+ cells with BCR-ABL lentivirus alone or in conjunction with mutant-βCatenin (mβCat) or GSK3β shRNA expressing lentiviruses and transplanted them into highly immunocompromised mice. CD34+ CP CML patient samples, carrying endogenous BCR-ABL, were also transplanted into highly immunocompromised mice with or without prior transduction with mutant-βCatenin or GSK3β shRNA lentiviruses. Through this work we aimed to compare engraftment capacities of these cell types expressing different CML relevant oncogenes. βCatenin and GSK3β are involved in self-renewal pathways and thus may enhance engraftment of BCR-ABL+ cells through overexpression or knock-down respectively[10,13,23,73-78]. BCR-ABL+ cells from CP CML patient samples had a very low engraftment potential compared to their BCR-ABL- normal counterparts and were unable to confer secondary engraftment, where BCR-ABL- CD34+ cells from patients did. Furthermore, we found that neither constitutively active-βCatenin nor GSK3β

shRNA enhanced the engraftment or self-renewal of BCR-ABL+ or BCR-ABL- CP CML patient sample cells. BCR-ABL+ hESC derived CD34+ cells were able to engraft in primary transplanted animals at a similar level to those that did not express BCR-ABL or expressed mβCat or GSK3β shRNA in addition to BCR-ABL. However, BCR-ABL+ cells co-expressing mβCat were able to confer secondary transplantation where no other BCR-ABL+ cells, from hESC or CP CML samples, had this capacity. This suggests that hESC derived CD34+ cells may express factors that enhance their ability to engraft when expressing BCR-ABL alongside mβCat compared to CP CML patient samples. Although BCR-ABL is more highly expressed in the hESC CD34+ cells over the endogenous expression in patient samples, through our CB colony forming studies was saw a reduction in self-renewal from BCR-ABL. Therefore something inherent in the hESC CD34+ cells and not in CP CML patient cells may be overcoming this when mβCat is present.

Future studies could examine cell type specific functional and molecular differences that may further elucidate BCR-ABL transformation in human cells. Our gene expression profiling studies on the different CD34+ and CD34- populations derived from hESCs may lead to potential clues that could explain why hESC derived CD34+ cells could confer self-renewal when expressing BCR-ABL and mβCat though CP CML patient samples were not, such as differential survival or self-renewal gene expression. Furthermore, mirroring these engraftment experiments with CD34+ CB samples would be interesting. CB CD34+ cells readily engraft and confer secondary transplantation, whereas only low levels of hESC derived CD34+ have been shown to engraft in our experiments and those carried out by others[79-84]. Therefore, BCR-ABL's effect on self-renewal, localization, and differentiation could be further

examined in cells that can be identified and characterized by flow cytometry after engraftment in different organs of the mouse. BCR-ABL+ LSC localization may be particularly interesting as we observed that the hESC derived CD34+ BCR-ABL+ cells expressing mβCat that were found in secondary transplanted animals as well as BCR-ABL+ cells found in primary transplanted animals, were located only in the spleen or the liver, which was the site of injection. All BCR-ABL- cells that conferred primary or secondary transplantation were found only in the livers and BM. Differences in BCR-ABL+ cells that reside in the spleen versus the BM of a transgenic CML mouse model have also been reported, suggesting this may have important implications in the study of BCR-ABL+ leukemic stem cells (LSCs) and how treatments are developed[26,85]. Furthermore, using induced pluripotent stem cell (iPSC) technology to create CML patient specific iPSC lines that express endogenous levels of BCR-ABL in CP and contain other mutations in BC may be beneficial. These experiments may aid in teasing apart which cell types localize to which organs and how endogenous levels of BCR-ABL alter self-renewal, survival, and proliferation compared to transduced hESC derived or CP CML CD34+ cells. The homing, surface marker expression, genetic changes, and environmental cues necessary to maintain the LSC population are characteristics that treatment could potentially exploit, thus making identifying these as they relate to stage specific LSCs of the utmost importance. Utilizing new human cell based models may lead us to discoveries that mouse models have not identified thus far.

In summary, hESC derived CD34+ cells can be used for both modeling of hematopoietic development and CML development, as they have unique properties. These cells show a more primitive genetic and surface expression profile compared

to that of CB HSCs, yet have the capacity to engraft along the hematopoietic lineage in highly immunocompromised animals and express important hematopoietic regulatory genes. Further identifying how these cells differ from fully functional HSCs may lead to clues as to how to create these highly sought after hESC derived HSC from the limitless source that hESCs offer. With this knowledge, better hESC based LSC models can be developed to expand our understanding of how BCR-ABL causes transformation of specific populations of human cells and how CP CML progresses to the currently untreatable phase of CML, BC.

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