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Establishing a Robust In Vitro Embryonic Stem Cell Differentiation Assay to Monitor the Hematopoietic Potential of DELES Clones

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Résumé

Afin d'effectuer des études fonctionnelles sur le génome de la souris, notre laboratoire a généré une bibliothèque de clones de cellules souches embryonnaires (ESC) présentant des suppressions chromosomiques chevauchantes aléatoires – la bibliothèque DELES. Cette bibliothèque contient des délétions couvrant environ 25% du génome murin.

Dans le laboratoire, nous comptons identifier de nouveaux déterminants du destin des cellules hématopoïétiques en utilisant cet outil. Un crible primaire utilisant la benzidine pour démontrer la présence d'hémoglobine dans des corps embryoïdes (EBS) a permis d'identifier plusieurs clones délétés présentant un phénotype hématopoïétique anormal. Comme cet essai ne vérifie que la présence d'hémoglobine, le but de mon projet est d'établir un essai *in vitro* de différenciation des ESC permettant de mesurer le potentiel hématopoïétique de clones DELES. Mon hypothèse est que l'essai de différenciation hématopoïétique publié par le Dr Keller peut être importé dans notre laboratoire et utilisé pour étudier l'engagement hématopoïétique des clones DELES. À l'aide d'essais de RT-QPCR et de FACS, j'ai pu contrôler la cinétique de différenciation hématopoïétique en suivant l'expression des gènes hématopoïétiques et des marqueurs de surface comme CD41, c-kit, RUNX1, GATA2, CD45, β-globine 1 et TER-119. Cet essai sera utilisé pour valider le potentiel hématopoïétique des clones DELES candidats identifiés dans le crible principal.

Mon projet secondaire vise à utiliser la même stratégie rétro-virale a base de Cre-*lox*P utilisée pour générer la bibliothèque DELES pour générer une bibliothèque de cellules KBM-7 contenant des suppressions chromosomiques chevauchantes. Mon but ici est de tester si la lignée cellulaire leuémique humaine presque haploïde KBM-7 peut être exploitée en utilisant l'approche DELES pour créer cette bibliothèque. La bibliothèque de clones KBM-7 servira à définir les activités moléculaires de drogues anti-leucémiques potentielless que nous avons identifiées dans le laboratoire parce qu'elles inhibent la croissance cellulaire dans plusieurs échantillons de leucémie myéloïde aiguë dérivés de patients. Elle me permettra également d'identifier les voies de signalisation moléculaires qui, lorsque génétiquement perturbées, peuvent conférer une résistance à ces drogues.

Mots-clés: cellules souches embryonnaires, différenciation hématopoïétique, cellules KBM-7, suppressions chromosomiques.

Abstract

To carry out functional studies on the mouse genome, our laboratory has generated a library of Embryonic Stem Cell (ESC) clones harboring random nested chromosomal deletions – DELES library. This library contains deletions covering ~ 25% of the mouse genome.

In the lab, we are interested in identifying novel hematopoietic cell fate determinants using this resource. A primary screen using benzidine to demonstrate the presence of hemoglobin in embryoid bodies (EBs) was able to identify several DELES clones exhibiting abnormal hematopoietic phenotype. Since this assay only tested for the presence of hemoglobin, the goal of my project is to establish a robust *in vitro* ESC differentiation assay to monitor the hematopoietic potential of DELES clones. My hypothesis is that the hematopoietic differentiation assay published by Dr. Keller can be used to observe hematopoietic commitment of the DELES clones. Using QRT-PCR and FACS assays I was able to monitor the kinetics of hematopoietic differentiation by observing the expression of hematopoietic genes and surface markers including CD41, C-KIT, RUNX1, GATA2, CD45, β-GLOBIN 1 and TER-119. This assay will be used to validate the hematopoietic potential of the candidate DELES clones identified in the primary screen.

My secondary project aims to use the same retro-viral Cre-*lox*P strategy used for the DELES library, in order to generate a library of KBM-7 leukemic cells harboring nested chromosomal deletions. My goal here is to test if the human near haploid KBM-7 cell line can be exploited using the DELES approach to create this library. The library of KBM-7 clones will be used to delineate the molecular activities of potential anti-leukemic drugs that we have identified in the lab to inhibit cell growth in several patient-derived acute-myeloid leukemia specimens. It will also allow me to identify molecular signaling pathways that, when genetically disrupted, can confer resistance to these drugs.

Keywords: Embryonic stem cells, hematopoietic differentiation, KBM-7 cells, chromosomal deletions.

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AGM - Aorta Gonad Mesonephros

APC/Cy7 - Allophycocyanin/Cyanine7

BAC - Bacterial Artificial Chromosomes

BM - Bone Marrow

BMP4 - Bone Morphogenetic Protein 4

bp - basepair

BSA - Bovine Serum Albumin

CCC- Compound Correlation Clusters

cDNA - complementary DNA

CLP - Common Lymphoid Progenitors

CML – Chronic Myeloid Leukemia

CMP - Common Myeloid Progenitors

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

Dctn6 - dynactin 6

DELES – Deletion in Embryonic Stem Cells

DMEM - Dulbecco's Modified Eagle Medium

DNA – deoxyribonucleic acid

DSB – double stranded break

dsRNA – double stranded Ribonucleic Acid

EB – Embryoid Body

EPO - Erythropoietin

ESC – Embryonic Stem Cell

EUCOMM - European Conditional Mouse Mutagenesis Program

FACS - Fluorescence-activated cell sorting

FBS - Fetal bovine serum

FDA - Food and Drug Administration

FSC – Forward Scatter

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GATA 1- GATA Binding protein 1

GATA2 - GATA Binding protein 2

GFP - Green fluorescent protein

GM-CSF - Granulocyte-macrophage colony-stimulating factor

Gsr - glutathione-disulfide reductase

Gtf2e2 - General transcription factor IIE subunit 2

HAT - Hypoxanthine-Aminopterin-Thymidine

HDR - Homology Directed Repair

HEK293 - Human Embryonic Kidney 293 cells

HPRT - Hypoxanthine-guanine phosphoribosyltransferase

HPRT - Hypoxanthine-guanine phosphoribosyltransferase

HSC – Hematopoietic Stem Cell

ICM – Inner Cell Mass

IKMC - International Knockout Mouse Consortium

IL-3 - Interleukin 3

IL-6 - Interleukin 6

IMDM - Iscove's Modified Dulbecco's Medium

INDELs - Insertions and/or Deletions

I-PCR - Inverse - Polymerase Chain Reaction

iPS - Induced Pluripotent Stem Cell

JAK/STAT - Janus kinase/signal transducers and activators of transcription

KDR - Kinase Insert Domain Receptor

KOMP - Knockout Mouse Project Repository

Leprotl1 - Leptin Receptor Overlapping Transcript-Like 1

LIF - Leukemia inhibitory factor

Mboat4 - Membrane Bound O-Acyltransferase Domain Containing 4

MEF – Mouse Embryonic Fibroblast

mESC – Mouse Embryonic Stem Cell

MMLV-RT - Moloney Murine Leukemia Virus Reverse Transcriptase

MPP - Multi-Potent Progeny

mRNA – messenger RNA

MSC – Mesenchymal Stem Cell

MSCV - Murine Stem Cell Virus

NHEJ - Non Homologous End Joining

NorCOMM - North American Conditional Mouse Mutagenesis Project

PBS - Phosphate-buffered saline

PFHM II - Protein Free Hybridoma Medium II

PGC – Primordial Germ Cell

PIGF- Placental Growth Factor

qRT-PCR - Quantitative Real Time Polymerase Chain Reaction

Rbpms - RNA Binding Protein with Multiple Splicing

RISC - RNA-induced silencing complex

RNA - Ribonucleic Acid

ROS - Reactive oxygen species

RUNX1- Runt-related transcription factor 1

S.D – Standard Deviation

SCF - Stem Cell Factor

shRNA – Short hairpin RNA

siRNA – Small Interfering Ribonucleic Acid

SSC - Side Scatter

T - Brachyury

TALEN- Transcription activator-like effector nucleases

TALEs - Transcription activator- like effectors

TGF-β - Transforming Growth Factor- β

Tmem 66 - Transmembrane Protein 66

TPO - Thrombopoietin

VEGF – Vascular Endothelial Growth Factor

WT – Wild-type

ZFN – Zinc Finger Nuclease

Liste des abréviations

µg - microgram

℃ - Celsius

et al - et alia

mL – millilitre

mm - millimetre

ng - nanogram

nm - nanometre

Rpm - Revolutions per minute

T.U - Transducing Unit

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1. Introduction

Embryonic stem cells (ESCs) have two main characteristics – self renewal and pluripotency. Self-renewal is a mechanism by which a cell can generate a daughter cell identical to itself. ESCs undergo symmetric cell division to generate two identical daughter cells. Pluripotency refers to the ability of a cell to differentiate into the three primary germ layers – ectoderm, mesoderm and endoderm. [1]

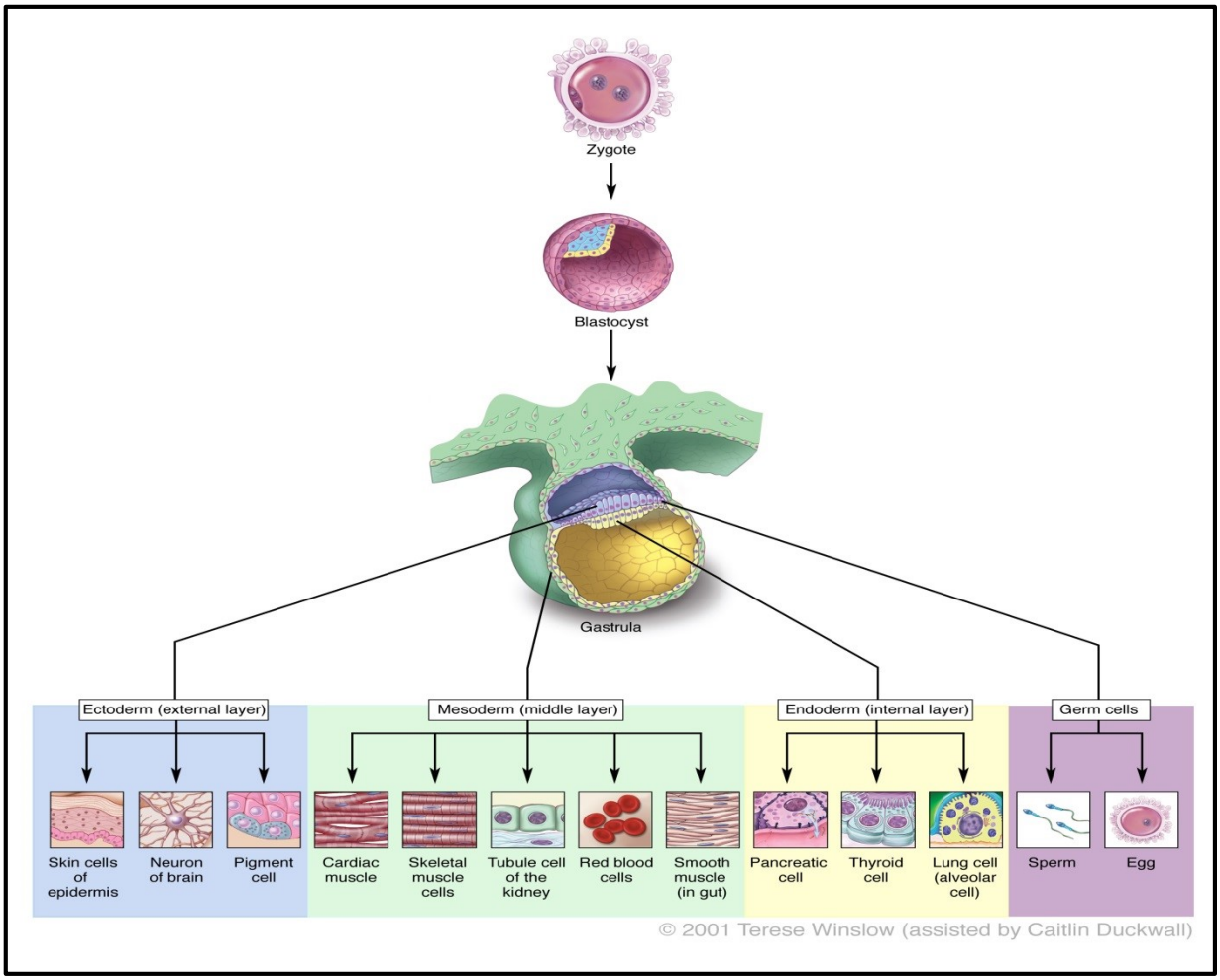

Figure 1: Illustration of the origin and differentiation of the three germ layers.

(Permission obtained from Terese Winslow

http://stemcells.nih.gov/StaticResources/info/scireport/images/figure11.jpg)

Mouse Embryonic Stem Cells (mESCs) are pluripotent cells isolated from the inner cell mass (ICM) of day 3.5 preimplantation blastocyst. The ICM isolated from the blastocyst has the potential to give rise to all the three germ layers. It is however unable to contribute to the trophectoderm and primitive endoderm lineages. [2] When maintained under appropriate conditions these ICM cells give rise to ESC cell lines that have normal karyotype and high telomerase activity. Each cell line is defined by colonies derived from a single blastocyst; therefore all ESC cell lines are genetically unique. Most ES cell lines are 40XY, this is because in XX ES cell lines there is a global reduction of DNA methylation due to the presence of two active X chromosomes (one X chromosome eventually becomes inactive). This hypomethylation affects the long term maintenance of these cell lines in culture. [3, 4] One of the most debated questions in the embryonic stem cell field is whether ES cells are artifacts of tissue culture or/and whether they relate to a specific cell type *in-vivo*? ESCs exhibit some characteristics that are not shown *in vivo* by cells within an embryo; an example of one such property is that, no cell in the embryo demonstrates long-term self-renewal and pluripotency. When embryonic cells are brought to culture and exposed to specific signals (to which they wouldn't have been exposed to *in vivo*), they adapt and procure novel functions that enable them to maintain their pluripotency and self-renewal abilities indefinitely. Studies have suggested that within the embryo there is a transient population of cells that are pluripotent and possess self-renewal capabilities, however, this population is present *in vivo* for only a limited period of time. These cells become more restricted in their potency as development progresses. Therefore, one can say that ES cells are a tissue culture artifact. [5]

ES cell lines can be derived from the ICM, primitive ectoderm and possibly from early germ cells. (Figure 3) mESCs are not equivalent to ICM cells because the ICM cells are capable of differentiating into the trophoblast whereas mESCs cannot. [5] mESCs are not entirely equivalent to germ cells as early germ cells do not self-renew for an extended period of time. It is shown that primordial germ cells (PGCs) do not contribute to chimeras when injected into blastocysts. [5] Studies have shown a founder population of cells that emerges from the primitive ectoderm before gastrulation that passes through the primitive streak to give rise to many structures of the extraembryonic mesoderm and the germ cells. The inductive signaling

involved in this process might be reproducible *in vitro*. ESCs might be related to this founder population. [5]

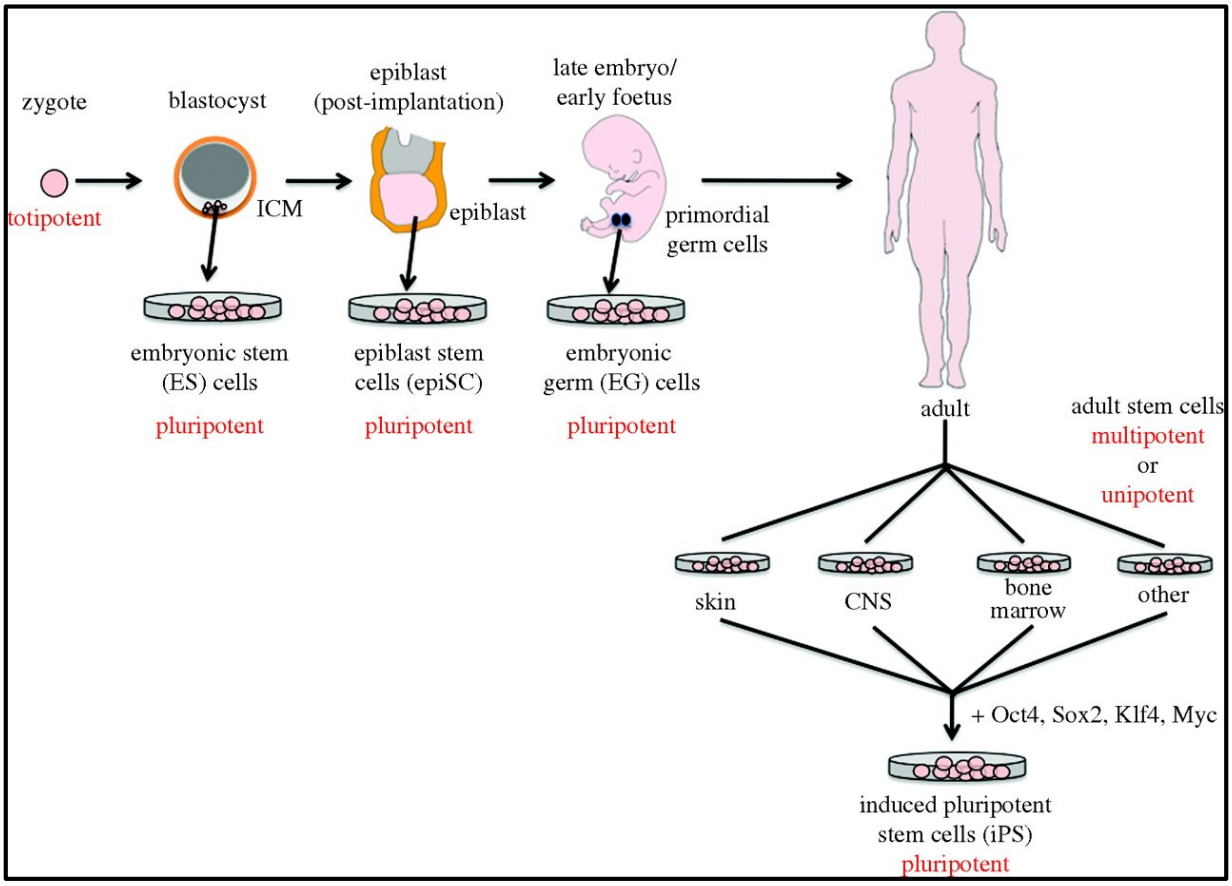

Figure 2: Illustration of the origin of stem cells. (Fiona M. Watt, Ryan R. Driskell. Phil. Trans. R. Soc B (2009).365 155-163, Permission obtained from the journal).

Embryonic stem cells are an interesting model in research for several reasons. Due to their self-renewal ability, they can be cultured *in vitro* for an extended period of time - serving as a constant supply of starting material. [6] mESCs can be used to generate mouse chimeras when re-introduced into a blastocyst. The pluripotency of ESCs enable them to differentiate into the three germ layers, making them an excellent model to study most developmental processes. Finally their genome is well characterized and easily modifiable using different mutagenesis strategies. [6, 7]

1.1 Genomic approaches used to conduct functional studies on ESCs.

Over the years, several genome editing strategies have been developed to conduct functional studies on the mouse genome. In this section, I will briefly explain a few strategies that have gained popularity in the past decade.

1.1.1 Gene targeting

Gene targeting is an approach that relies on homologous recombination to modify an endogenous target gene. [8] This strategy requires the creation of a vector that contains a part of the gene to be modified, a reporter gene and a selection marker gene flanked by two homology arms. [8] To prevent unwanted effects caused by the presence of selection marker genes, Cre-*lox*P or Flp-*frt* technologies can be used to excise these genes.

The type and exact location of the mutation is known to us when we use this strategy. This offers us an advantage when we wish to recover these mutations. One of the drawbacks associated with this approach is that it requires germ-line competent ES cells. When recombinant ES cells are injected into the blastocoel cavity of 3.5 day old embryos to generate chimeric mice, it is important they colonize the germ cells of the animal to propagate the targeted mutation to subsequent generations. When using any genetic mutation strategy we must keep in mind that genetic redundancy can result in compensatory effects thereby possibly impeding the functional analysis of the mutation. [9] Sometimes the targeted gene of interest is involved in embryonic viability in the early stages of development and has several different functions at the later stages of development. [9] Abrogation of such genes results in embryonic lethality preventing the study of the genes' function in later developmental processes. The design of conditional recombinase systems, allowing the abrogation of gene expression in a spatio-temporal controlled manner has helped overcome this limitation. [10]

The International Knockout Mouse Consortium (IKMC), Knockout Mouse Project Repository (KOMP), European Conditional Mouse Mutagenesis Program (EUCOMM) and North American Conditional Mouse Mutagenesis Project (NorCOMM) are some of the international efforts in place that employ gene targeting to study the functional mammalian genome.

1.1.2 siRNA and shRNA screens

Though RNA interference technology is not a genome editing strategy, it allows us to study loss of function effects of specific genes in the mammalian genome by degrading the specific gene mRNAs. [11] Two approaches that have gained interest for achieving gene silencing are ds-small interfering RNA (siRNA) and vector based short hairpin RNA (shRNAs). While both approaches result in gene silencing, they differ in their mechanisms of action. In siRNA screens, dsRNA is synthesized complementary to the gene of interest and is introduced into the cytosol of the cells through transfection. Upon introduction to the cell, a ds-RNA specific enzyme Dicer forms a complex with long dsRNAs and cleaves them into shorter fragments of 20-25bp. The cleaved fragments are then incorporated into the RISC complex which is comprised of Arogonaute 2 (Ago-2), Dicer and TAR-RNA binding protein (TRBP). The RNA duplex is dissociated, the strand with the lowest duplex stability remains in complex with the RISC while the other strand is removed.[12]

shRNAs can be delivered to the cells by infection with virally produced vectors or through transfection with plasmid vectors encoding them. shRNAs consists of two complementary 19-22 bp RNA sequences linked by a loop of 4-11 nucleotides. shRNAs are transcribed by RNA polymerase II or III based on the promoter driving their expression. Drosha and its dsRNA-binding partner DGCR8 processes these initial precursors to pre-shRNAs which is then exported to the cytoplasm by Exportin-5. Dicer and TRBP/PACT cleaves these pre-shRNAs, removing the hairpin and creating a 20-25 nucleotide ds-siRNA which is then loaded onto the RISC complex.[12]

Once the fragments are loaded onto the RISC, the process by which siRNA and shRNA recognizes and degrades the target mRNA is the same. As part of the RISC, siRNA binds to the target mRNA mediated by complementary base pairing. This leads to the cleavage of the target RNA phosphate backbone around the center of the duplex by the action of Ago-2. RNAi-mediated cleavage products are degraded by the same enzymes responsible for degrading the bulk cellular mRNA.

Factors such as cell type and requirement for a transient or stable integration determine the method of choice to be employed. Off-target effects need to be considered when working with siRNAs and shRNAs. These off-target effects can be mitigated by using more than 3

siRNAs/shRNAs for the gene of interest. [12] Additional controls include non-targeted/scrambled siRNAs and shRNAs.

Several siRNA and shRNA screens have been carried out in ESCs using different readouts such as reporter assays, cell morphology and proliferation assays to study ESC pluripotency and self-renewal. [13, 14]

1.1.3 ZFNs, TALENs and CRISPR

Previously, Zinc Finger Nucleases (ZFNs) and Transcription activator-like effector nucleases (TALENs) were the most widely used targeted genome editing strategies. Both these technologies use engineered nucleases, i.e. artificial proteins that are composed of a customizable sequence-specific DNA binding domain that is fused to a nuclease that cleaves DNA non-specifically. [15]

ZFN is a heterodimer, where each subunit consists of a zinc finger DNA binding domain and a Fok1 endonuclease domain. To achieve a double strand break (DSB) the Fok1 domains must dimerize. Each Zinc Finger (ZF) motif folds into a ββα structure that consists of ≃ 30 amino acids. The binding of ZF motifs to DNA takes place by the insertion of the α- helix into the major groove of the DNA double helix. Each zinc finger binds to a triplet within the substrate DNA. The amino acid residues at -1,+1,+2,+3,+4,+5 and +6 positions to the start of the α-helix contribute to the sequence-specific interactions between the ZF motifs and the DNA site. To generate ZF motifs with different sequence specificities, amino acids at these positions can be changed while maintaining the remaining amino acids as a consensus backbone. Several ZF motifs can be linked in tandem to form Zinc Finger Proteins (ZFPs) to enable binding to longer DNA sequences.[16]

Specificity and affinity of ZF sets can be a challenge. To obtain adequate affinity, atleast 3 fingers in each ZFNs are required. Addition of fingers can improve specificity as well as affinity , however, there is a possibility that they might mediate binding to off-target sites. Zinc finger technology is relatively expensive and can be tricky to synthesize. [15, 17]

The TALEN system is similar to ZFN but more flexible. TALENs too contain the Fok1 endonuclease domain that is fused to a DNA binding domain. The DNA binding domain

consists of highly conserved repeats derived from transcription activator- like effectors (TALEs). TALEs can be designed to bind to any target DNA sequence. TALEs are proteins secreted by the Xanthomonas bacteria to alter transcription genes in host plant cells. The DNA binding domain consists of 33-34 amino acid sequence which is highly conserved. Within this sequence, the 12^{th} and the 13^{th} amino acid location is highly variable and is involved in specific nucleotide recognition. TALENs, like ZFNs function as dimers, thereby it requires two unique DNA binding domain sites in the genome with the correct orientation and spacing to induce a DSB. ZFNs and TALENs induced DSB can be repaired by cellular Non Homologous End Joining (NHEJ) or Homology Directed Repair (HDR) pathways. NHEJ-mediated repair of a DSB in a gene results in insertions and/or deletions (INDELs) causing frameshift mutations that disrupts gene function. HDR repair pathway can also be exploited to introduce specific nucleotide modifications by supplying the double stranded DNA of interest as template. [15, 17]

Currently, clustered regularly interspaced short palindromic repeats (CRISPR) is the most popular targeted genome editing tool, allowing to edit genomes with unprecedented efficiency, precision and flexibility. CRISPR/Cas9 is part of the bacterial adaptive immune system. Cas9 is an endonuclease that makes a double stranded break in the DNA. The site for DNA break is determined by a guide RNA of ~20 nucleotides. Cas9 induced DSB are repaired via the NHEJ pathway. The NHEJ pathway is more error prone compared to the HDR pathway and thereby introduces INDELs resulting in impaired gene function. Alternatively, precise replacement mutations can also be made if a donor template having a mutation and homology to the targeted locus is supplied. [18]
Although genome wide specificities of the CRISPR-Cas9 system has yet to be entirely defined, several modifications of this system such as inducible Cas9 strategies are being employed to provide a platform to study loss of function phenotypes. [19]

1.1.4 Cre-*lox*P

The Cre-*loxP* recombination strategy of Coliphage P1 provides a unique tool to manipulate the mouse genome. The site specific DNA recombinase Cre (**c**yclization **re**combination) is a 38 kDa protein recognizing 34bp *lox*P sites and catalyzes inter and intramolecular recombination

between them. The *loxP* site consists of a nonpalindromic 8bp core region flanked by two palindromic 13bp sequences. [20]

This strategy brings about chromosomal rearrangements between two *lox*P sites, referred to as endpoints. The recombination between two *lox*P sites results in chromosomal deletions, inversions and duplications if the *lox*P sites are located on the same chromosome whereas recombination between *lox*P sites located on different chromosomes results in translocations. To create a deletion, two regions on the same chromosome are successively targeted by homologous recombination using different vectors. Each vector contains a sequence for a *lox*P site. Cre recombinase expression results in the excision of DNA sequences between the *lox*P sites that have been integrated. [21] To isolate ESC clones in which successful recombination and deletion of genes have occurred, two non-functional halves of a selection marker gene are inserted in each of the vector containing the loxP site. [22, 23] For example the Conlon group previously used a vector that included a non-functional "split" Hprt1 cassette along with the first *lox*P site. The first *lox*P site was introduced into a particular locus by homologous recombination whereas the second *lox*P site and the second half of the Hprt1 cassette was introduced using retro-viral gene transfer. Cre recombinase expression resulted in deletion of chromosomal regions between the *lox*P sites and reconstitution of the functional Hprt1 gene. These recombinants were selected in Hypoxanthine-aminopterin-thymidine (HAT) medium. [23] HAT is a selection medium for mammalian cell culture. Aminopterin blocks DNA *de novo* synthesis which is essential for cell division. Hypoxanthine and thymidine provide the cells with essential components required for them to evade the blockage and proceed with the salvage pathway, provided the cells have functional copies of the gene encoding the HPRT enzyme.

The Cre-*lox*P system can be used to conditionally modulate tissue-specific gene expression. [24] The *lox*P sites can be introduced around a functionally essential genomic region followed by a tissue-specific Cre recombinase mediated excision of the *lox*P flanked sequence. A similar strategy can be used for tissue specific overexpression of a transgene, where a strong expressing promoter is separated from the coding region of a gene by "stop" sequences flanked by *lox*P sites. Although in both cases Cre recombinase transgene allows spatial

control, once Cre expression is induced and recombination has occurred, the resultant gene expression is mostly irreversible.

Animal models for spatial and temporal gene regulation have been generated by combining the Cre-*lox*P technology with inducible systems. Transgenic mice containing the gene of interest surrounded by *lox*P sites are crossed with transgenic mice that have tissue-specific expression of the Cre gene. The resulting mice will have the target gene deleted in tissues expressing Cre whereas the target gene will be present and function normally in tissues that do not express the Cre gene. These animals could be used to study specific tumor signature profiles and signaling pathways relevant to tumor supressors and oncogenes during tumor progression. Additionally, studies combining the Cre-*lox*P inducible system with RNAi have been used to efficiently knock down endogenous genes. [25]

1.1.5 DELES approach

In our laboratory, we used a Cre-*lox*P strategy to carry out functional studies on the mouse genome. [26, 27] Simon Fortier and Melanie Bilodeau employed a retro-viral based Cre-*lox*P approach to create a library of ESC clones harboring hemizygous nested chromosomal deletions – the DELES (**Del**etion in **ES** cells) library. This strategy was different from the Cre-*lox*P strategies used by other groups as it did not use homologous recombination to deliver *lox*P sites to specific sites in the genome. The sequential addition of two retroviruses – anchor virus and saturation virus were used to deliver the *lox*P sites. The anchor virus consisted of a *lox*P site, a functional puromycin gene and a truncated ATG-less neomycin gene. The saturation virus consisted of a *lox*P site, a functional hygromycin gene and a PGK-ATG promoter cassette that on Cre recombination would drive the expression of a functional neomycin gene. (Figure 3) The retrovirus design used to generate the library is as illustrated below:

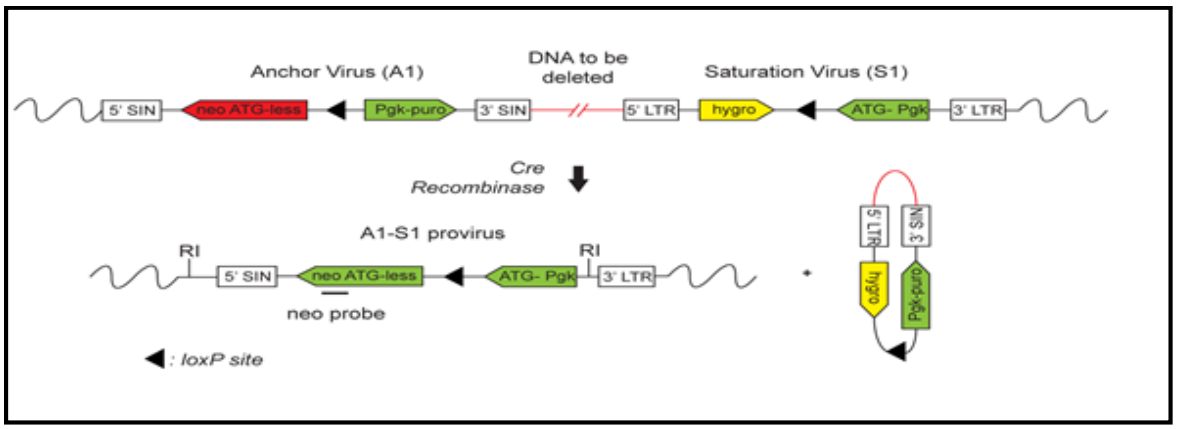

Figure 3: Retro-viral design of the primary A1 and secondary S1 Saturation virus.

(Fortier, S., et al., Genome-wide interrogation of Mammalian stem cell fate determinants by nested chromosome deletions. PLoS Genet, 2010. **6**(12): p. e1001241. , Permission obtained from Simon Fortier.)

R1 ESCs were first infected with the A1 anchor virus to generate primary clones. The ESCs were infected with an infection efficiency of < 1% to achieve single provirus integration per cell. The puromycin resistant clones were next infected with low titer saturation virus to generate hygromycin resistant secondary clones. Cre-recombinase induced gene deletions were carried out by introducing a Cre-plasmid in ESCs through electroporation. Puromycin selection was again performed on $G418^R$ tertiary clones to identify clones that harbor gene deletions rather than other re-arrangements such as inversions and duplications. Inverse-PCR was used to map the deletions obtained. This library contains chromosomal deletions covering ~ 25% of the mouse genome with a 3Mb average deletion size. The average genome coverage per autosome is 23% with no deletions on 19, X and Y chromosomes.

To evaluate genome coverage in DELES, the cohorts of mapped deletions were categorized according to their molecular function based on gene ontology analysis. The 25.4% genome coverage of DELES includes 7083 genes, 4265 CpG islands, 128 miRNAs, 470 lincRNAs, 241 ultraconserved elements, 648571 LINE/SINE elements and 108 cancer related genes. Clusters of protein-coding and non-coding elements were also deleted in this library of ES clones, enabling us to analyze synthetic interactions and functional redundancies between

family members (clones that have the anchor virus integration at the same point in the genome but different saturation virus integrations) of DELES. [26]

Advantages of DELES strategy:

I. Integration of the *lox*P sites was achieved by retro-viral gene transfer and not gene targeting, thereby the library was generated quickly and was less labor-intensive.
II. Nested chromosomal deletions were generated enabling us to interrogate protein coding and non-coding regions associated with a phenotype.
III. Alleles of the genes are permanently deleted and not just silenced.

The DELES clones can be used to generate homozygous mutant mice to perform *in vivo* phenotypic studies of dominant and recessive mutations. For example, in our lab, Melanie Bilodeau wanted to assess the *in vivo* biological roles of two cystatin proteins Stfa2l1 and Csta, the dysregulation of which has been observed in cancers such gastric cancer, head and neck carcinomas. To this end, she generated chimeric mice by injecting the DELES clone 7-30 harboring a 95-kb chromosomal deletion whose deletion spanned four genes (*Fam162a, Ccdc58, Csta and Stfa2l1)* into C57BL/6J morulas and blastocysts. To achieve germline transmission of the mutant allele, the two chimeric mice were crossed with C57BL/6J females. Homozygous mutant mice were generated by intercrossing heterozygous mutant mice. PCR studies were conducted on the genomic DNA isolated from mouse tail clips to confirm the loss of the genomic regions covered by the chromosomal deletion in the homozygous mice. She reported that Csta and Stfa2l1 were dispensable for viability, fertility and hematopoietic activity. [27]

Additionally, Simon Fortier exploited the DELES library to identify ES cell fate determinants. The goal of the study was to identify minimal genetic regions which when deleted blocked normal embryoid body development. LIF and BMP signaling maintains ESCs in their undifferentiated state in vitro. Upon removal of these signals, ESCs differentiate into aggregated structures called embryoid bodies (EB). These 3-D aggregate structures contain cells derived from the three germ layers and their formation seems to recapitulate several

differentiation processes observed *in vivo* in a spatio-temporal manner. Melanie Bilodeau provided evidence that within our DELES library, there is a co-relation between EB formation competency *in vitro* and contribution to chimeras *in vivo*. Clones which showed normal in vitro EB differentiation contributed to the generation of chimeras which was documented by coat color whereas clones with abnormal EB differentiation showed undetectable ES-derived contribution in the pups. She also observed that most of the clones with EB differentiation anomalies show formation of EBs at low frequency.[28]

The criteria that a DELES family of clones should meet to determine defects in EB formation are:

I. A clone should have at least 5% of seeding density: Clones were plated in two 96-well plates in parallel, one containing semi-solid differentiation media and the other coated with gelatin. EBs were counted after 8 days of differentiation while the colonies propagated on the gelatinized surface was stained with methylene blue 24 hours after seeding. Metamorph software that analysed methylene blue stained area was used to analyze cell input that produced the corresponding EB number. Clones with methylene blue staining < 5% was excluded. This low value could be the result of a defect in proliferation, cell adhesion or maybe just a technical issue.

II. An abnormal tertiary clone should produce less than $1/5^{th}$ the number of EBs compared to the primary clone.

III. An abnormal family should include clones with a phenotype that co-relates with deletion sizes.

Simon Fortier reported that three ribosomal protein genes *Rps5, Rps14* and *Rps28* are haplo-insufficient for EB formation in a p53-independent manner. [29]

Disadvantages of the DELES strategy:

I. Large deletions could involve removal of haplo-insufficient regions that are detrimental to ES cells.

II. Efficiency of Cre-recombination between two *lox*P on the same chromosome decreases with increasing genetic distance.

III. Physical constraints such as chromatin organization might affect the efficiency of Cre-*lox*P recombination.

We propose to use cDNA and BAC complementation approaches to re-introduce deleted regions and identify the deletions associated with an abnormal phenotype. Annotated cDNA libraries that can be manipulated easily are commercially available. These libraries can be used to identify determinants involved in relevant pathways; however, potential roles of non-coding elements cannot be determined. Additionally, cDNA expression is mostly driven from a ubiquitous promoter, which may not reflect the endogenous expression level, temporally or specific to a cell type. To overcome these limitations, different cDNA isoforms might have to be tested using an inducible promoter. BAC complementation is advantageous when large regions (100 ~ 250kb) need to be re-introduced. They contain regulatory elements such as promoters and they also help in identifying non-coding elements that may be associated with an abnormal phenotype. However, due to their large sizes, their transfection in cells is more challenging. A protocol for BAC complementation has been optimized in the laboratory.[28]

Since we are a lab whose focus is hematopoiesis, we would now like to use the DELES library to identify novel determinants of hematopoietic cell fate commitment. A preliminary hematopoietic screen conducted in the lab identified several DELES clones with an abnormal hematopoietic phenotype. To identify chromosomal regions essential for hematopoietic commitment, DELES clones were seeded into 96-well plates and subjected to EB differentiation. Embryoid bodies are able to spontaneously differentiate into cells of the three germ layers, including the hematopoietic cells. To test the presence of primitive hematopoietic cells, day 8 EBs were directly stained with benzidine – a highly selective and efficient procedure to demonstrate the presence of hemoglobin in EBs.

Principle of Benzidine staining:

The “haem” moiety of hemoglobin possesses peroxidase activity which can bring about the decomposition of hydrogen peroxide and liberate nascent oxygen. This nascent oxygen reacts with a chromogen such as benzidine to produce a blue colour product. The equation is as follows:

$$H_2O_2 + \text{Chromogen} \xrightarrow[\text{Peroxidase activity}]{\text{haem}} \text{oxidized chromogen} + H_2O$$

The intensity of the blue colour is proportional to the amount of hemoglobin present. Benzidine can also be used to test the presence of erythrocytes. Erythrocytes contain an enzyme called catalase which also possesses peroxidase activity and catalyses the oxidation of hydrogen donors. [30]

Though benzidine staining is a specific, inexpensive and a quick assay to test for the presence of haemoglobin, benzidine is a carcinogen. Skin absorption or inhalation of the powder has shown to lead to bladder cancer, therefore utmost care must be taken when handling benzidine.

Using this assay, DELES clones were screened for the presence of benzidine positive EBs. Out of 88 clones screened, several clones exhibited abnormal phenotype i.e. % of benzidine-positive EBs < 30 % compared to the WT R1 ESCs. Figure 4 shows the DELES families with the highest reduction in benzidine staining.

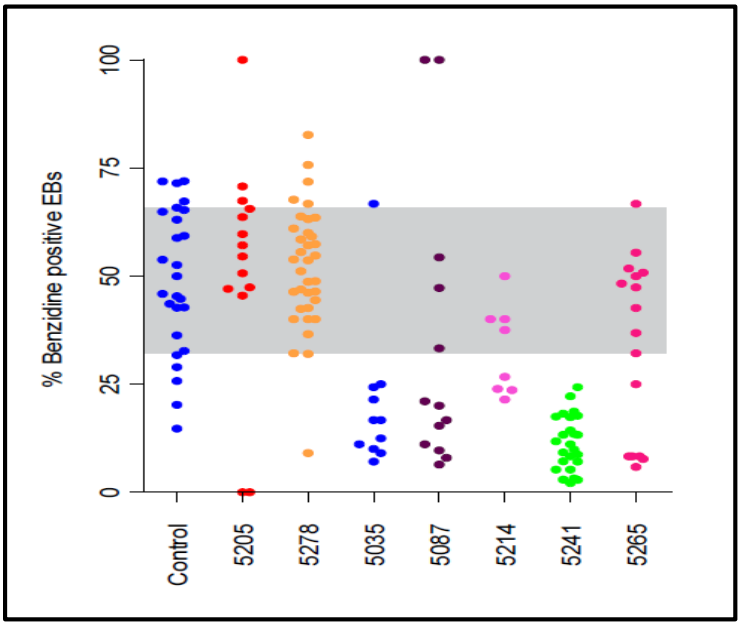

Figure 4: Preliminary hematopoietic screen using benzidine staining assay - The graph represents the percentage of benzidine positive EBs. The DELES clones were plated in duplicates in a 96-well plate to form embryoid bodies in the absence of LIF. Direct benzidine staining was performed on day 8 EBs. Controls are R1 ESCs. Each dot represents a tertiary clone belonging to the family

mentioned on the x-axis. The grey region represents the standard deviation for the average of percentage of benzidine positive EBs for control R1 ESCs.

To identify the stage at which hematopoietic specification is arrested in these DELES clones, we need to screen these clones using a hematopoietic assay that would differentiate ESCs to form hematopoietic cells in a stepwise manner.

1.2 Hematopoiesis

Blood cells are responsible for the maintenance of the immune system of the body. This relentless work requires that these blood cells have the ability to replenish themselves. The production and maturation of blood cells is termed hematopoiesis. [31] The Hematopoietic Stem Cells (HSCs) are ultimately responsible for the constant renewal of blood. [32] HSCs produce differentiated effector cells through a series of increasingly more committed intermediate progenitors. The Long-Term HSCs give rise to Short-Term HSCs which in turn produce multi-potent progeny (MPP) that have limited self-renewal capacity. The MPPs give rise to oligopotent progenitors which contain the Common Lymphoid Progenitors (CLP) and Common Myeloid Progenitors (CMP). CLP and CMP then give rise to lineage-restricted effector cells. [33] (Figure 5)

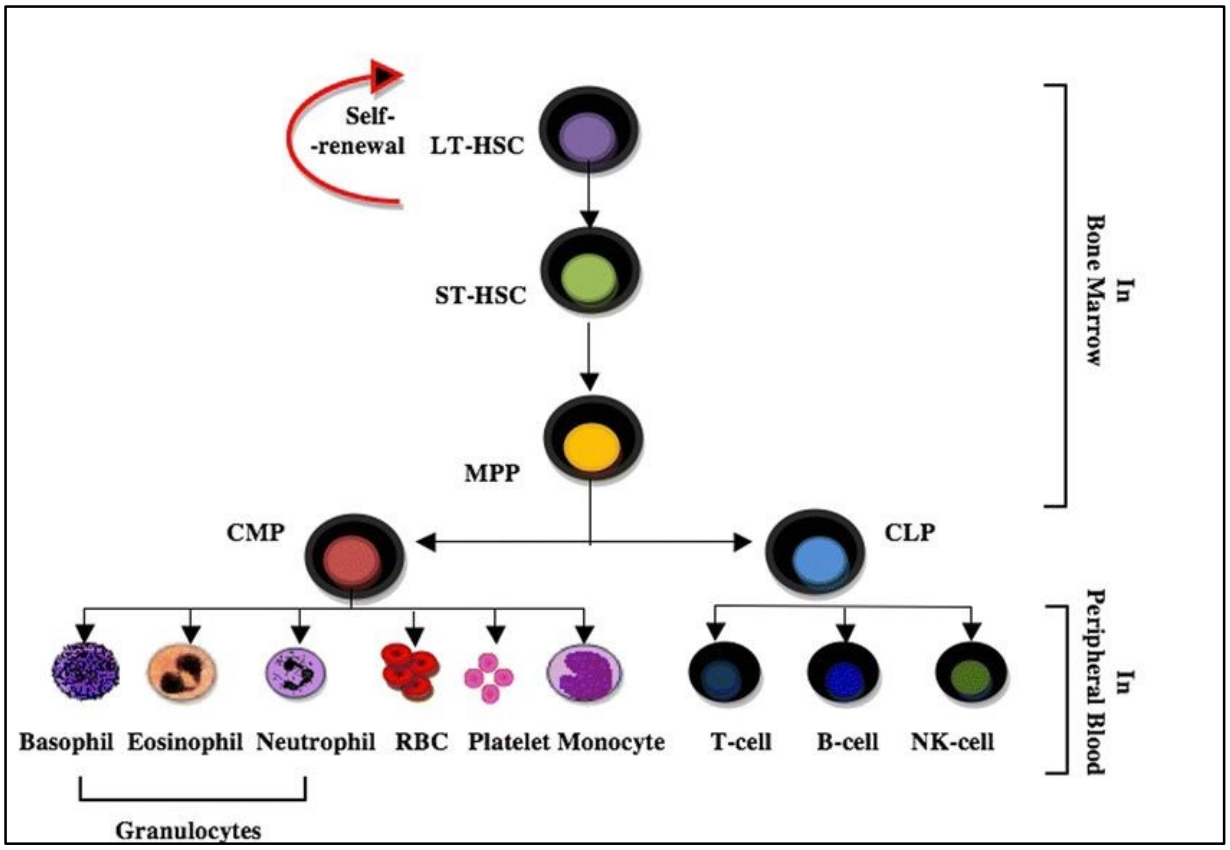

Figure 5: Hierarchy of hematopoietic stem cells - Illustration of the hierarchy of HSPCs based on self-renewal and differentiation capacity (Permission obtained, .Maha M. Bakhuraysah Christopher Siatskas and Steven Petratos. Hematopoietic stem cell transplantation for multiple sclerosis: is it a clinical reality? *Stem Cell Research & Therapy*20167:12, (http://creativecommons.org/licenses/by/4.0/)

HSC transplantation is one of the common cell-based therapies applied in clinical settings today. HSCs can be isolated from umbilical cord blood, peripheral blood and bone marrow but their use is limited due to the need for compatibility between donor and recipient and low HSC content. [34] Although, somatic HSCs can be expanded, after *ex vivo* culture their *in vivo* potential might be compromised. Pluripotent stem cells are potential sources of HSCs and can serve as an *in vitro* model to study mechanisms regulating embryonic hematopoietic development. Additionally, human ES cells are more amenable to genetic manipulation compared to the somatic HSCs that are currently used for bone marrow transplantations. Eventually we should be able to derive ES cell lines from patients with inherited disorders

using somatic cell nuclear transfer (SCNT). The genetic defects in these cell lines could be treated by genetic manipulation using siRNA, lentiviral and homologous recombination techniques and can be used to generate transplantable HSCs via *in vitro* differentiation.

Mature hematopoietic lineages derived from human ES cells can be used for replacement therapies. For example, human dendritic cells (DCs) manipulated *in vitro* are being explored for the treatment of tumors and autoimmune diseases, however using current techniques, the number of DCs that can be isolated from patients is a limiting factor. Generating DCs from human ES cells can help circumvent this problem. [35] Likewise, it may be possible to generate mature red blood cells from ES cells that could help in erythrocyte transfusions especially in cases where there is limited donor availability for rare blood cell types. [36]

To enable the transit of ES-derived hematopoietic cells from bench to bedside, we will have to understand the 3-D microenvironment composed of stromal contacts and paracrine factors present *in vivo* during hematopoietic development and be able to recapitulate them *in vitro* to efficiently produce cells of the hematopoietic lineage.

1.2.1 Ontogeny of hematopoiesis

During vertebrate development, two waves of hematopoiesis can be found at distinct anatomical sites and they contribute to both embryonic and adult hematopoiesis. The first wave, known as primitive hematopoiesis, originates in the yolk sac at about E7.5-E11 [37] of mouse development and week 3-6 of human development. [38] At this stage, primitive erythrocytes are formed but the lymphoid lineages are absent. These primitive erythrocytes pool into blood islands soon after the start of gastrulation and these blood islands become enveloped by endothelial cells to form the vascular plexus of the yolk sac. [39] Primitive erythrocytes fulfill the functions necessary for the survival and growth of the post-implantation embryo, however as the embryo grows, it requires more specialized cells to meet the demands of growth and development in later stages. To cater to this demand, the onset of definitive hematopoiesis occurs in the Aorta Gonad Mesonephros (AGM) and then transitions around E10.5 to mouse fetal liver, or between 6-22 weeks to human fetal liver. Definitive erythrocytes are formed during this wave. The definitive erythroid progenitors transition from

the fetal liver towards the newly developed bone marrow at the end of gestation. [37] The generation of all cells of the immune system occurs here, with the exception of T cells, which although originate from precursors derived from the bone marrow, develop in the Thymus. [39]

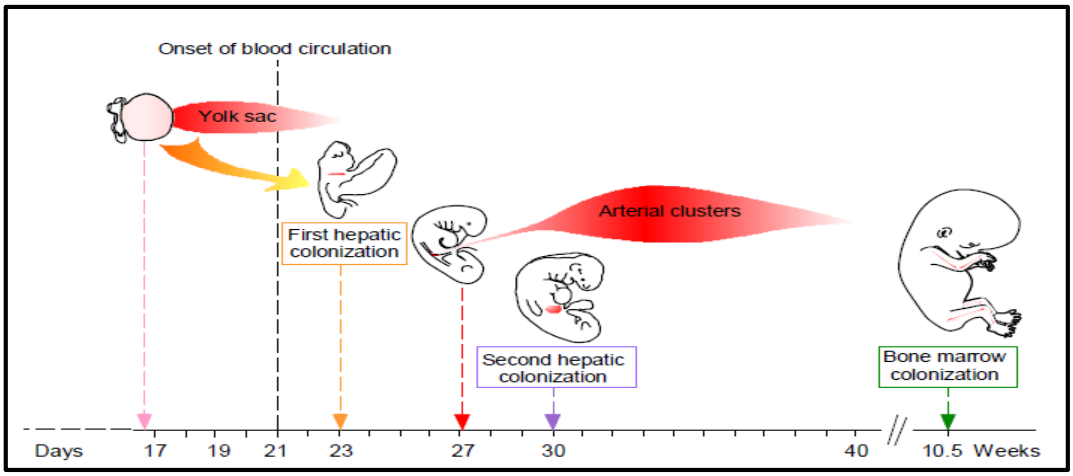

Figure 6: Ontogeny of hematopoiesis – Illustration of the developmental ontogeny in humans. Reproduced with permission from The International Journal of Developmental Biology (Int. J. Dev. Biol.) (2005) Vol: 49 page numbers 243-250.

1.2.2 *In- Vitro* derivation of hematopoietic cells

Hematopoietic differentiation from ES cells can be induced by two types of approaches:

a) Genetic approaches
b) Non-genetic approaches

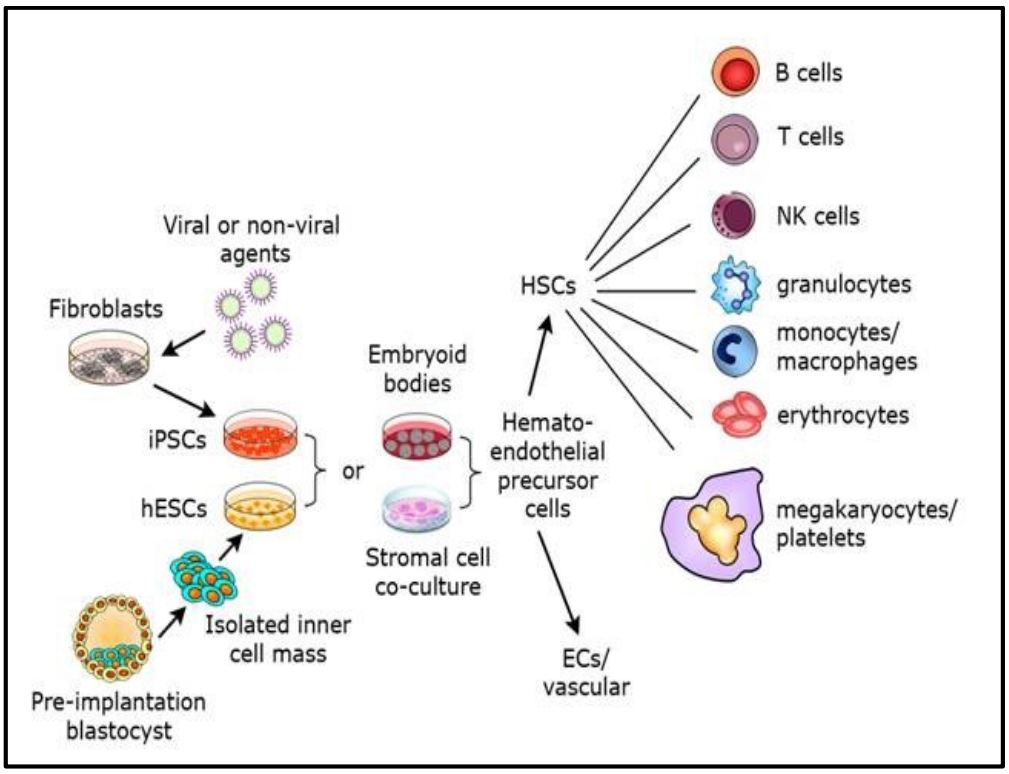

Figure 7: Methods for deriving hematopoietic cells in-vitro. (Dan S Kaufman, toward clinical therapies using hematopoietic cells derived from human pluripotent stem cells2009, Blood. Permission obtained from the journal.)

Genetic approaches: Studies carried out by the Humphries and Daley group have shown that ectopic expression of certain genes into ES cells can be used to coax these cells to differentiate into HSCs/HPSCs in an *in vitro* system. [40, 41]

Ectopic expression of HoxB4 in ES-derived hematopoietic cells in the presence of stromal cells has shown to promote expansion of hematopoietic populations with definitive HSC potential. [42] Studies have shown that these ESC –derived hematopoietic progenitors were rendered competent for engraftment and long-term multilineage reconstitution.

In mouse ES cells ectopic expression of Cdx (family of caudal-related homeobox-containing transcription factors) induced mesodermal specification and enhanced HPC production. Expression of HoxB4 and Cdx on EB-derived cells cultured on OP9 stromal cells had a

synergistic effect on HPC production, enabling engraftment of all hematopoietic lineages in irradiated adult mice. [43]

Recapitulating cellular interactions and paracrine effects occurring *in vivo* during hematopoietic development will help in the optimal development of ES-derived HPSC/HSCs. Towards this end, 3-D EB formation and stromal feeder co-culture differentiation systems are used in an effort to mimic the *in vivo* niches that support hematopoietic lineage specification.

Non-genetic approaches: Different protocols are used for generating hematopoietic progenitors from embryonic stem cells. The most commonly used are by:

- Formation of Embryoid bodies
- Culture on supportive stromal layers (eg. OP9 stromal cells)

Differentiation from embryoid bodies recapitulates many aspects of embryogenesis, it takes advantage of the principle that three-dimensional EBs can spontaneously differentiate into any cell type including ones of the hematopoietic lineage. [44] Gene expression analysis and studies on kinetics of hematopoietic lineage development within EBs have shown that it accurately reflects the early stages of yolk sac hematopoiesis.

Several techniques are used to generate EBs from cultured ESCs: 1) liquid cultures in non-adherent dishes; 2) methylcellulose or other semi-solid media; 3) hanging drop cultures and 4) porous scaffolds. [44]

The 3-dimensional EB method of hematopoietic differentiation is more efficient than the adherent method of ESC hematopoietic differentiation on a gelatinized surface whereas the frequency of hematopoietic progenitors obtained using any method of EB culture is similar. [45] Liu group has shown that dynamic conditions and biomaterial-based scaffolds enhance the efficiency of ESC differentiation. To evaluate the spontaneous differentiation of ESCs, EBs can be cultured in ESC differentiation media without cytokines. In the absence of cytokines, cells within the EB are able to secrete proteins such as CSF, IL-3 and Epo to support the development of early erythroid and macrophage precursors. [46] To induce the differentiation of a specific hematopoietic lineage , single-cell suspension of EBs are directed

to differentiation media or methylcellulose culture containing hematopoietic cytokines such as SCF, GM-CSF, Epo and interleukins such as IL-1, IL-3,IL-4 and IL-6. [46] The emergence of hematopoietic cells in this system can be tracked through gene expression analysis of lineage specific markers and immunohistochemistry. Additionally, single-cell suspensions obtained by dissociating EBs with trypsin or collagenase can be analyzed by flow cytometry. [47, 48] Within the EBs a transient primitive erythroid population emerges first. The lymphoid progenitors and the HSCs are not generated in the early stages of EB hematopoiesis, further reflecting yolk sac hematopoiesis. Though lymphoid progenitors are not generated in the early stages of EB differentiation, B and T cell lineages have been generated when ES cells are cultured for extended periods of time in the presence of stromal cells. [45]

The bone marrow stroma acts as a three dimensional framework for hematopoiesis. It contains a heterogeneous population of cells including mesenchymal stem cells (MSCs) that play an important part in the bone marrow niche. It helps in the interactions of hematopoietic stem and progenitor cells (HSPCs) with the extra-cellular molecules and soluble factors that are essential for survival and proliferation. [49] With this in mind, primary stromal cell lines were used as feeders to expand HSPCs *ex-vivo*. The different stromal lines commonly used are OP9 [50], S17 [51] and AM20-1B4 [52]. OP9 is a cell line established from mouse calvaria and lack functional macrophage colony-stimulating factor (M-CSF). The absence of M-CSF enhances hematopoietic differentiation by minimizing preferential differentiation towards the monocyte-macrophage lineage. [53, 54] ESC – derived erythroid, myeloid and B- lineage cells are obtained using OP9 stroma without the addition of exogenous growth factors; however cytokine supplementation influences lineage output. The onset of definitive hematopoiesis and expansion of HSCs takes place in the AGM region; cell lines derived from the AGM region like UG26-1B6 are also used with the aim of providing signals unique to this micro-environment to help with the hematopoietic differentiation. [55]

Culturing on feeder layers have resulted in successful hematopoietic differentiation, however, use of cells in human therapy requires their culture conditions to be defined and “animal-free”. Serum contains several undefined components and we cannot exclude the contamination of the desired hematopoietic cells with the feeder cells or antigens transmitted by the feeder cells even with processes such as cell sorting. Also, since the serum contains undefined factors, the

efficiency of the differentiation assay may differ from batch to batch of the serum used and call into question the robustness of the differentiation protocol. [56]

To overcome these issues, I have adopted a "serum-free, feeder-free" hematopoietic differentiation protocol published by the Keller group. [57] The hematopoietic cells derived here are free of undefined components and interactions.

Growth factors and cytokines constitute an important part of the regulatory niche; therefore it is important to know the effect of different cytokines on the developmental pathways in order to nudge the differentiation of mESCs to the desired lineage. In the protocol I set up in the lab, Wnt3a, Activin A, BMP4 and VEGF were used during appropriate time windows to bring about lineage specification step by step.

Transforming Growth Factor – β (TGF-β), Bone Morphogenetic Protein 4 (BMP4) and Activin are members of the TGF-β family of ligands that signal though SMAD pathways to regulate hematopoietic stem and progenitor cells (HSPCs) during hematopoiesis. Upon binding of TGF- β ligands to their receptors, the receptor regulated R-SMADs get activated and bind to SMAD4. This complex translocates to the nucleus and activates several target genes responsible for regulating many signaling pathways. BMP4 is a morphogen that induces ventral mesoderm and promotes hematopoietic commitment. [58] Apart from its role in specification of HSC during development, it also regulates proliferation of adult HSCs [59] and addition of BMP4 in culture has shown to increase the percentage of CD45+ population. [60] BMPs are mainly produced by the osteoclasts in the HSC niche. [61]

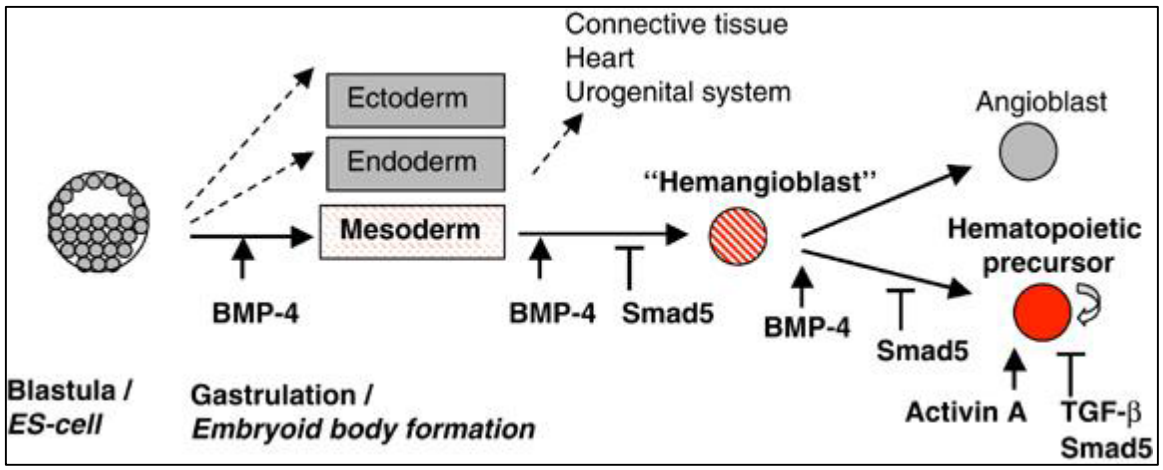

Figure 8: SMAD signaling at different stages during hematopoietic commitment - SMAD signaling regulates HSC specification and its subsequent expansion critically at different stages (Jonas Larson et al, 2005, Oncogene. Permission obtained from the journal.)

While BMP4 appears to have a crucial instructive role for the induction and formation of blood cell precursors, other factors are also involved in their regulation and subsequent expansion. Vascular Endothelial Growth Factor (VEGF), a KDR ligand is known to induce the proliferation and differentiation of the BMP4-induced hematopoietic progenitors. [62, 63] VEGF and its homologue PIGF produced by HSCs and erythroblasts influence the bone marrow microenvironment in many ways. They induce bone marrow endothelial cells to express E- selectin and thereby play an important role in HSC migration. Additionally they also act as survival and proliferation factors as VEGF-R1 and VEGF-R2 are expressed on immature HSCs that possess repopulating capacity. [64] Studies have shown that elimination of VEGF genes leads to decreased HSC survival, colony forming ability and hematopoietic repopulation following transplantation. [65] VEGFA is known to promote HSC survival, whose expression levels are dramatically increased in hypoxic conditions. [66]

The Wnt pathway has a varied influence on hematopoiesis. This pathway has 19 different ligands and about 10 different receptors. The different receptor-ligand combination can result in either canonical or non-canonical downstream signalling. [33] Evidence that Wnt signaling plays a role in hematopoiesis came from studies demonstrating the presence of Wnt ligands and receptors in different hematopoietic anatomical sites such as murine yolk sac, aorta-gonad-mesonephros, fetal liver and adult bone marrow. [33] Studies have shown that the

addition of Wnt3a in mESC cultures increases the generation of hematopoietic cells and promotes proliferation of hematopoietic progenitors. Wnt signaling not only plays a role in HSC survival and proliferation but is also involved in the maturation and maintenance of lymphoid progenitor cells such as pro-B cells, pre-B cells, immature thymocytes and mature T-cells. [67]

As ES cells differentiate towards the hematopoietic lineage, they express several transcription factors in an orderly manner. Some of the transcription factors and surface markers that can be used to assess the differentiation at specific stages are illustrated in Figure 9.

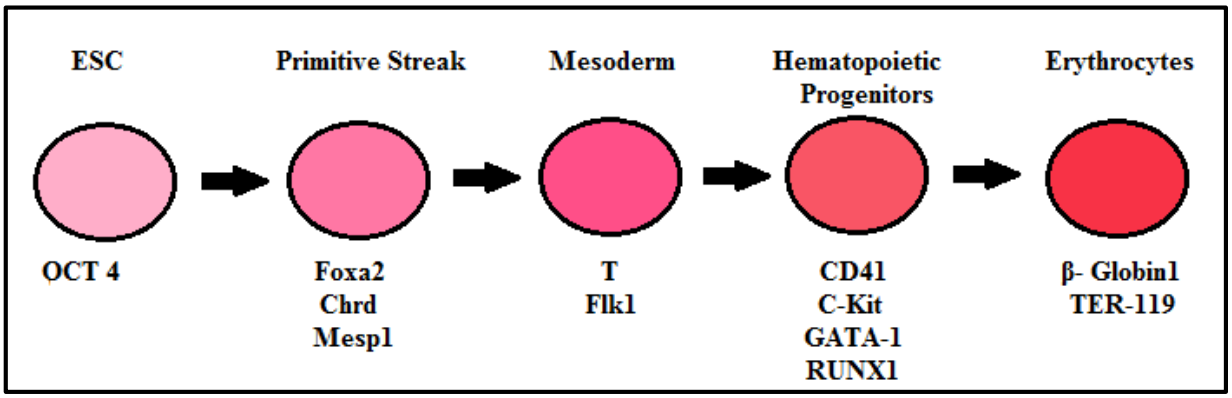

Figure 9: Lineage-specific gene markers used to assess hematopoietic differentiation from ES cells.

1) Brachyury (T) – It is a transcription factor that binds to a specific DNA palindromic site. Binding occurs through a region called the T-box present in the N-terminus region. On binding, Brachyury regulates the transcription of several genes required for mesoderm differentiation. Brachyury expression is critical for posterial mesoderm development in the mouse. During development, Brachyury expression is observed in early gastrulae next to primitive streak cells destined to form the mesoderm. $T^{-/-}$ embryos die at E.10 due to the absence of a notochord and allantois. [68] 2) CD41 – expression of CD41 in murine development marks the onset of definitive hematopoiesis. CD41 expression precedes that of CD45. In adult hematopoiesis CD41 expression is associated with megakaryocytes and also plays a role in platelet function. Hematopoietic cells emerge from a CD41+ population; these cells then express CD45 on maturation. It can thus be said that in murine development, the

expression of CD41 separates the hemogenic endothelium from the hemangioblast - which is the common precursor for hematopoietic and endothelial cells. [69] 3) Runt-related transcription factor 1 (RUN1X) plays a crucial role in definitive hematopoiesis. It is involved in the transition of hemogenic endothelial cells to hematopoietic cells. [70] 4) GATA-1 and GATA-2 are members of the GATA family of transcription factors. GATA-1 is involved in erythroid development and is highly expressed in erythroid cells whereas GATA-2 is expressed on early hematopoietic progenitors and plays a role in HSC production in the AGM region and HSC proliferation throughout the ontogeny. [71, 72] 5) Beta-Globin is part of the hemoglobin protein and is involved in erythropoiesis. Mutations in the β-globin gene results in blood disorders such as β-thalassemia and anemias such as sickle cell anemia. [73] 6) C-kit – also known as cytokine tyrosine kinase receptor. C-kit, the receptor for SCF, is present on HSCs and the stromal cells in the bone marrow secrete SCF. Upon binding to the receptor, the receptor homo-dimerizes and auto-phosphorylates at tyrosine residues activating pathways responsible for hematopoiesis and HSC maintenance such as JAK/STAT and RAS/ERK pathways. C-Kit is not essential for the expansion of HSCs in fetal development, although it plays an important role in maintaining the long-term steady state HSCs in adult BM. It also promotes HSC proliferation when in synergy with other hematopoietic cytokines. [74] 7) CD45 is a pan-hematopoietic marker expressed on most hematopoietic cells except erythrocytes and platelets. [75, 76] Due to variable exon splicing, different CD45 isoforms are expressed by different subsets of hematopoietic cells. For example CD45RA is expressed by naïve/resting T cells and medullary thymocytes and CD45RO is expressed on memory/activated T cells and cortical thymocytes. [77]

Goal, Hypothesis and Objectives:

Goal: As mentioned previously, one of our interests in the lab is to take advantage of the DELES library to identify genes or DNA fragments that are required for hematopoietic cell fate. Using clones from the DELES library, we expect to reveal several haploinsufficient and synthetic loci that contribute to the differentiation of ES cells to the hematopoietic lineage. Before this is possible we need to establish a robust hematopoietic differentiation assay which could be exploited to rescreen the DelES collection.

My hypothesis is that the *in vitro differentiation assay developed in Dr. Keller's laboratory could be imported in our laboratory and be used to better characterize the hematopoietic phenotype of DelES clones.*

The objectives of my project are to :

- Establish in the laboratory a hematopoietic differentiation assay from mouse ES cells which will serve as a more stringent screen to observe hematopoietic commitment of the candidate DELES clones. This will involve optimizing Q-RT-PCR and FACS parameters for this assay.
- Validate candidate DELES clones identified in the preliminary hematopoietic screen using this assay to monitor where the differentiation arrest is occurring.

In a secondary project I will test if a human haploid cell line, KBM-7, can be exploited using the DELES approach to create a library of clones comprising random chromosomal deletions. KBM-7 is a human near haploid Chronic Myeloid Leukemia (CML) cell line. Haploid cell lines such as the KBM-7 cell line serve as an excellent model system to carry out functional screens as the phenotype of the mutated allele will not be masked by the allele on the other homologous chromosome.[78]

This library of KBM-7 clones containing nested chromosomal deletions can serve as a platform on which various studies to understand the mechanisms contributing to leukemia development and treatment of leukemia can be conducted. For example, in our lab, this library will be used to delineate the molecular activities of potential anti-leukemic drugs as well as allow me to identify molecular signaling pathways that, when genetically disrupted, can confer resistance to novel and existing drugs.[79]

2. Materials and Methods

Embryonic Stem Cell maintenance and differentiation

R1 ESCs were maintained on irradiated mouse embryonic fibroblasts (MEFs) in the presence of LIF and serum. Hematopoietic differentiation of mESCs was induced using the protocol published by Keller *et al. (2012).* To set up the differentiation assay, the ES cells previously grown on MEFs were split on dishes coated with 0.1% gelatin. ESCs were treated with trypsin and seeded at a density of 2.5x10^5/mL in a serum-free differentiation medium (75% IMDM, 25% Ham's F12, supplemented with 1% N2, 0.5% B27, 0.05% BSA, 50 µg/mL ascorbic acid, 4.5 x 10^{-4} M 1-thioglycerol, 2 mM glutamine and penstrep) for 48 hours in 60mm bacterial dishes. The embryoid bodies formed were dissociated using 0.25% Accutase for 5 minutes at 37˚C and then re-aggregated at the same density in the serum free media containing 3ng/mL Wnt3a and 1ng/mL Activin A for an additional 48 hours to induce mesoderm differentiation. The aggregates formed were once again dissociated and re-suspended in the serum free medium, this time in the presence of 1ng/mL BMP4 and 5ng/mL VEGF to induce hematopoietic commitment. Recombinant Mouse Activin A (R&D systems, Ref # 338-AC-010), recombinant mouse Wnt3a (R&D systems, Ref # 1324-WN-002), recombinant human BMP-4 (R&D systems. Ref # 314-BP-010) and recombinant human $VEGF_{165}$ (R&D systems, Ref # 293-VE-010) were used.

Embryoid body differentiation

To improve the differentiation efficiency by getting rid of the MEFs, ES cells were split on 0.1% gelatin prior to the differentiation. A 5X Master mix of the differentiation media was prepared containing IMDM+glutamine, 15% heat-inactivated Fetal Calf Serum, 50µg/mL Ascorbic acid, 5% PFHM II , 0.3Mm 1-thioglycerol and Penstrep. To prepare the semi-solid media, half the media volume was replaced with methylcellulose. Tubes containing methylcellulose and differentiation media were vortexed to attain a homogeneous formulation. The ES cells were trypsinized for 5 minutes at 37˚C and neutralized using the differentiation media. ESCs were seeded at a density of 1250cells/mL in 60mm bacterial- grade tissue culture

dishes. After adding the cells into the semi-solid media, the cells were mixed manually. 5mL syringes and blunt needles were used to slowly transfer the mixture to the dishes. Each dish was placed in a larger dish humidified with sterile water.

Benzidine Staining Assay

A benzidine (Sigma, Cat # B1883-100mg) stock solution of 3% was made by diluting 4, 4'-Diaminobiphenyl in 90% glacial acetic acid and 10% water solution. A fresh working solution of 1 part benzidine stock solution, 1 part hydrogen peroxide (Sigma, Cat # H3410) and 5 parts of sterile water was prepared prior every staining. Day 8 embryoid bodies were stained for the presence of hemoglobin by adding 0.2mL of the working solution per 2mL of methylcellulose culture directly onto the dish.

RNA Extraction

The cell suspension from one 60 mm dish was collected and spun down at 800rpm for 5 minutes. Media was aspirated and the cells were washed with PBS and spun down again. The PBS was aspirated and the cell pellet was re-suspended in 1mL TRIzol reagent (Life Technologies, Cat # 15596-026). The samples were stored at -80 ℃ for subsequent RNA isolation.

- **RNA Isolation:**

RNA was extracted from TRIzol stored samples according the manufacturer's instructions.

- **Measurement of RNA concentration :**

The concentration (260nm) and purity of RNA was assessed using an Implen nanophotometer.

cDNA synthesis

1.5 µg of RNA was used to synthesize DNA using MMLV-RT (Invitrogen, Cat # 28025-013) according to manufacturer's instructions. The following program was used:

Table 1: cDNA synthesis program

Temperature	Time
25˚C	10 minutes
37˚C	50 minutes
70˚C	15 minutes

QRT-PCR

Here the DNA was further quantified and simultaneously amplified. The qRT-PCR was carried out using TaqMan® Fast Advanced Master Mix (Applied Biosystems, Cat # 4444558)

- Master Mix preparation :

A master mix was prepared and used at a final volume of 8.5µL for each well of a 384- well plate.

Table 2: Composition of Master Mix for qRT-PCR.

Component	Volume (µL)
Taqman Mix	5
Primers (F+R)	0.1
Exiqon probe	1
Water	2

- cDNA (1/5 dilution) = 2µL was used for each reaction.

The primers used for qRT-PCT were obtained from the genomic platform at IRIC.

Table 3 – Sequence of primers used for qRT-PCR

GAPDH	**F - TGTCCGTCGTGGATCTGAC**
	R - CCTGCTTCACCACCTTCTTG
HPRT	**F -TCCTCCTCAGACCGCTTTT**
	R-CCTGGTTCATCATCGCTAATC
OCT 4	**F-GAGGCTACAGGGACACCTTTC**
	R-GTGCCAAAGTGGGGACCT
T	**F-CGACCACAAAGATGTAATGGAG**
	R-CCAGCACCAGGAACAAGC
CD41	**F-TGCTGCTGACCCTGCTAGT**
	R-GTCGATTCCGCTTGAAGAAG
C-KIT	**F-GGAGCCCACAATAGATTGGTAT**
	R-CACTGGTGAGACAGGAGTGG
RUNX1	**F-CTCCGTGCTACCCACTCACT**
	R-ATGACGGTGACCAGAGTGC
GATA2	**F-TCACCCCTAAGCAGAGAAGC**
	R-TGTGGCACCACAGTTGACA
β-GLOBIN	**F-TGCATGTGGATCCTGAGAAC**
	R-AGCAGGGGTGAAATCCTTG
CD45	**F-AGTTAGTGAATGGAGACCAGGAA**
	R-TCCATAAGTCTGCTTTCCTTCG

- **Calculation for relative fold expression** :

For each reaction, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Hypoxanthine guanine phosphoribosyltransferase (HPRT) were used as internal primer controls. Relative fold expression was calculated by subtracting Ct (GAPDH/HPRT) values from the Ct (sample) values. The resulting values were used to calculate fold expression by comparing it to the experimental control which in my case was day 0 of differentiation. The following formula was used to calculate relative fold expression:

$\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (Day 0)

Relative fold expression = $2^{\wedge - \Delta\Delta Ct.}$

The qRT-PCR program used is as follows:

Table 4: qRT-PCR program

	Temperature	**Time**	**Stage**
Step 1	95 ˚C	20 seconds	Hold stage
Step 2	95 ˚C	1 second	PCR stage
	60 ˚C	20 seconds	

Number of cycles = 40

Flow cytometry

Cells from a dish were collected and spun down at 800rpm for 5 minutes. This was followed by washing with PBS for 5 minutes at 800rpm. The cell pellet was treated with 500µL of Accutase at 37˚C for 5 minutes. The Accutase was neutralized with 7mL of serum-free differentiation media. Antibody staining was conducted for 1x10^5 cells in 100µL media for 20 minutes. The cells were then washed by adding 2mL PBS and spun at 800 rpm for 5 minutes. The PBS was aspirated leaving behind 50µL. The samples were read using BDFACS Canto. The antibodies used were anti-mouse APC/Cy7 CD45.2 (BioLegend, Cat # 109824) and anti-mouse APC Ter-119 (BioLegend, Cat # 116212). To

gate out dead cells, Molecular probes Live/dead Fixable Violet Dead cell Stain Kit was used (Life technologies, Ref # L3L955).

Methylcellulose preparation used in viral stock titration

MethoCultTM (StemCell Technologies Inc.)

- **Methylcellulose preparation used to plate wild type KBM-7 cells :**

Table 5: Methylcellulose Mix

Components	Volume for 30mL prep	Final Concentration
Methylcellulose stock 2%	17mL	1.13%
IMDM	6.4mL	
FBS – Heat Inactivated	6mL	20%
Glutamine (200Mm)	300µL	2mM
B- Mercaptoethanol (10^{-2})	300µL	10^{-4} M

- **Methylcellulose + cytokines preparation used to plate wild type KBM-7 cells and titrate MSCV- GFP and A1 viral stock:**

Table 6: Methylcellulose Mix with cytokines

Components	Volume for 30mL prep	Final Concentration
Methylcellulose stock 2%	17mL	1.04%
IMDM	4.5mL	
FBS – Heat Inactivated	6mL	20%
Glutamine (200Mm)	300µL	2mM
B- Mercaptoethanol (10^{-2})	300µL	10^{-4} M

Deionized BSA, 30% stock	1mL	
Holo-transferrin (30 mg/mL)	0.192µL	
SCF (60ng/µL)	60µL	100 ng/mL
1L-3 (80ng/µL)	4µL	10 ng/mL
GM-CSF (300ng/µL)	1µL	10 ng/mL
EPO (280 U/mL)	320µL	3 U/mL
IL-6 (10ng/µL)	30µL	10 ng/mL
TPO (8ng/µL)	200µL	50 ng/mL

Transfection

HEK293 (expressing stably VSV-G envelope and gag and pol genes) were cultured to be 75 - 80% confluent on the day of transfection. For transfection in a 10 cm dish the following mixes were prepared:

Mix A:

- DNA – 20 µg
- Gag-Pol - 5µg
- VSV-G - 5µg

The volume was made up to 1mL with Opti-MEM.

Mix B:

- Lipofectamine - 80µL
- Opti-MEM - 1920µL

The Mix B was incubated for 5 minutes at room temperature. 1mL of A and B were mixed and incubated for 20 minutes at room temperature. The media from the plates were collected and fresh media (DMEM + 10% Heat inactivated FBS) was added very slowly. 5µg/mL of tetracycline was added to the plate.

The cells were incubated for 5 hours, after which the media was changed and fresh media was added. The viral supernatant was harvested after 48 hours and continued for 5 days.

- Formula to calculate viral titer:

Titre (TU/mL) = {(F x Cn)/V} x D.F

Where,

F – Frequency of target cells (GFP+ cells) or {colonies (+ puromycin) / colonies (- puromycin)}y

Cn – the total number of target cells infected

V – The volume of the inoculum

D.F – dilution factor

3. Results

3.1.1 Establishment of a hematopoietic differentiation protocol.

In the lab, we had conducted a preliminary hematopoietic screen using a benzidine staining assay to test the hematopoietic phenotype of the DELES clones. Benzidine is a compound that stains blue in the presence of hemoglobin. 5000 ES cells were plated in a 60mm dish and allowed to differentiate to EBs for 8 days.0.4mL of working benzidine solution was prepared as described in the Material and Methods section and was used to directly stain EBs to test for the presence of hemoglobin. The percentage of benzidine-positive EBs was calculated using the formula:

$$\frac{\text{(Number of EBs that stained blue in the presence of benzidine)} \times 100}{\text{Total number of EBs}}$$

Based on this preliminary assay, we identified several DELES clones that showed an abnormal hematopoietic phenotype. However, since this assay only tested for the presence of hemoglobin, we needed to establish a more stringent (and more sensitive) screen to study stepwise hematopoietic commitment of the candidate DELES clones using q-PCR and flow cytometry techniques. To achieve this, I worked on setting up a feeder-free, serum-free differentiation assay published by the Keller group [57] .

Figure 10 illustrates the differentiation protocol. Based on literature review, I decided to examine the expression of hematopoietic genes such as CD41, C-KIT, RUNX1, GATA1, β-globin1 and CD45, which will provide a good insight on hematopoietic commitment and differentiation.

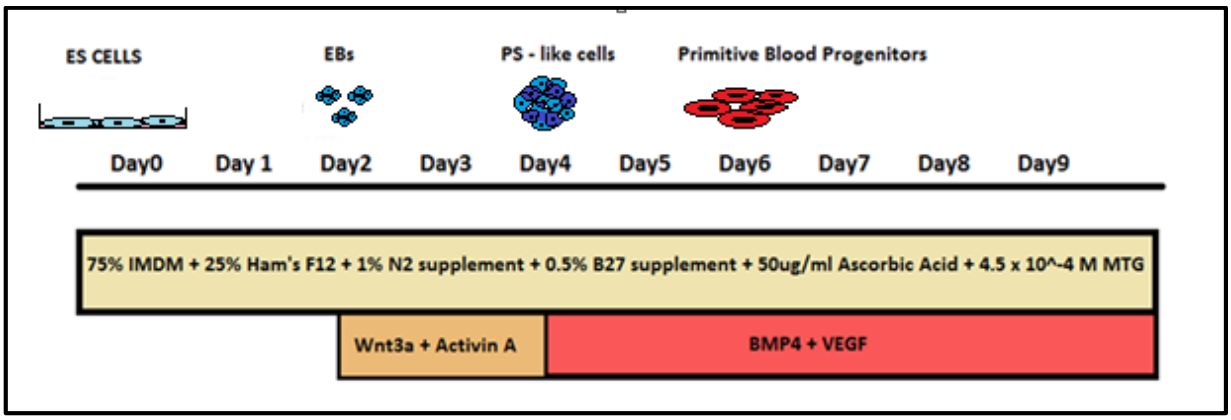

Figure 10: Schematic of the hematopoietic differentiation assay. R1 ESCs split previously on 0.1% gelatin were trypsinized and re-suspended in a serum-free differentiation media at a density of 2.5x10^5 cells/mL for 48 hours to form EBs. Day 2 EBs were dissociated and re-aggregated in the presence of Wnt3a and Activin A to form primitive streak-like cells. Day 4 cell aggregates were dissociated and re-suspended in the presence of BMP4 and VEGF for an additional 48 hours to form primitive blood progenitors.

3.1.2 Kinetics of the hematopoietic differentiation.

Lineage commitment upon directed differentiation is associated with a decline in pluripotency and upregulation of lineage specific genes. Here, the differentiation time course was monitored by qRT-PCR.

To induce differentiation, different growth factors and cytokines were added in the appropriate time windows as displayed in Figure 10. The impact of these signals on differentiation can be seen by the decline in OCT4 expression (Figure 11) and concomitant upregulation of the mesodermal marker Brachyury (T) which peaks by day 5 and then declines over the next few days. Expression of hematopoietic genes like CD41, C-kit, RUNX1 and GATA1 is not detected until day 4 and is induced from day 5. This trend is expected, as in the protocol I am using, it is on day 4 of differentiation I add cytokines and growth factors that drive hematopoietic differentiation. CD45 and β-globin 1 expression is also induced, indicating hematopoietic differentiation and erythroid lineage commitment.

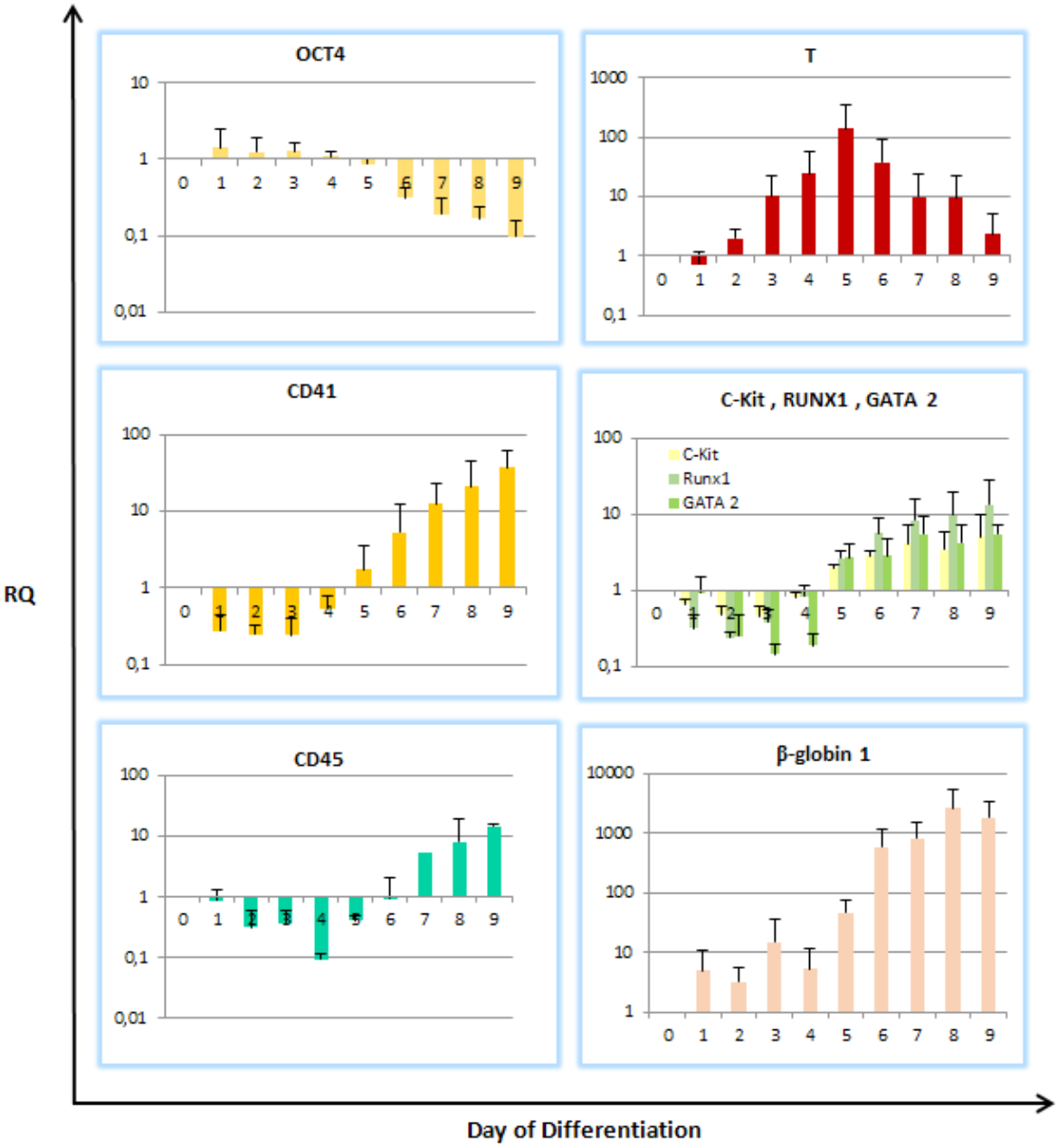

Figure 11: Kinetics of hematopoietic differentiation. – Sequential expression of OCT4, T and hematopoietic genes CD41, C-KIT, RUNX1, GATA2, CD45 and β-GLOBIN1 monitored by qRT-PCR. GAPDH and HPRT housekeeping genes are used as internal control. Relative Quantification (RQ) value is calculated using day 0 as a reference (described in the material and methods section), it depicts the expression of the target gene on a given day compared to its expression on day 0 of the differentiation assay. N=3, Error bars represent S.D

From the literature, we know that during hematopoietic ontogeny, mesodermal cells first acquire CD41 expression upon hematopoietic commitment. CD41 is one of the earliest mouse markers representative of hematopoietic commitment. As the cells continue to differentiate from hematopoietic stem cell (HSC) to a more progenitor-like fate, they gain CD45 expression as well, which is maintained in all cell lineages except erythrocytes. We observe a similar trend in our assay. We also know that the first lineage to emerge during primitive hematopoiesis is the primitive erythrocytes; thereby the presence of β-globin1 expression at earlier time points is as expected. From the above results, it was concluded that the hematopoietic differentiation assay was able to induce the expression of hematopoietic genes CD41, C-KIT, RUNX1, GATA2, CD45 and β-GLOBIN1 in a reproducible manner.

Studying the kinetics of the differentiation assay, I decided to choose day 6 (a time point where hematopoietic differentiation is just being induced) and day 8 (a later time point after the induction of hematopoietic differentiation) time points to conduct FACS analysis of hematopoietic surface markers CD45 (pan-hematopoietic marker, expressed in all hematopoietic cells except in erythrocytes) and Ter-119 (expressed on all erythroid cells). The first step in this direction was to evaluate the optimal concentration of the respective antibodies to be used in our assay.

3.1.3 Titration of CD45 and Ter-119 antibodies.

The process of identifying the optimum concentration of antibody to use for a given assay is known as titration. To titrate antibodies it is important that we use a cell population that expresses the target of interest. In our case, the R1 cells are Ly 5.2, I used cells isolated from the spleens of two mice – Pep3B and C57BL/6 which are Ly5.1 and Ly5.2 cells, respectively, to get a good positive and negative peak while titrating the antibodies.

Cells were isolated from the entire spleen of a mouse of each genetic background. The total numbers of cells were pooled together. An aliquot of 100,000 cells were taken and used as a positive control for the markers CD45 and Ter-119. Serial dilution of the antibodies was

prepared as to get five different concentrations of the antibodies -1/25, 1/50, 1/100, 1/200 and 1/400.

The hematopoietic system has a well-defined differentiation cascade comprised of distinct intermediates connecting the differentiation of naïve hematopoietic stem cells into mature hematopoietic cells.

In FACS, a scatter plot of FSC vs SSC will give us the distribution of cells based upon size and granularity respectively. From literature, and experience we expect to see the distribution of blood cells in a SSC vs FSC FACS plot as below:

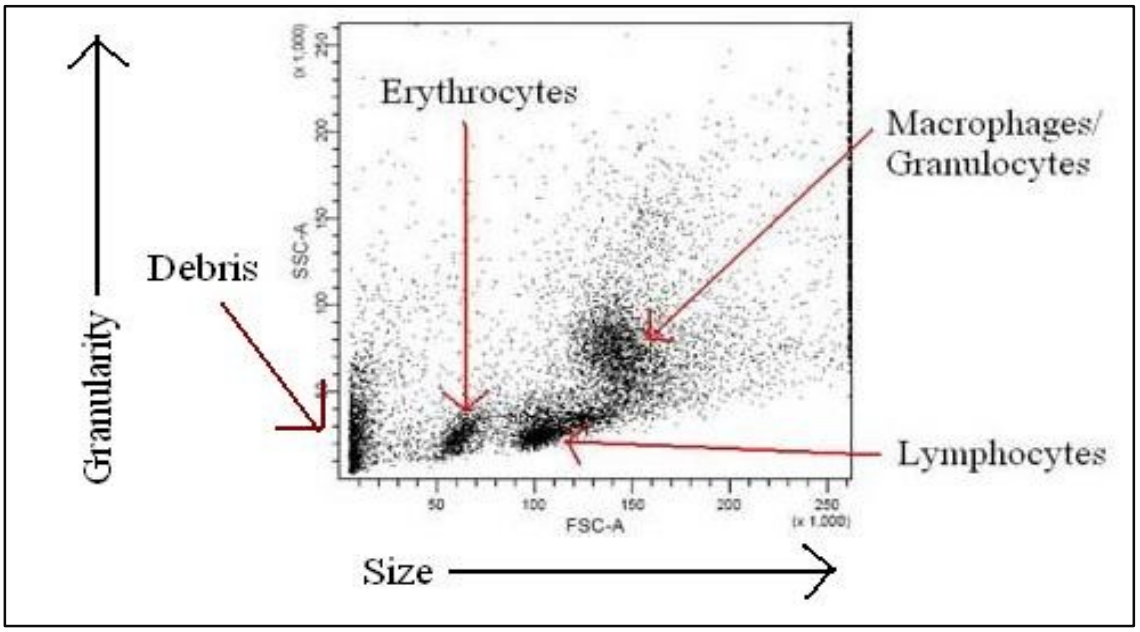

Figure 12- Illustration of the distribution of different blood cell types on a SSC vs FSC FACS plot.

(https://www.researchgate.net/post/What_FACS_parameters_to_consider_when_I_want_to_count_MEFs_cells_and_differentiate_between_live_and_dead_cells**)**

- **Ter-119 Antibody Specificity:**

The following analysis was conducted to test whether the Ter-119 antibody is specific for erythrocytes as expected.

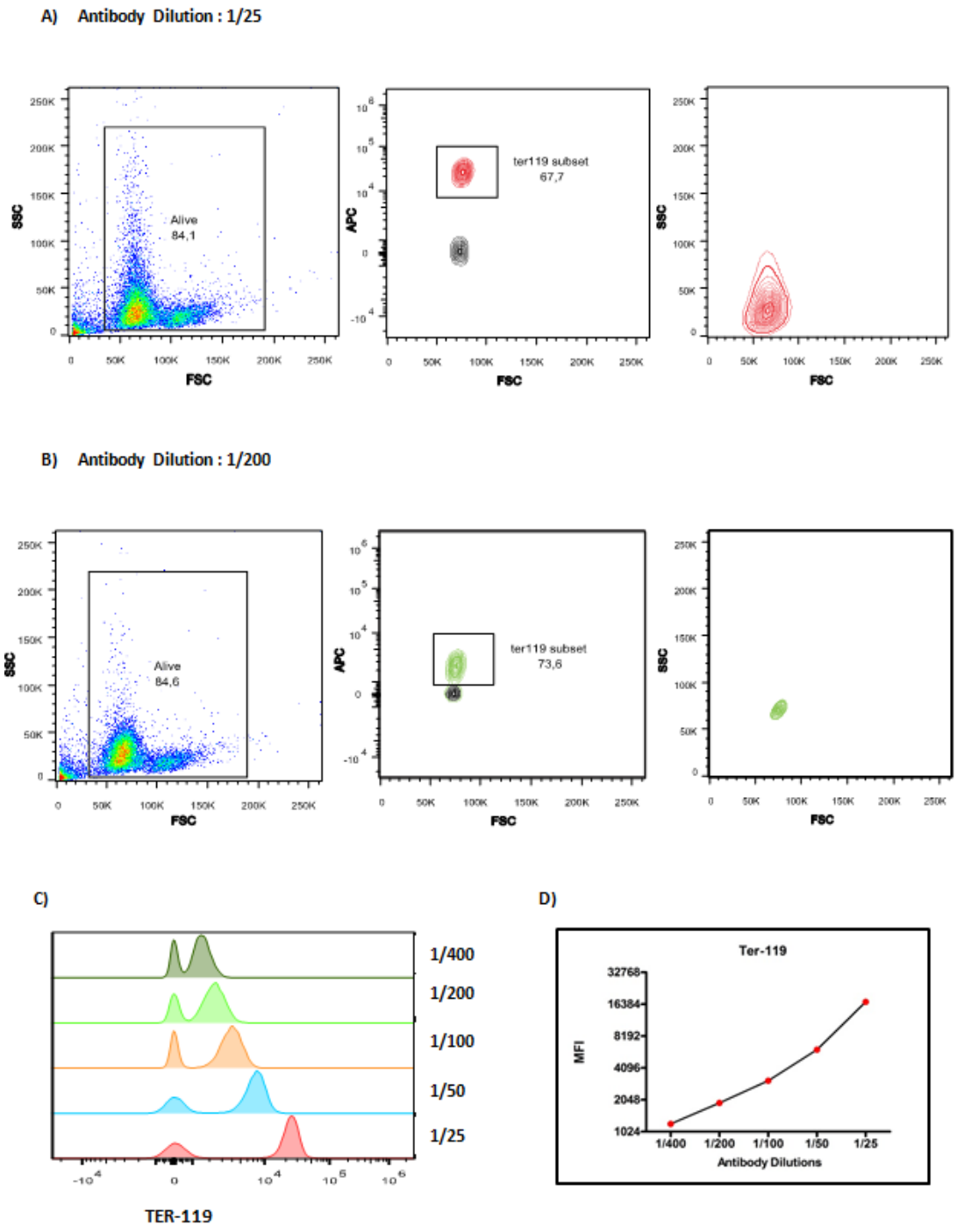

Figure 13: Ter-119 Antibody titration - Spleens isolated from C57BL/6 and Pep 3B mice were used to as positive control for the titration of Ter-119 antibody. A and B panels show the

antibody specificity for the 1/25 and 1/200 dilution of the antibody respectively. The living population was first gated on the SSC-FSC plot. The TER-119 + population correspond to the region where the erythrocytes are expected on the SSC-FSC FACS plot. C) Figure showing the separation of negative and positive population for different Ter-119 antibody dilutions. D) Graph showing the Mean Fluorescent Intensity for different Ter-119 antibody dilutions.

The plots on panel A and B shows that, with higher antibody concentration I get a better separation of the positive and negative populations. I also see that the TER-119 positive population corresponds to a region where we expect to see erythrocytes in a SSC vs FSC plot. (Figure 12)

- **CD45.2 Antibody Specificity :**

The following analysis was conducted to test if the CD45.2 antibody being used is specific for cells expressing CD45.2 – hematopoietic cells except erythrocytes.

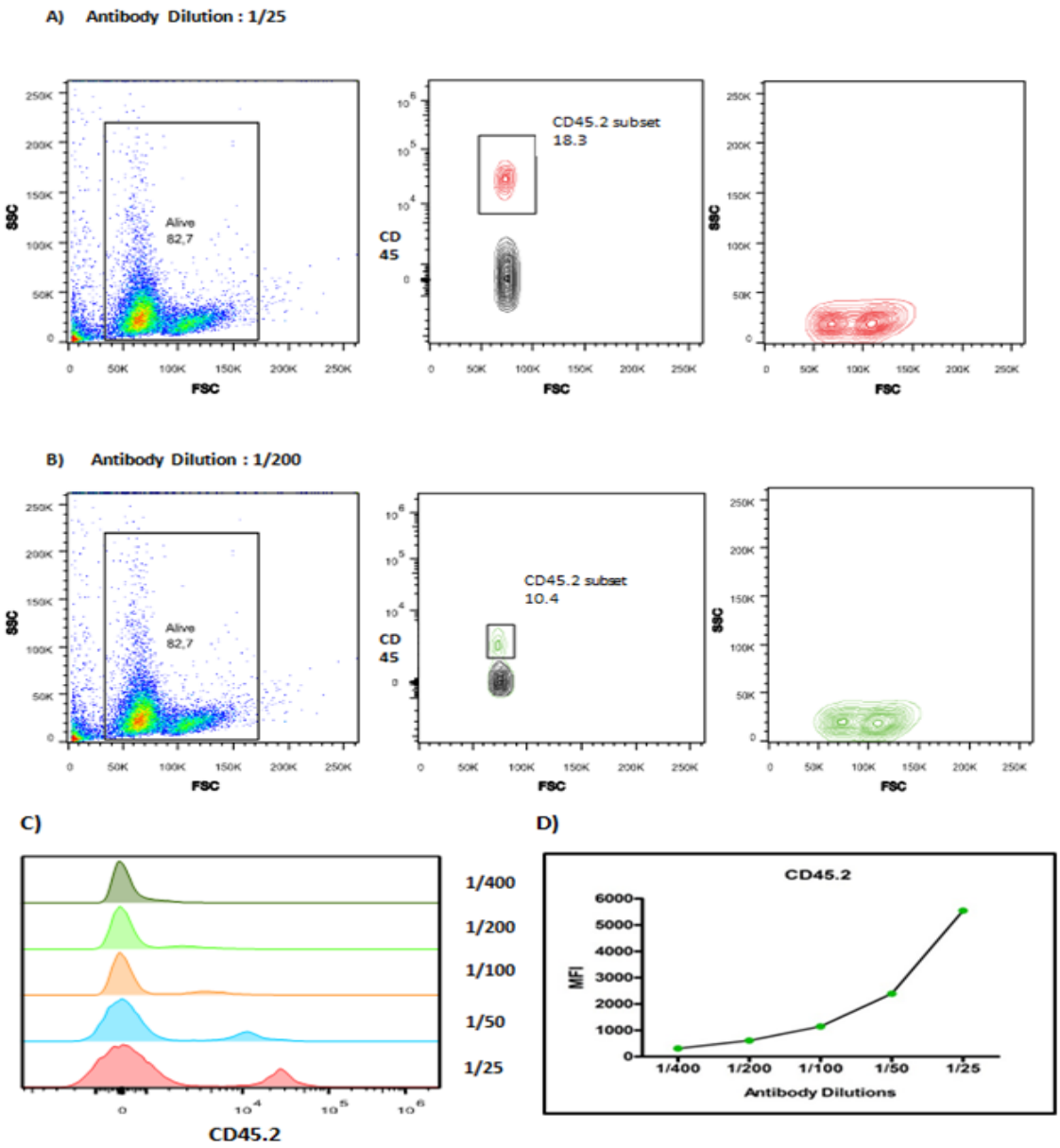

Figure 14: CD45.2 Antibody titration - : Spleens isolated from C57BL/6 and Pep 3B mice were used as positive control for the titration of CD45.2 antibody. A and B panels shows the antibody specificity for the 1/25 and 1/200 dilution of the antibody respectively. The living population was first gated on the SSC-FSC plot. The CD45.2 + population correspond to the region where the hematopoietic cells are expected on the SSC-FSC FACS plot. C) Figure showing the separation of negative and positive population for different CD45.2 antibody dilutions. D) Graph showing the Mean Fluorescent Intensity for different CD 45.2 antibody dilutions.

Given that in a titration assay, the optimal antibody concentration will be the one resulting in specific staining of the target population and the best separation of the positive and negative populations , I used the 1/25 dilution of TER-119 and CD45.2 for my flow cytometry assays.

To determine the efficiency of the differentiation assay, I assessed the percentage of CD45+ and TER-119+ population in the differentiation cultures. On FACS analysis, I observed about 10% CD45+ cells on day 6 which slightly increased to 15% on day 8 whereas the percentage of TER-119+ cells was about 1.5% on day 6 which increased to 30% on day 8. (Figure 15).

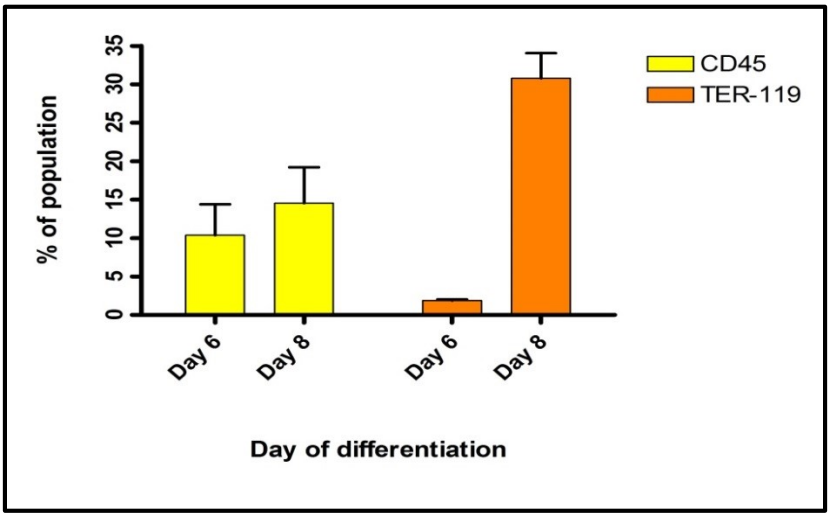

Figure 15: FACS analysis of the differentiation assay - Percentages of CD 45.2+ and TER-119 + populations on day 6 and day 8 of the hematopoietic differentiation assay. N=2, Error bars represent S.D.

From this result , I concluded that the readout fror CD45+ and Ter-119+ populations on day 6 and day 8 time points of the differentiation assay will be used for screening the hematopoietic phenotype of the DELES clones.

Q-PCR and FACS results indicated that hematopoietic differentiation of R1 ESCs was occurring and the differentiation was skewed to the erythroid lineage. The assay I set up is robust and will be used to accurately screen the hematopoietic potential of the candidate DELES clones.

3.2 Validation of the DELES clones using the hematopoietic differentiation assay.

3.2.1 Preliminary screen of DELES library identified clones having reduced hematopoietic potential.

In the preliminary hematopoietic screen conducted using benzidine assay (Figure 16), reduction or absence of benzidine coloration in some clones strongly suggests the deletion of gene(s) important for hemoglobin biosynthesis and/or deletion of chromosomal regions playing a critical role in hematopoietic commitment.

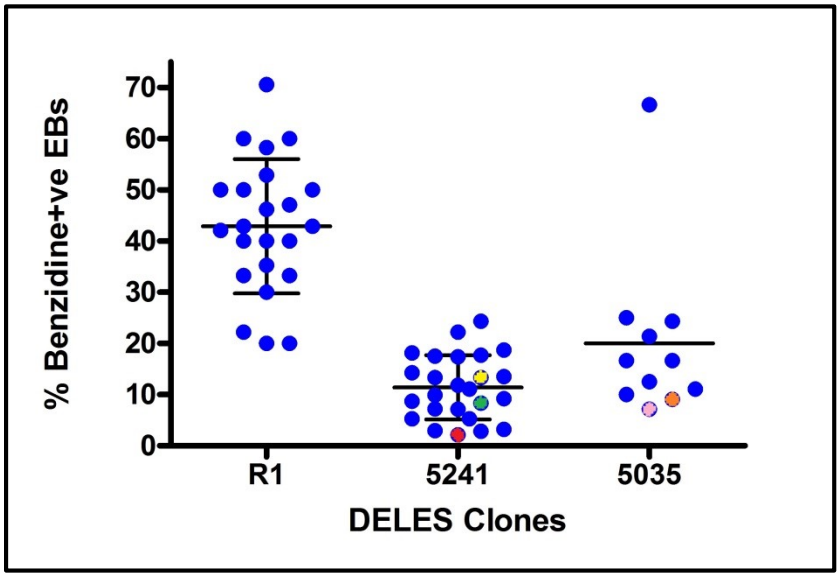

Figure 16: Benzidine Staining Assay - The graph represents the percentage of benzidine positive EBs. The DELES clones were plated in duplicates in a 96-well plate to form embryoid bodies in the absence of LIF. Direct benzidine staining was performed on day 8 EBs. Controls are R1 ESCs. Each dot represents a tertiary clone belonging to the family mentioned on the x-axis. Error bars represent the standard deviation for the average of percentage of benzidine positive EBs for each family. The clones selected for validation are red dot – 5241.16, green dot – 5241.09, yellow dot – 5241.42, pink dot – 5035.10 and orange dot – 5035.41.

Out of the 7 families which had clones exhibiting reduced benzidine staining (Figure 4), I decided to pursue the 5241 and 5035 families further. To confirm the robustness of the benzidine staining assay, I repeated the assay for the two families in a larger 60mm dish format.

3.2.2 Validation of the 5241 and 5035 DELES families.

The 5241 family contains nested deletions in chromosome 15. Within this family, I selected one primary clone (clone having only the anchor virus integration and no deletions) – **5241 1°** and three tertiary clones (clones having the same anchor virus integration and different saturation virus integrations.) – **5241.16** (*Rims2)*, **5241.09** (*Klf10, Azin1, Atp6v1c1, Fzd6, Cthrc1, Stc25a32, Wdsof1, Rims2*) and **5241.42** (*Zfp706, Grhl2, Ncald, 4930447A16Rik, Rrm2b, Ubr5, Odf, Klf10, Azin1, Atp6v1c1, Fzd6, Cthrc1, Stc25a32, Wdsof1, Rims2*) with increasing deletion sizes as shown in Figure 17. The three tertiary clones were selected based on their chromosomal deletion size and their high proliferation state as determined by Ki67 staining. High proliferation state clones were selected as they can be propagated easily. Clones 5241.16, 5241.09 and 5241.42 had 99.3%, 94.3% and 93.4% of cells positive for Ki67 respectively.

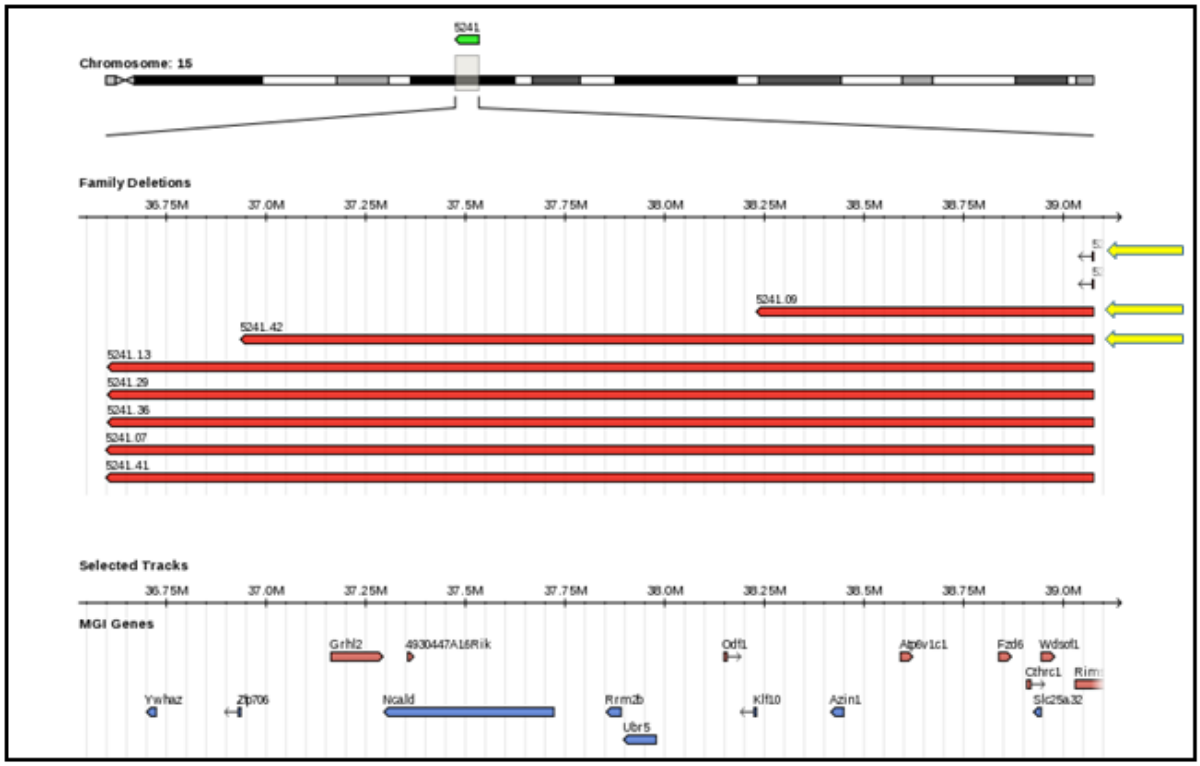

Figure 17: Representation of the nested chromosomal deletions in family 5241 -The deletions were mapped using I-PCR. The location of the anchor virus integration (green arrow) is Chr 15: 39,076,241. The 1st, 2nd and 3rd yellow arrows indicate the selected clones 5241.16, 5241.09 and 5241.42 having 1, 8 and 15 genes deleted in them respectively. (http://bioinfo.iric.ca/deles/Families)

Benzidine staining assay was performed on day 8 of EB differentiation. The percentage of benzidine positive EBs in clones 5241.16, 5241.09 and 5241.42 were 5% ± 0.9, 4 % ± 0.3 and 10%±1 respectively compared to the primary clone which was 13 % ±1. (Figure 18, left panel) We observed that the number of EBs formed for each of the 5241 clones and the R1 ES cells are comparable (Figure 18, right panel). As mentioned in the literature review, to state a tertiary clone has a defect in EB formation, it should meet the following criteria:

a) A clone should have at least 5% of seeding density.
b) An abnormal tertiary clone should produce less than 1/5th the number of EBs compared to the primary clone.

c) An abnormal family should include clones with a phenotype that co-relates with deletion sizes.

5241 tertiary clones do not meet these criteria.

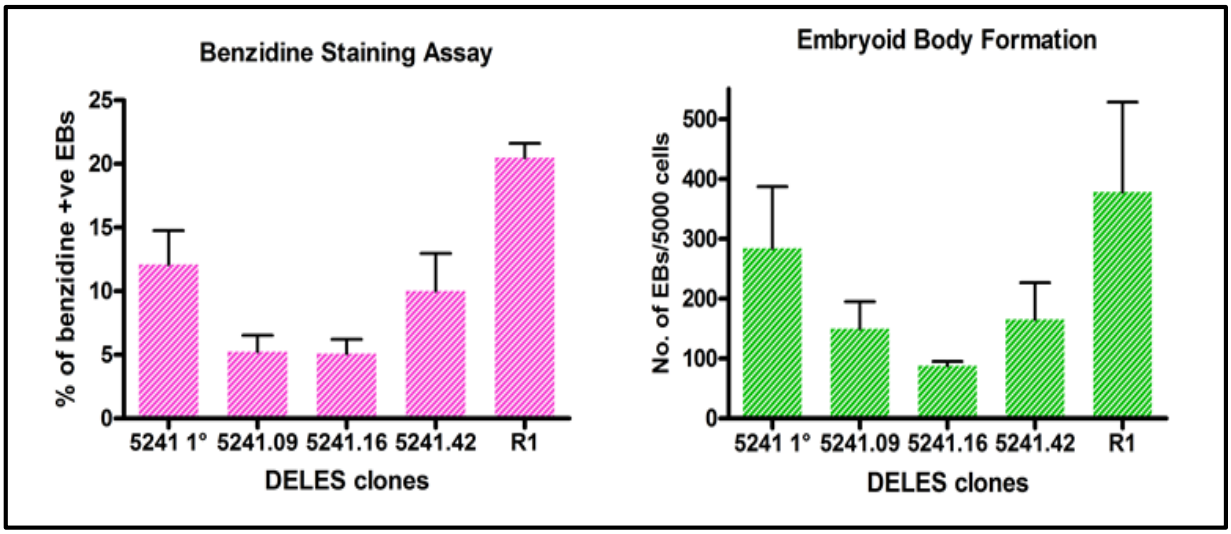

Figure 18: Embryoid body formation and Benzidine staining assay of 5241 family : 5241 clones were seeded at a density of 1250cells/mL in semi-solid differentiation media and allowed to form EBs. 5241 1° is the primary clone without any chromosomal deletions. 5241.16, 5241.09 and 5241.42 are tertiary clones containing 1, 8 and 15 genes deleted in them respectively. Day 8 EBs were stained with benzidine and scored, n=3. Error bars represent S.D

The benzidine staining results showed that there is indeed reduced benzidine coloration among the tertiary clones compared to the primary clone. I also observe a reduction in the percentage of benzidine staining between the control primary clone and the R1 ESCs, here I hypothesized that the infection and integration of the primary anchor virus may itself have contributed to the reduced hematopoietic potential of this family. An interesting result here is that the tertiary clone 5241.16 containing a single gene (Rims2) deletion already exhibits an abnormal hematopoietic differentiation. Though I see a reduction in the number of EBs formed in clone 5241.16, I do not see the same extent of reduction in clone 5241.09 and 5241.42 (criteria c) although I do observe the same extent of reduced benzidine staining in clone 5241.16 and 5241.09. Therefore, I suggest that the reduction in the percentage of benzidine positive EBs observed in these clones are not due to defects in the formation of embryoid bodies.

The Rims2 gene is highly expressed in reproductive tissues such as testis and moderately expressed in hematopoietic myeloid and lymphoid cells. Mice homozygous for this deletion have reduced body size, aberrant insulin granule exocytosis, impaired secretion of hormones associated with glucose homeostasis, however, their hematopoietic phenotype is undetermined.

Next, I proceeded with the validation of another DELES family clone – 5035 that looked interesting based on the initial preliminary screen data. (Figure 16)

The 5035 family have nested chromosomal deletions on chromosome 8. Within this family I selected the 5035 primary clone and two tertiary clones 5035.10 (*Gsr, 1700104B16Rik, Gtf2e2, Rbpms)* and 5035.41 (*Gsr, 1700104B16Rik, Gtf2e2, Rbpms, Dctn6, Mboat4, Leprotl1 and Tmem66)* harboring 4 and 8 gene deletions respectively. (Figure 20) These tertiary clones were selected based on the number of genes deleted and the percentage of Ki67 positive cells indicating proliferation potential. 5035.10 had a Ki67 staining of 74.5% and 5035.41 had a Ki67 staining of 87.1%. However, the 5035.10 clone was not recovered due to viability issues, and hence not pursued further.

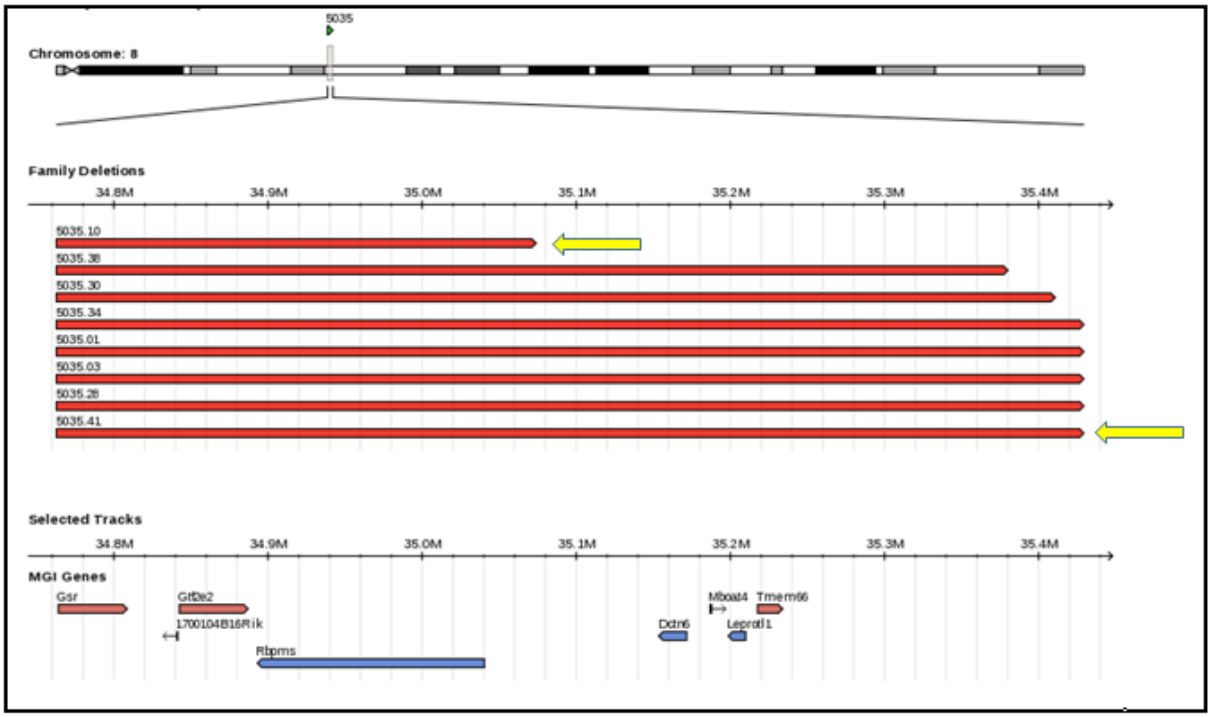

Figure 19: Representation of the nested chromosomal deletions in family 5035 : The deletions were mapped using I-PCR. The location of the anchor virus (green arrow) is Chr 8: 34,762,701. The 1st and the 2nd yellow arrow indicate the selected tertiary clones 5035.10 and 5035.41 having 4 and 8 gene deletions respectively. (http://bioinfo.iric.ca/deles/Families)

The percentage of benzidine positive EBs in clones 5035.41 was 7 %±0.4 compared to 31%±1 of the primary clone. (Figure 20, left panel). This family too doesn't meet the criteria for EB formation defects. In Figure 20, right panel, I do see quiet a difference in the number of EBs formed in the tertiary clone compared to the 5035 primary clone. However, to either confirm or rule out EB formation defects, other clones from the same family comprising the same deletions as 5035.41 will have to be tested for their EB formation potential (criteria c). Additionally, Simon Fortier had tested the 5035 family for EB differentiation defects and concluded no abnormal phenotype for this family.

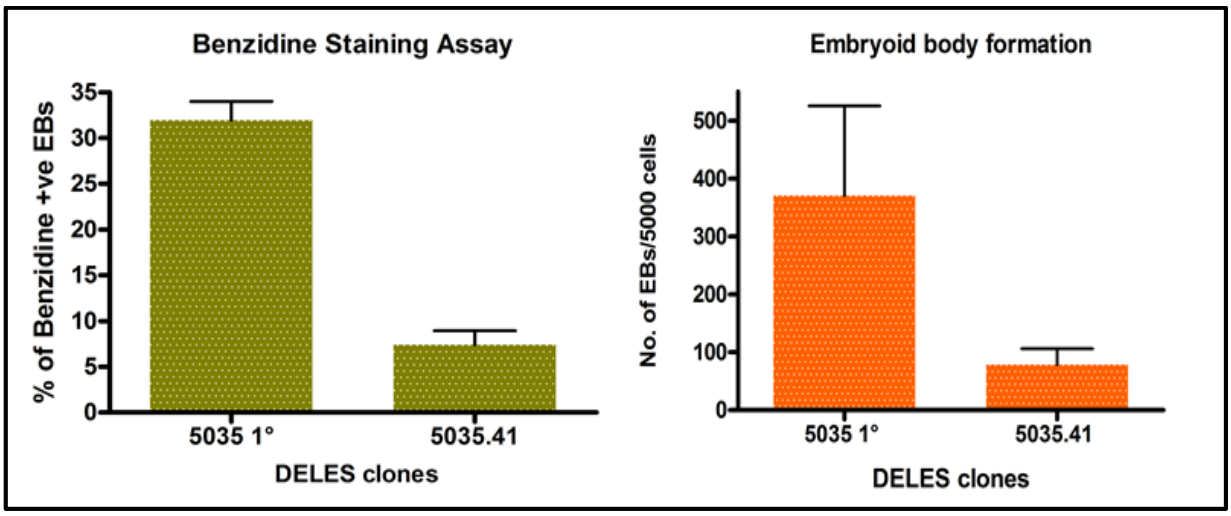

Figure 20 – Embryoid body formation and Benzidine staining assay of 5035 family: 5035 clones were seeded at a density of 1250cells/mL in semi-solid differentiation media and allowed to form EBs. 5035 1° is the primary clone without any chromosomal deletions. 5035 is a tertiary clone containing 8 gene deletions. Day 8 EBs were stained with benzidine and scored, n=2. $p < 0.05$. Error bars represent S.D

The difference in the percentages of benzidine positive EBs in the primary and tertiary clone implied the absence of chromosomal regions potentially responsible for the hematopoietic defects in these clones.

3.2.5 Validation of 5035 family using hematopoietic differentiation assay.

The kinetics of hematopoietic differentiation assay was determined for the different hematopoietic genes in wild-type R1 ES cells. I observed and concluded that hematopoietic genes were induced systematically and reproducibly for every biological repeat. I next decided to test the hematopoietic differentiation potential of the 5035 family using this asasy.

A dramatic decrease in the pluripotency marker OCT4 compared to the control was observed suggesting a pluripotency defect in these clones. I also observed a reduced expression of hematopoietic genes like CD41 and GATA2. Compared to the wild-type ES cells (Figure 11) a delay in RUNX1 expression in the primary clone (induced on day 8 compared to day 5 in wild-type cells) and no expression in tertiary clone was detected. This suggests the integration

of the anchor virus at a locus that may be involved in the trans regulation of this gene. Additionally , β-Globin1 expression in the tertiary clone of 5035 was not detected. (Figure 21)

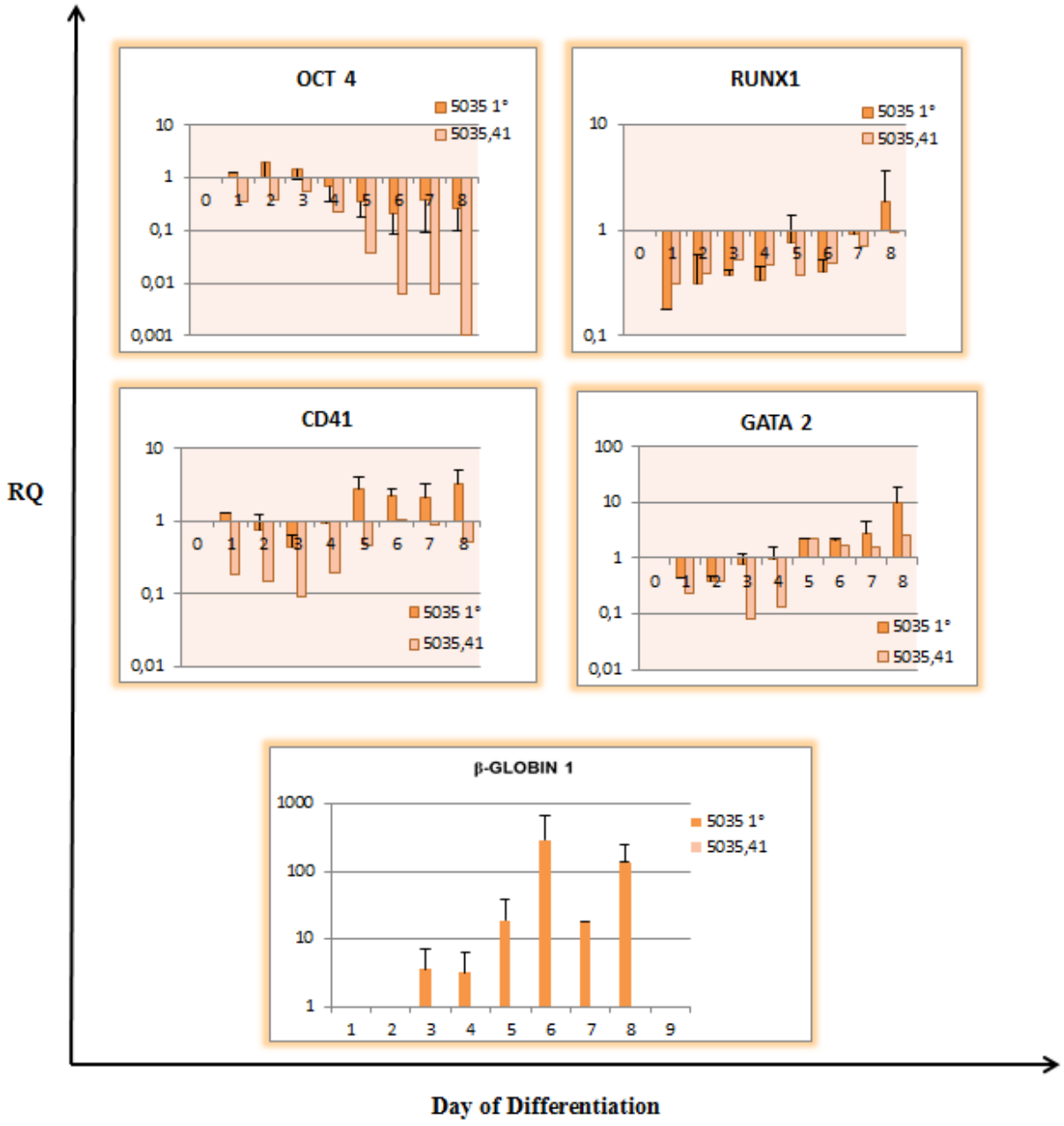

Figure 21: Kinetics of hematopoietic differentiation for 5035 family. – Sequential expression of OCT4 and hematopoietic genes CD41, RUNX1, GATA2, and β-GLOBIN1 monitored by qRT-PCR. Control = 5035 1° (n=2) and tertiary clone 5035.41 having 8 gene deletions. Relative Quantification (RQ) value is calculated using day 0 as a reference, it

depicts the expression of the target gene on a given day compared to its expression on day 0 of the differentiation assay. Error bars represent S.D

The Q-RT-PCR results indicate a defect in the expression of hematopoietic genes in the 5035.41 clone. This may be explained by the deletion of Gsr and Rbpms genes that have been known in the literature to play a role in hematopoiesis.

In the next steps of this project I will confirm the qPCR results obtained for the 5035 family by FACS using the method I set up. BAC complementation and cDNA complementation methods that have already been optimised in the lab will be used to rescue the phenotype observed in the 5035 clone and will help me identify networks that govern hematopoietic cell fate.

Altogether my results show that :

- ✓ The established differentiation assay can reproducibly induce hematopoietic commitment.
- ✓ qPCR and FACS analyses can reliably monitor the differentiation kinetics.
- ✓ DELES library has helped us identify candidate genes (*Rims2*, *Gsr, 1700104B16Rik, Gtf2e2, Rbpms, Dctn6, Mboat4, Leprotl1 and Tmem66*) with potential roles in ES to hematopoietic cell differentiation.
- ✓ We do observe a reduction in the number of EBs formed in clone 5035.41. This suggestes that hematopoietic defects observed in this clone may be the consequence of EB differentiation defects. To confirm EB formation defects, this clone must satisfy the criterias that an abnormal tertiary clone should produce less than $1/5^{th}$ the number of EBs compared to the primary clone and an abnormal family should include clones with a phenotype that co-relates with deletion sizes. 5035.41 does not meet the first criteria. Clones from this family have been previously tested for EB formation defects and was concluded to have no phenotype.

- ✓ The established differentiation protocol and Q-PCR/FACS analyses can be used to investigate hematopoietic defects in the 5035 and 5241 DELES families in more detail.

3.3 Towards the generation of a KBM-7 cells library harboring nested chromosomal deletions using DELES methodology.

A recently performed drug screen in the lab has tested 5,120 FDA-approved drugs for their ability to inhibit the growth of twenty genetically diverse patient-derived leukemia samples. In this screen we have identified groups of drugs that have the ability to inhibit selective subsets of the tested leukemia specimens. This suggests that these compounds target very specific pathways, which are important for the growth of some leukemia specimens. We have functionally classified selectively inhibitory compounds into groups that we termed *Compound Correlation Clusters (CCCs*). Identified CCCs comprise a varying number of compounds with diverse chemical structures (chemo-types). Their functional overlap with respect to their ability to inhibit leukemia growth suggests that drugs in CCCs act on the same or converging molecular pathways. As such, CCC-classified drugs represent promising pharmacological tools to develop novel anti-leukemia agents.

To elucidate genetic determinants of leukemia drug resistance, my project focuses on utilizing the DELES retrovirus-based Cre-*lox*P approach to introduce chromosomal deletions in haploid KBM-7 leukemia cells.

Rationale: Using the human KBM-7 CML cell line as a model system and employing the retrovirus based Cre-*lox*P strategy as a genetic approach to create nested chromosomal deletions in these cells, molecular pathways relevant to CCC drug sensitivity and resistance can be identified.

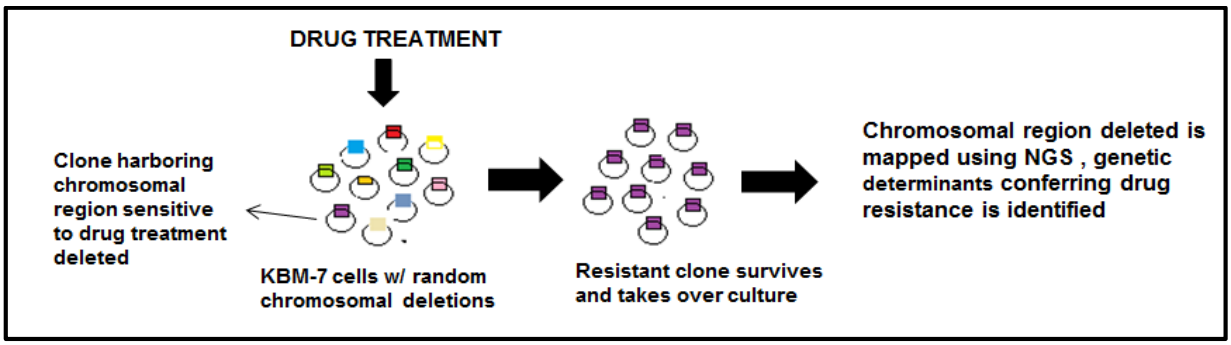

Figure 22: Rationale - Screening a library of clones with various chromosomal deletions will help identify genes/chromosomal regions that when deleted confer drug resistance to CCCs.

The goal of my project is to test if the human haploid KBM-7 cell line can be exploited using the DELES approach to create a library of clones comprising random chromosomal deletions. The advantage of using a haploid cell line is that the DELES approach would result in homozygous deletions resembling complete knockout phenotypes.

Objectives : To test if the KBM-7 cells can be exploited to create a library of clones containing random chromosomal deletions, I will have to determine if :

a) KBM-7 cells can be expanded rapidly.
b) KBM-7 cells are clonogenic.
c) KBM-7 cells are selectable using appropriate antibiotics.
d) KBM-7 cells are amenable to retrovirus infection.

3.3.1 Growth kinetics of KBM-7 cells.

As an initial preparation to creating the library of KMB-7 cells harboring nested deletions, I wanted to determine the doubling time of these cells. To do this, KMB-7 cells were plated at a density of 2.5x10^5 cells/mL in a 96-well format and the cells were counted on each day for 4 days. Figure 23 shows us the doubling time for these cells is ~ 24 hours.

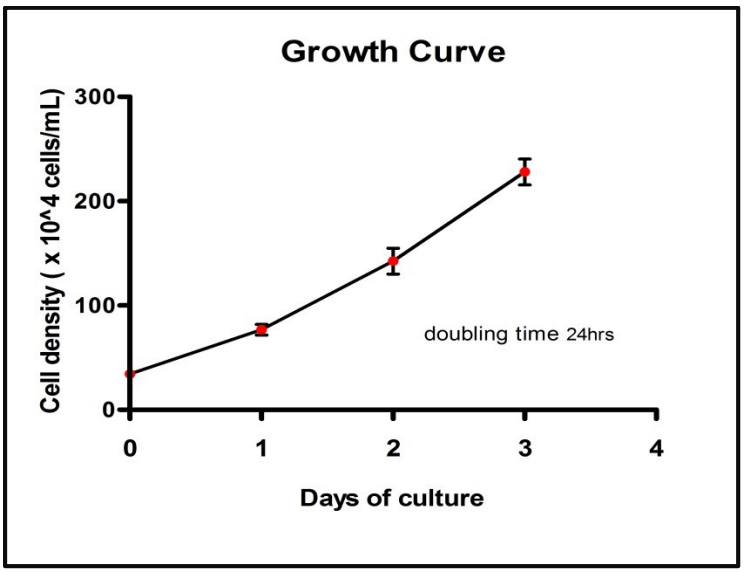

Figure 23: Growth curve of KBM-7 cells - Cells were plated at a density of 2.5 x 10^5 cells / mL. Cells were counted for 4 days using Trypan blue. Error bars represent S.D.

This indicates that the KBM-7 cells can be expanded rapidly.

3.3.2 Clonogenicity of KBM-7 cells.

Next, I wanted to evaluate the clonogenicity of the KBM-7 cell line. Clonogenicity is the ability of a single cell to form a colony. The clonogenicity of these cells will allow us to determine the number of cells needed to be infected with the primary anchor virus and saturation virus to achieve the final desired complexity of our library. To determine the clonogenicity of KBM-7 cells I conducted a limiting dilution assay (LDA). I performed serial dilutions to get different cell doses of 1000, 333, 111, 37, 12, 4, and 1 cells /well. This assay was done in a 96-well plate where each row was replicates for a single cell dose. I scored the wells for each cell dose for the absence of colonies after 7 days. Using poisson distribution , $F_0 = e^{-m}$, where F_0 is the frequency of wells receiving no cells, e = 2.7183 is a base of natural logarithm and m= is the average number of cells per well. The frequency of cells giving us 37% negative cultures gives us an estimate of the frequency of cells having clonogenic capacities. (Figure 24) The graph below determined that the clonogenicity of KBM-7 cells is 33% (1/3 * 100).

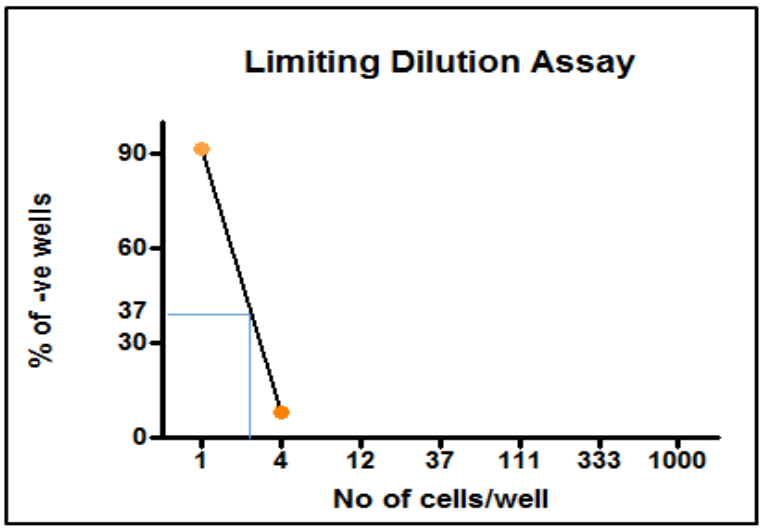

Figure 24: Limiting diluting assay - Serial dilutions to get cell doses of 1000, 333, 111, 37, 12,4 and 1 cells/well were performed. Each row of a 96-well plate was replicates for a single cell dose. For each cell dose, wells were scored for the absence of colonies formed after 7 days. The percentage of wells without colonies was plotted on the y axis and the cell dose was plotted on the x-axis.

The graph above determined that the clonogenicity of KBM-7 cells is 33% (1/3 * 100). This means that in order to have 1000 clones, we will have to plate 3000 KBM-7 cells.

3.3.3 Antibiotic selection tests.

The selection of primary, secondary and tertiary clones will be based on their resistance to puromycin, hygromycin and neomycin antibiotic resistance respectively (Figure 3). To determine the appropriate dose of these antibiotics for selection, I conducted dose response tests on KBM- 7 cells. KBM-7 cells were plated at a density of 2.5x10^5 cells /mL and selection for puromycin, hygromycin and neomycin were initiated after 48 hours. (Figure 25)

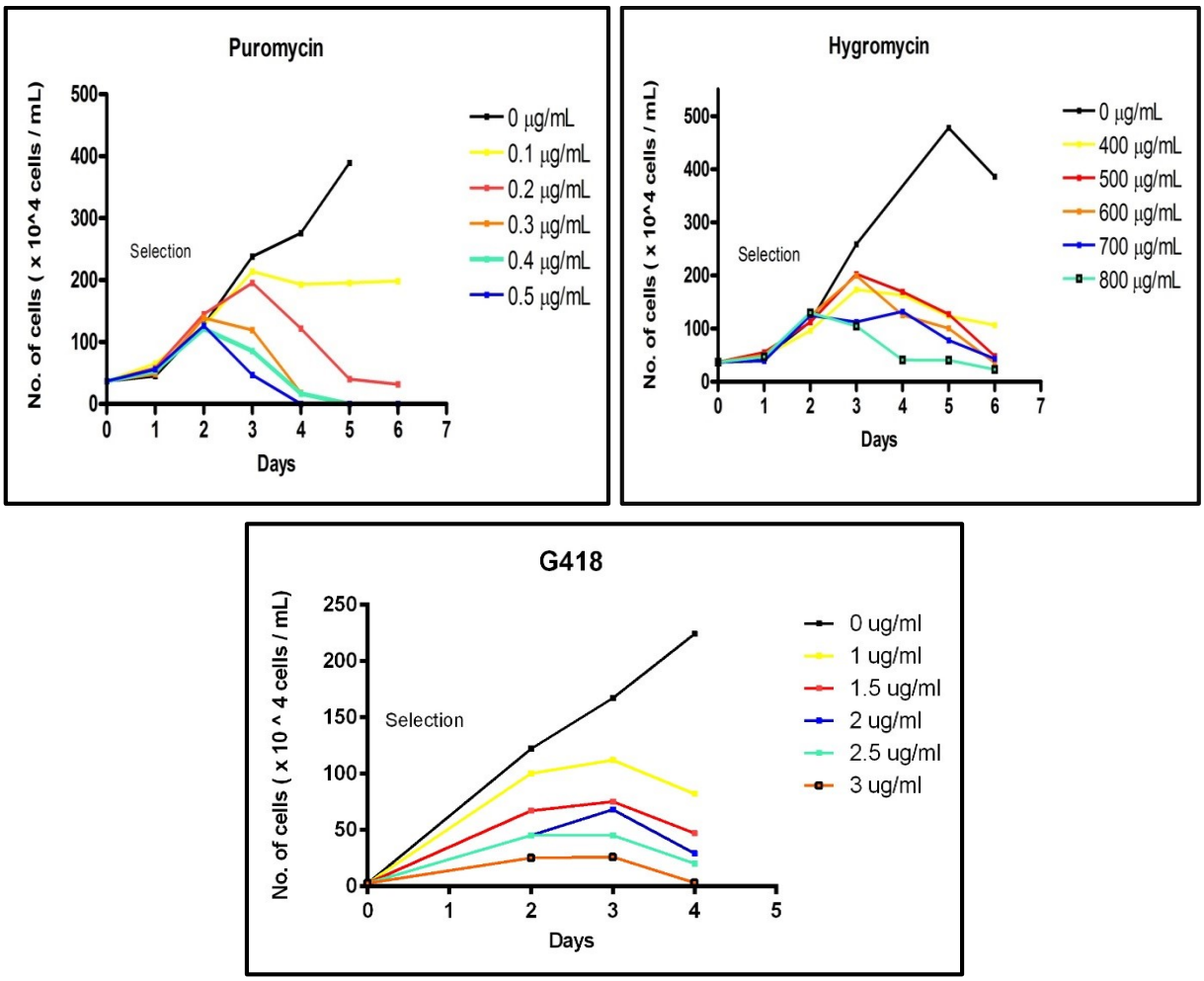

Figure 25: Dose response selection tests for puromycin, hygromycin and G418 - KBM-7 cells were plated at a density of 250,000 cells/mL in a 96 well plate. The selection for each antibiotic was initiated after 48 hours. The selection for each dose was carried out in duplicates and the line plotted for each dose represents the mean value of the cell count for each duplicate.

Five different doses for each antibiotic were used. The dose for puromycin ranged from 0 – 0.5 µg/mL with 0.1 µg/mL increments. For hygromycin we tested a range of 0 – 800 µg/mL, with 100 µg/mL increments. The dose for G418 ranged from 0 – 3 µg/mL, with increments of 0.5 µg/mL.

Looking at the data obtained, I estimated that the appropriate dosages for puromycin, hygromycin and G418 to be used would be the lowest concentration of the drug that will bring

about selection in two days' time. The concentration for puromycin, hygromycin and G418 to be used for selection will be 0.3µg/mL, 700 µg/mL and 1.5µg/mL respectively.

3.3.4 Retrovirus infection of KBM-7cells.

Transfection of producer HEK293 (expressing stably VSV-G envelope and gag and pol genes) cells with A1 virus was conducted according to the protocol discussed in the Material and Method section. Titration of the viral supernatant was done by infecting the KBM-7 cells as illustrated below in Table 8.

Table 7: Serial Dilution of Viral supernatant.

TUBE #	1	2	3	4	5
Volume of virus	80µL stock	600 µL	120 µL	120 µL	120 µL
Volume of media with 2X polybrene	1920 µL	600 µL	1080 µL	1080 µL	1080 µL
Final dilution	**1/50**	**1/100**	**1/1000**	**1/10,000**	**1/100,000**

Since the A1 virus did not have a fluorescent marker we could not use FACS to determine the titer. Instead we decided to titrate the virus using methylcellulose. KBM-7 cells infected with various viral supernatant dilutions in methylcellulose were plated in the presence and absence of puromycin. The viral titer could then be determined by comparing the colonies formed in both cases.

To do this, I first plated non-infected KBM-7 cells in methylcellulose to see if they could grow and form colonies. I initially plated two cell doses of 2500 and 5000 cells in 1mL of methylcellulose. I obtained 7 and 2 colonies for the respective cell dose. I next repeated the assay this time increasing the cell dose to 3000, 6000 and 10,000 cells in 1mL of methylcellulose. I did not observe any colonies in these cases.

We know that the doubling time of these cells is ~ 24hours but in methylcellulose they did not grow and form colonies as well as expected. I decided to test if supplementing methylcellulose with human cytokines (used in the lab to propagate human myeloid clonogenic progenitors) would make a difference in the maintenance of KBM-7 cells in methylcellulose. In the

presence of these cytokines, the KBM-7 cells proliferated as expected and formed robust colonies in methylcellulose. (Figure 26)

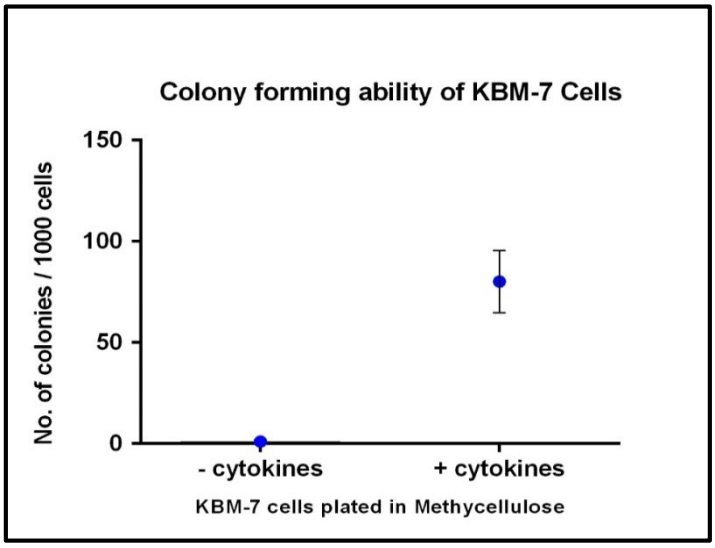

Figure 26: Colony forming ability of KBM-7 cells - 1000 KBM-7 cells were plated in 1mL methylcellulose in the presence and absence of cytokines such as SCF, EPO, IL-3, IL-6, GM-CSF and TPO. Each cell dose Mere plated in duplicates. Error bars represent S.D.

- Titration of the A1 virus :

5x10^5 cells were infected using the ½ dilution of the viral titre. 1000 cells were plated in methylcellulose in the presence and absence of puromycin. Colonies were scored after 12 days. (Figure 27)

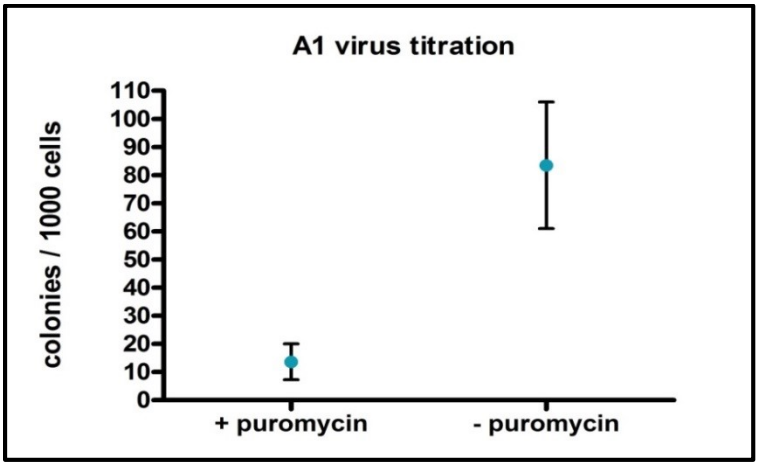

Figure 27: Titration of A1 virus in methylcellulose - 1000 cells were plated in 1 mL methylcellulose in duplicates in the presence and absence of puromycin. Colonies were scored under the microscope after 12 days. n=1. Error bars represent S.D.

Using the formula to calculate viral titer, we calculated the titer for the A1 virus as 20,434.13 TU/mL.

This result implies that the KBM-7 cells are amenable to infection and selection. In order to obtain maximum genome coverage in the KBM-7 library, we require one anchor virus integration per cell. From literature we know this can be attained by infecting the cells at an infection efficiency less than 15%. From this assay we attained an infection efficiency of 12.5% ((10 colonies/80 colonies)*100). This is within the desired range of 15-20% infection efficiency that enables us to achieve 1 viral integration per cell.

Altogether my results have allowed me to conclude that the human near haploid KBM-7 CML cell line can be exploited by the DELES approach to create a library of clones containing random chromosomal deletions.

Table 8: Criteria for the candidate cell lines for the DELES approach to be feasible.

	R1 ESCs	KBM-7
Rapid Expansion	✓	✓
Clonogenic	✓	✓
Selectable using Puromycin, Hygromycin and G418	✓	✓
Amenable to retrovirus infection	✓	✓

4. Discussion

Clinicians have been using hematopoietic stem cells to cure malignant and non-malignant diseases for over a decade now. Therefore, the use of stem cells is not a new concept. However, derivation of Embryonic Stem Cells (ESCs) and their remarkable ability to differentiate into cells of the three germ layers have captured the imagination of the public and the scientific communities. A major thrust of basic hematopoietic stem cell (HSC) research since the late 90's has been to characterize these stem cells and identify determinants responsible for the constant renewal of blood.

The DELES library is a collection mESCs harboring random nested chromosomal deletions. In the lab, we are interested in identifying novel determinants for hematopoietic cell fate using the DELES library. To identify clones exhibiting reduced hematopoietic potential, we screened 88 DELES clones for the presence of benzidine positive embryoid bodies (EBs). Benzidine is a compound that is highly selective and stains blue in the presence of hemoglobin. In this primary screen, we identified several DELES clones exhibiting an abnormal hematopoietic phenotype. The defects in hematopoietic phenotype here in some clones could be a result of EB formation defects. In order to conclude abnormal hematopoietic differentiation in a family of clones, the benzidine assay was validated by repeating the assay in a larger format and the candidate clones would have to be subjected to a second round of screening.

The objective of my project was to establish a robust hematopoietic differentiation assay to monitor the hematopoietic differentiation potential of the candidate DELES clones. The idea was to set up a secondary screen using qPCR and flow cytometry approaches that would help me identify the stage at which hematopoietic differentiation is arrested by observing changes in mesodermal and hematopoietic genes and surface marker expression when these clones differentiate to the hematopoietic lineage.

Here, I used a protocol published by Gordon Keller's group, which allowed the generation of hematopoietic cells in a step-wise manner without interference from serum and stromal feeders.

I started by looking at the kinetics of hematopoietic differentiation through qRT- PCR. Stepwise addition of growth factors and cytokines induced hematopoietic differentiation from mESCs. This was demonstrated by the upregulation of gene transcripts marking hematopoietic commitment. The qRT-PCR results showed the downregulation of pluripotency marker OCT4 with the concomitant upregulation of the mesodermal marker Brachyury (T). As the differentiation towards the hematopoietic lineage occurs, I observed a decrease in T expression with the concomitant increase in hematopoietic gene markers CD41, C-KIT, RUNX1, GATA2, CD45 and β-GLOBIN 1 from day 5 onwards. This is expected with the protocol I am using as it is on day 4 I add cytokines and growth factors that drive hematopoietic differentiation. Before I screened the DELES clones using this assay, I needed to set up a differentiation protocol that was robust, reproducible and that had minimum variability between each repeat. From the qRT-PCR data, the expression of the hematopoietic genes tested is reproducible and observing the error bars, the variability between the biological repeats is in an acceptable range.

Next, I wanted to determine the expression of hematopoietic cell surface markers such as Ter-119 and CD45. Studying the kinetics of the differentiation assay by qRT-PCR, I decided to conduct FACS on day 6 and day 8 time points. When I performed FACS on day 6 and day 8 of the differentiation protocol, I obtained ~ 10% and 15% CD45+ population on day 6 and day 8, respectively. I observed a 1.5 % of Ter-119 + population on day 6 which increased to 30% on day 8. On FACS analysis of the differentiation assay, I concluded that percentages of CD45+ and TER-119+ populations on day 6 and day 8 time points can be used as the readout to screen candidate DELES clones.

The hematopoietic system involves distinct developmental steps from the induction and patterning of primitive streak cells and the mesoderm to the specification of the earliest blood cell progenitors. We can observe this developmental progression by studying the expression of genes indicative of each stage. Numerous protocols have been published for generating *in vitro* derived HSCs, but most of them make use of serum and stromal feeder layers. These protocols are compromised by the undefined components in the serum and the robustness of these protocols is dependent on the stromal feeder layers. The protocol used here is serum-free and feeder-free and overcomes these limitations. I conclude that the protocol from the Keller

group worked successfully in my hands and enables observation of the expected kinetics of hematopoietic differentiation. As with any biological system, variation between the biological and technical repeats was observed but overall this differentiation protocol was robust. Additionally, the advantage of this protocol over others is that it induces the differentiation of ES cells to hematopoietic cells in a stepwise manner. This helps us identify at which step the differentiation is compromised in the candidate clones by studying the expression of genes associated with that developmental stage.

Out of the DELES clones screened in the primary assay, the 5241 and 5035 families looked interesting to study, with an average of 10 % and 20.05% benzidine positive EBs respectively compared to 46% for the wild-type R1 ES cells. I chose these families for further validation studies.

I picked 4 clones within the 5241 family - the primary clone of 5241 as the control and three tertiary clones with increasing chromosomal deletion sizes – 5241.16, 5241.09 and 5241.42 having 1, 8 and 15 genes deleted in them respectively. The clones 5241.16, 5241.09 and 5241.42 had 5.09 %, 4.27% and 10 % benzidine positive EBs respectively compared to 13.4% in the control. Though the primary clone of 5241 had no deletions, it had 13.4 % benzidine positive EBs compared to the wild type R1 which had an average of 22% benzidine positive EBs. I hypothesize that the infection and integration of the anchor virus may itself have contributed to the reduced hematopoietic potential of this family. Surprisingly I did not observe a more pronounced hematopoietic differentiation defect with a larger deletion size in clone 5241.42 (10% benzidine positive EBs). Clone 5241.42 has 15 genes deleted compared to 5241.16 and 5241.09 which have 1 and 8 genes deleted respectively. Less pronounced hematopoietic defect in clones with bigger deletions could be due to the deletion of a gene (or region) that acts as a negative regulator of the hematopoietic differentiation. Other tertiary clones from the same family will have to be examined to confirm these observations. Interestingly, we observe clone 5241.16 with only one gene (Rims2) deleted exhibited a reduction in benzidine staining. Though I observe a lower frequency of EBs formed in this clone, I do not observe the same extent of reduction in EB frequency in clone 5241.09 (which also has the Rims2 gene deleted). However, I do observe a similar reduction in the benzidine staining in these two clones. Therefore, I hypothesize that the abnormal hematopoietic defect observed in these clones is not due to EB formation defects. In addition, this clone does not

meet the criteria that in order to confirm a EB formation defect, an abnormal tertiary clone should produce less than $1/5^{th}$ the number of EBs compared to the primary clone. Regulating synaptic membrane exocytosis 2 (Rims2) is a gene that is 486.26 kb in length. It is highly expressed in reproductive tissues such testis and has a low to moderate expression in lymphoid and myeloid hematopoietic cells. Rims2 is known to be involved in glucose homeostasis and insulin secretion pathways. Mice homozygous for this deletion show reduced body size and abnormal maternal behavior however, their hematopoietic phenotype is undetermined. In humans, mutations in Rims2 are associated with lung cancer. BAC and cDNA complementation experiments will be conducted to identify if the phenotype observed is solely due to the deletion of the Rims2 gene or due to the deletion of other non-coding elements within the gene.

Results from the primary screen also showed an interesting phenotype for the 5035 family. The percentage of benzidine positive EBs was 7% in the tertiary clone compared to 32% of the 5035 primary clone.

Once the gene expression profiles for the different hematopoietic markers were characterized for the wildtype R1 ESCs by Q-RT-PCR, I proceeded to test the hematopoietic differentiation potential of the 5035 primary and tertiary clone (5035.41) using this assay. qPCR results obtained showed a decrease in OCT4 expression in the tertiary clone compared to the primary 5035 clone. I also observed a reduced expression of hematopoietic genes such as CD41 and GATA2. The expression of RUNX1 is similar in the primary and tertiary clones but is reduced compared to the wildtype R1 ESCs, suggesting the integration of the anchor virus at a locus involved in the trans-regulation of the RUNX1 gene. Additionally, I did not observe β-globin1 expression in the 5035.41 clone. Clones from the 5035 family have nested gene deletions on chromosome 8. The 5035.41 clone has 8 genes deleted – *Gsr, 1700104B16Rik, Gtf2e2, Rbpms, Dctn6, Mboat4, Leprotl1 and Tmem 66.* From the literature we know Gsr plays a critical role in reducing oxidative stress. [80] ES cells in their undifferentiated state have low levels of reactive oxygen species (ROS) which increases on differentiation. [81] The deletion of this gene may result in an accumulation of ROS in these clones causing them to lose their pluripotency state and differentiate. We also know that Gtf2e2 gene is differentially expressed in the ICM. [82] The deletion of the Gsr and Gtf2e2 alleles might explain the low OCT4 expression of the 5035.41 clone. Deletions of the Gsr and Rbpms genes in 5035.41 might

explain the reduced expression of hematopoietic genes like β-globin1, CD41 and GATA2. Gsr is known to protect hemoglobin against oxidative stress induced damage. [80] Rbpms plays a role in SMAD signaling [83], thereby loss of this allele might impede BMP4 signaling affecting hematopoietic commitment. Nevertheless, we will conduct BAC complementation for all the 8 genes to identify which genes are haplo-insufficient to induce hematopoietic differentiation.

The idea behind my secondary project was to employ the same retro-viral Cre-*lox*P strategy used to generate the DELES library, to generate a library of KBM-7 cells harboring nested chromosomal deletions. The advantage of using the near haploid KBM-7 cell line is that the DELES approach would result in homozygous deletions resembling complete knockout phenotypes.

With the successful completion of the human genome sequencing project, one challenge now is to understand the functional annotation of these genes in the cell. One approach to this would be to study phenotypes in mutants which lack the gene of interest or mutants in which the expression of the gene is altered. Studying the phenotype when specific cellular pathways are compromised or disrupted in these mutants will elucidate the biological role of the gene. Mutagenesis studies on near haploid human CML cell line-KBM7 have been conducted to screen for phenotypes such as proliferative defects or sensitivity to pathogen infection when human genes have been disrupted. [84] In gene-trap mutagenesis screens, not all trapped genes result in complete inactivation of the gene transcripts. In some cases, the inactivation of a gene transcript can be masked by alternative transcripts. [84] In studies, these individual mutagenized clones have to be isolated and expanded for DNA isolation to map the gene-trap insertions by sequencing or I-PCR , which is labor intensive. Additionally, it may not produce a reliable and robust genome-wide overview of genes contributing to the phenotypes of interest. [85]

Our approach is to use a retroviral based Cre-*lox*P strategy to create random nested chromosomal deletions. The advantage of our method over other mutagenesis methods is: 1) it will help us understand the contribution of both protein coding regions and non-protein coding regions to a phenotype. 2) This approach does not favor small deletions as there is no significant difference in the efficiency of Cre-induced recombination as the distance between

the two *lox*P sites increases from a few kilobases upto a few megabases. [86] 3) Deletions are permanent, rapidly obtained and can be mapped easily.

In order to create a library of KBM-7 cells harboring nested chromosomal deletions, my objective was to test if the KBM-7 cells can be exploited using the DELES approach. To determine the feasibility of our approach on the KBM-7 cell line, I had to evaluate the growth kinetics, clonogenicity of these cells and also determine if they are ammenable to retrovirus infection and selection. I observed that the KBM-7 cells double every 24 hours. Clonogenicity of a cell line is the degree to which the cells can form clones. I observed that the clonogenicity of KBM-7 cells is 1/3 when plated in methylcellulose supplemented with cytokines used to propagate human myeloid progenitors. This is an important factor to consider when designing the library. The clonogenicity of the cell line and infection efficiency of the primary, secondary and Cre- virus helps us calculate the number of cells to be infected and expanded at each stage to achieve the desired complexity of the library. For example, the human genome is 3x10^9 bp , the average deletion size obtained by our approach in the DELES library was 3Mb, therefore to obtain a 10X genome coverage , we require 10,000 primary clones. With a clonogenicity of 1/3, we will have to plate 30,000 primary clones to obtain 10,000 primary clones. We know that in order to obtain a library of high complexity, we need to infect KBM-7 cells at an infection efficiency of 10% to obtain one anchor virus integration per cell, thereby to obtain 30,000 infected cells, we will have to infect 300,000 KBM-7 cells with the primary anchor virus. Here, I was able to determine that KBM-7 cells are clonogenic, can be rapidly expanded, are amenable to infection and selection by puromycin (0.3µg/mL), hygromycin (700µg/mL) and neomycin (1.5µg/mL) and thereby the DELES strategy can be used on this cell line to obtain a library containing clones with nested chromosomal deletions.

This library of KBM-7 clones can serve as a platform on which various studies to understand the mechanisms contributing to leukemia development and to the treatment of leukemia can be conducted. For example, in our lab, this library will be used to delineate the molecular activities of potential anti-leukemic drugs that we have identified to inhibit cell growth in several patient-derived acute-myeloid leukemia specimens. It will also allow us to identify molecular signaling pathways that, when genetically disrupted, can confer resistance to these

drugs. The objective is to functionally link these deleted regions and potential targets for the drugs that will be screened.

Despite the advantages and possible uses of our approach, one of the limitations of this methodology is the complexity to demonstrate that an identified determinant is solely responsible for a phenotype observed. To validate the candidate determinants, CRISPR/Cas9 techniques can be used to knockdown the candidate genes in wildtype cells; this will help determine if the phenotype observed is solely due to the absence of the identified gene and not due to other genetic elements also deleted.

5. Conclusion

In conclusion, our approach to screen DELES clones for defects in hematopoietic commitment first involves screening the clones using the primary benzidine assay and then monitor with the Keller protocol where the differentiation arrest is occurring. The results show that the hematopoietic differentiation assay I set up in the laboratory can reproducibly induce hematopoietic commitment and Q-RT-PCR and FACS analyses can reliably monitor the differentiation kinetics. We also observe that the DELES library can help us identify candidate genes (*Rims2, Gsr, 1700104B16Rik, Gtf2e2, Rbpms, Dctn6, Mboat4, Leprotl1 and Tmem66*) with potential roles in ES to hematopoietic cell differentiation. I conclude that the established differentiation protocol and Q-PCR/FACS analyses can be used to investigate hematopoietic defects in the 5035 and 5241 DELES families in more detail. BAC and cDNA complementation experiments will now be conducted to identify which gene or combination of genes will be able to rescue the hematopoietic defects observed in clones 5241.16 and 5035.41.

For the second part of my project, I have determined initial parameters such as doubling time of the KBM-7 cells, their clonogenicity, the appropriate dosages for the antibiotic selection tests and the appropriate MOI for infection of the cells that I require to generate a library of KBM-7 cells harboring random chromosomal deletions. A pilot experiment will be performed to evaluate all the parameters determined and make the appropriate calculations required for the scale-up experiment. By setting up this library, I will be developing a valuable research tool for drug target identification studies. I anticipate that the utility of this approach will be broad and will allow us and other researchers to explore the genetic basis of drug activities in leukemia cells.

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