Dissecting the Role of Trisomy 21 in Childhood Acute Lymphoblastic Leukaemia

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Declaration

I, Sean David Holohan, confirm that all the work completed in this thesis is my own unless otherwise stated. Information that has been taken from other sources in this thesis is referenced.

Abstract

Children with Down's Syndrome (DS) have a twenty fold increased risk of developing acute lymphoblastic leukemia (ALL) and a five hundred fold increased chance of developing acute megakaryoblastic leukemia (AMKL). This suggests a role for trisomy 21 as a 'first hit' event, predisposing these children to leukaemogenesis. Childhood leukaemias differ from adult leukaemias in that they have an increased incidence, improved prognosis and have a distinct mutational spectrum. I hypothesized that T21 distorts DS foetal hematopoiesis inducing a predisposition to B-ALL and that DS iPSCs could recapitulate these effects providing an in-vitro approach to identify likely target cells for 2^{nd} hits. The objective of this project is to utilize human DS induced pluripotent stem cells (DS-iPSCs) to model B-cell development in children with DS and to elucidate the effects of trisomy 21 on B-cell differentiation in-vitro and relate this to the characteristics of DS-ALL. In-vitro B-cell differentiation of isogenic DS iPSCs produced both pro and preB cells and an immature cellular compartment termed the LM cell immunophenotypically identified as $CD34^{+}$ $CD38^{-}$ $CD33^{hi}$ $CD45Ra^{hi}$ that displayed misexpression of the immature myeloid marker CD33. I hypothesised misexpression of CD33 induced a failure in lineage resolution and a partial block in B cell commitment at the LM cell. Transcriptomic analysis of LM, pro and preB compartments revealed enhanced stem, myeloid, cell cycle and protein synthesis gene expression pathways in the T21 LM progenitor suggestive of pre-leukaemic development. This suggests a DS hematopoietic hierarchical model in which a failure of lineage resolution in the T21 LM progenitor results in impaired B-cell commitment and conflicting lympho-myeloid signature observed in the B-cell compartment. I propose the LM cell is a potential in-utero target cell for DS pre-

leukaemic initiation and that this DS-iPSC system offers a platform for targeting potential therapeutic candidates of DS-ALL.

Impact Statement

Acute lymphoblastic leukaemia (ALL) is the most common malignancy observed in children. Childhood ALL have a small mutational spectrum relative to adult ALL that make them a good model to study the mechanisms that govern leukaemic initiation and development. Children with Down syndrome (DS) present with a range of haematological abnormalities postnatally and have a twenty and five hundred-fold increased risk of developing ALL and acute megakaryoblastic leukaemia (AMKL) respectively. This suggests a role for trisomy 21 as a 'first hit' oncogenic event.

There is growing evidence that childhood leukaemias initiate during foetal development. The increased risk of children with DS developing leukaemia may be due to its origins in a foetal specific cell that is susceptible T21 induced aberrations. Furthermore, chr 21 abnormalities have been observed in a number of childhood leukaemic subtypes therefore necessitating a developmentally relevant model in which the impact of trisomy 21 (T21) on foetal development can be investigated.

B-cell differentiation of human induced pluripotent stem cells (hiPSCs) has been demonstrated to recapitulate early foetal B-lymphopoiesis. The model of early DS B-cell development presented in this thesis provides further insight into the role of T21 on pre-leukaemic initiation and lends support to the discovery that T21 impairs foetal B-cell development. The findings presented provide evidence for pre-leukaemic initiation in the previously unidentified T21 LM progenitor that displays lineage dysregulation and enhanced cell-cycle and protein synthesis pathways. The impaired lineage resolution in the T21 preB

compartment may provide insight into the foetal specific vulnerabilities that render the B-cell compartment susceptible to secondary mutations.

These findings could aid in the characterisation of chr 21 induced foetal specific vulnerabilities, identification of novel therapeutic targets and the development of more efficacious and targeted therapies for DS B-ALL. Future studies utilising the inducible expression of known DS B-ALL secondary mutations cytokine receptor-like factor 2 (CRLF2) and interleukin-7 receptorα in this DS hiPSC model could facilitate identification of the leukaemic cell-of-origin and characterisation of molecular mechanisms that induce DS B-cell transformation.

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Abbreviations

HSA21	Human chromosome 21
DS	Down syndrome
DSCVHD	Down syndrome cardiovascular heart defects
T21	Trisomy 21
DSCR	Down syndrome critical region
MMU16	Mouse chromosomes 16
iPSC	Induced pluripotent stem cell
hiPSC	Human induced pluripotent stem cell
TAM	Transient abnormal myeloproliferative
HSC	Haematopoietic stem cell
ESC	Embryonic stem cell
Lin	Lineage
MPP	Multipotent progenitor
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
MEP	Megakaryocyte erythroid progenitor
GMP	Granulocyte macrophage progenitor
MkP	Megakaryocyte progenitor
EP	Erythroid progenitor
GP	Granulocyte progenitor
MacP	Macrophage progenitor
DC	Dendritic cell
NK	Natural killer
Neg	Negative
SLAM	Self-renewing multipotent progenitors
FOG1	Friend of GATA-1
BMP	Bone morphogenic protein
SCF	Stem cell factor
CXCL12	chemokine ligand 12
MPN	Myeloproliferative neoplasm
LSC	Leukaemic stem cell
dHSC	Definitive haematopoietic stem cell
VEGF	Vascular endothelial growth factor
SCGF	Stem cell growth factor
CFU-E	Colony forming unit erythroid
SCL	Stem cell leukaemia
Mk	Megakaryocyte
Er	Erythroid
My	Myeloid
Er-Mk	Erythroid-megakaryocyte
My-Er	Myeloid-erythroid
BCR	B-cell receptor
RAG	Recombination activating gene
AID	Activation induced cytidine deaminase

TdT	Terminal deoxynucleotidyl transferase
IZKF	Ikaros zinc family transcription factors
IL7R	Interleukin 7 receptor
Ig	Immunoglobulin
Flt3	Fms-like tyrosine 3
IL3	Interleukin 3
HSPC	Haematopoietic stem progenitor cell
T21	Trisomy 21
D21	Disomy 21
CSF-1R	Colony stimulating factor-1 receptor
GATA1	GATA binding protein-1
CRLF2	Cytokine receptor like factor 2
AMKL	Acute megakaryoblastic leukaemia
ALL	Acute lymphoblastic leukaemia
ML-DS	Myeloid leukaemia of Down syndrome
OICDS	Oxford imperial cohort Down syndrome
PCR	Polymerase chain reaction
TSLP	Thymic stromal lymphopoietin
OSKM	Oct4 sox2 klf4 c-myc
PSC	Pluripotent stem cell
HE	Haemogenic endothelium
HVMP	Heamtovascular mesodermal precursors
LIC	Leukaemia initiating cell
AML-CFU	Acute myeloid leukaemia colony forming unit
DMEM	Dulbecco's modified medium
MEF	Mouse embryonic fibroblast
DSCR3	Down syndrome critical region 3
SSC	Saline-sodium citrate buffer
PBS	Phosphate buffered saline
GSEA	Gene set enrichment analysis
CMP	Common myeloid progenitor
MLP	Mulitpotent lymphoid progenitor
MEP	Megakaryocyte erythroid progenitor
ETP	Early thymic progenitor
Lfc	Log fold change
M-CSFR	Macrophage-colony simulating factor receptor
GM-CSFR	Granulocyte macrophage-colony stimulating factor receptor
G-CSFR	Granulocyte-colony stimulating factor receptor
MPP	Multipotent progenitor
LMPP	Lymphoid primed multipotent progenitor
PCA	Principal component analysis
DEG	Differentially expressed gene
NES	Normalised enrichment score
DSCAM	Down syndrome cell adhesion molecule
APP	Amyloid precursor protein
RUNX1	Runt-related transcription factor 1
AML	Acute myeloid leukaemia

ERG	ETS transcription factor
HMGN1	High mobility group nucleosome binding domain 1
PRC2	Polycomb repressive complex 2
AIEOP	Associazione Italiana di Ematalogia e Oncologia Pediatrica
iAMP21	Intrachromosomal amplification of chromosome 21
DYRK1A	Dual specificity tyrosine phosphorylation regulated kinase 1
MPL	Thrombopoietin receptor
HOXB4	Homebox B4
CEBPα	CCAAAT enhancer binding protein a
CEBPβ	CCAAAT enhancer binding protein B
MAC-1	Macrophage-1 antigen
CFU	Colony forming unit
FACS	Fluorescent-activated cell sorting
hHSCs	Human haematopoietic stem cell
BCR	Breakpoint cluster region
ABL	Tyrosine-protein kinase
MSC	Mesenchymal stromal cell
dHSC	Definitive haematopoietic stem cell
AGM	Aorta gonad mesonephros
Mcl-1	Myeloid leukaemia initiating cell-1
EBF1	Early B cell factor 1
Pax5	Paired box 5
YAP1	Yes1 associated transcription regulator
TEAD1	TEA domain 1
TEAD2	TEA domain 2
CHIP	Chromatin immunoprecipitation
TSLP	Thymic stromal lymphopoietin
Lin28b	Lin-28 homolog B
GFP	Green fluorescent protein
FL	Foetal liver
MEF2C	Myocyte enhancer factor 2C
hESCs	Human embryonic stem cells
NFAT	Nuclear factor of activated T cells
cALL	Childhood acute lymphoblastic leukaemia
NGS	Next generation sequencing
JAK	Janus activated kinase
CRLF2	Cytokine receptor like factor 2
P2RY8	P2Y receptor 8
PAR1	Pseudoautosomal region 1
PDGFRα	Platelet derived growth factor a
TAL1	T-cell acute lymphoblastic leukaemia
HE	Hemogenic endothelium
LMO2	Lim domain only 2
CLDN5	Claudin-5
Hr	Human recombinant
UCB	Umbilical cord blood
Aldh1	Aldehyde dehydrogenase 1

ROS	Reactive oxygen species

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Go raibh maith agat.

Introduction

1 Chapter 1: Introduction

1.1 Down's Syndrome

1.1.1 Incidence and Prevalence

Down's syndrome (DS) was first described in 1887 by John Langdon Down and occurs in approximately 1:700 births. DS is caused by the presence of an extra copy of 324 protein coding and non-protein coding genes present on human chromosome 21 (HSA 21) thereby making the understanding of the over 80 different clinically defined phenotypes highly complex (Antonarakis *et al.*, 2020). Aneuploidy arises from missegragation of chromosomes during meiosis. Chromosome missegregation can be well tolerated in a number of organisms such as budding yeast and the Pacific oyster *Crassostrea gigas* while in humans the only tolerable aneuploidy chromosomes are 21, 18, 7 and 13 resulting in Down's, Edwards, Williams and Patau syndromes respectively (Leitão, Boudry and Thiriot-Quiévreux, 2001). Down syndrome has a median survival age of 60 years while <10% of children born with Edwards and Patau syndrome survive beyond their first year, indicating that human cells can adapt at the transcriptional and epigenetic level to compensate for the extra copy of chromosome 21 (Liu *et al.*, 2015). The physiological impact of extra chromosome copies results in a range of phenotypes between aneuploidy syndromes and individuals further highlighting their genomic complexity; alzheimers disease, congenital heart disease and premature death - in the majority of Edwards and Patau syndrome cases (Hassold and Hunt, 2001).

The severity of such phenotypes is dependent on the frequency of cells in which the extra chromosomal copy is present, termed mosaicsm, which can vary between individuals and tissues subsequently causing a range of phenotypic severity with cognitive abnormalities, craniofacial

defects and hypotonia being common to all DS individuals. Similarly the presence or absence of the full or partial extra chromosome copies varies - full trisomy of chromosome 21 accounts for 95% of Down's syndrome cases, with a translocation of chromosome 21 onto one of chromosome 13, 14, 15 or 22 and mosaicism accounting for the other 5% of cases (Kelminson, Elias and Goldson, 2011).

1.1.2 Phenotypes and Genetics

Chromosome 21 is the smallest human autosome containing 33.8Mb of genomic DNA. There is speculation within the field as to whether the DS phenotypes are associated with; 1) gene dosage imbalance hypothesis which states that specific HSA21 genes are directly inducing the observed phenotypes, 2) amplified developmental instability hypothesis implying that HSA21 genes induce a genome wide dysregulation of genes or 3) the critical region hypothesis whereby upon analysis of patients with partial trisomy 21 a region of 3.8-6.5Mb on 21q21.22 was identified to contain approximately 30 protein coding genes understood to be responsible for a number of DS phenotypes (Asim *et al.*, 2015).

Korbel at al,. utilised a tiling array analysis of a molecularly defined set of patients presenting with various DS phenotypes and segmental trisomy of chromosome 21 and challenged the current etiology of causative genes and identified new candidate regions specific for individual phenotypes such as transient myeloproliferative disorder (TMD), Down syndrome cardiovascular heart defects (DSCVHD) and Alzheimer's disease (Korbel *et al.*, 2009). Moreover the influence of T21 gene expression over development has been highlighted using microarray and qPCR analysis of adult and foetal brain tissue unveiling that upregulation of Hsa21 gene expression fits with the dosage sensitive model during foetal development (Mao *et al.*, 2005). In contrast, adult brain tissue displayed an increase in the expression of a number of chromosome 21 genes beyond the dosage sensitive model with a concomitant increase in transcriptional activity genome wide indicating that the 1.5 fold

expected increase in HSA21 genes may be developmental specific and that the strong increase of HSA21 genes observed in the adult brain may be due to a genome wide disruption of gene expression that increases during development (Lockstone *et al.*, 2007).

1.1.3 Genes on Chromosome 21

The Down syndrome critical region (DSCR), spanning 5.4Mb on HSA21q22 and HSA21q22.3, was discovered by Delabar et al, by correlating genotypes with phenotypes of 10 patients with partial amplification of chromosome 21 (Delabar *et al.*, 1993). Since then, numerous studies have described the DSCR to encode the genes responsible for the majority of Down syndrome features (Shapiro, 1999). Analysing various segmental T21 patient samples with differing DS phenotypes Korbel et al utilised in depth genomic analysis to develop a high resolution map of genomic regions spanning 1.8-16.3Mb that have duplicated regions encoding potential candidates for 8 different DS phenotypes such as DSCAM, located on chrm.21q22.2, in Hirschsprung disease and the APP gene, at chromosomal position 21q21.3, in Alzheimer's disease (Korbel *et al.*, 2009).

Interestingly, the haematopoietic regulator RUNX1, which is encoded within the DSCR, has tumor suppressive activity in acute myeloid leukaemia (AML) and has been reported to unexpectedly have lower expression in DS megakaryoblasts than non-DS (Bourquin *et al.*, 2006). Along with this another haematopoietic regulator, ERG, necessary for leukemic transformation in murine foetal liver progenitors is decreased in DS haematopoietic stem cells and leukemic cells (Stankiewicz and Crispino, 2009). Although both RUNX1 and ERG have not been directly linked to DS leukemic transformation, their role at a defined progenitor level may unveil new findings. Amplification of a minimal region overlapping the DSCR has been used to identify a subset of cALL, termed iAMP21 ALL, that occurs in 2% of paediatric B-ALL cases that, similar to DS cALL patients, display inferior outcome.

Amplification of this region occurs through dicentric chromosome formation that is followed by chromothripsis (Li *et al.*, 2014). The identification of this ALL subset suggests that irrespective of constitutive or somatic gain, enhanced dosage of genes in this region play a role in B-ALL development. More recently, whole genome and RNA sequencing of iAMP21 vs non-iAMP1 ALL suggests that iAMP21 displays a unique transcriptional profile that displays increased expression of DSCR genes DYRK!A, CHAF1B and SON (Ivanov Öfverholm *et al.*, 2020)

There have been several mouse models developed expressing an extra copy of different segments of HSA21, with the syntenic regions on mouse chromosomes 16 (MMU16), 17 and 18. Using the Ts1Rhr mouse model, which has triplication of a region of MMU16 that is orthologous to 33 genes within HSA21, Lane et al,. have shown that the overexpression of the nucleosome remodelling complex HMGN1, located within the DSCR, suppresses H3K27me3 of gene targets, specifically PRC2 resulting in the induction of B-ALL in vivo and B cell proliferation in-vitro (Lane *et al.*, 2014).

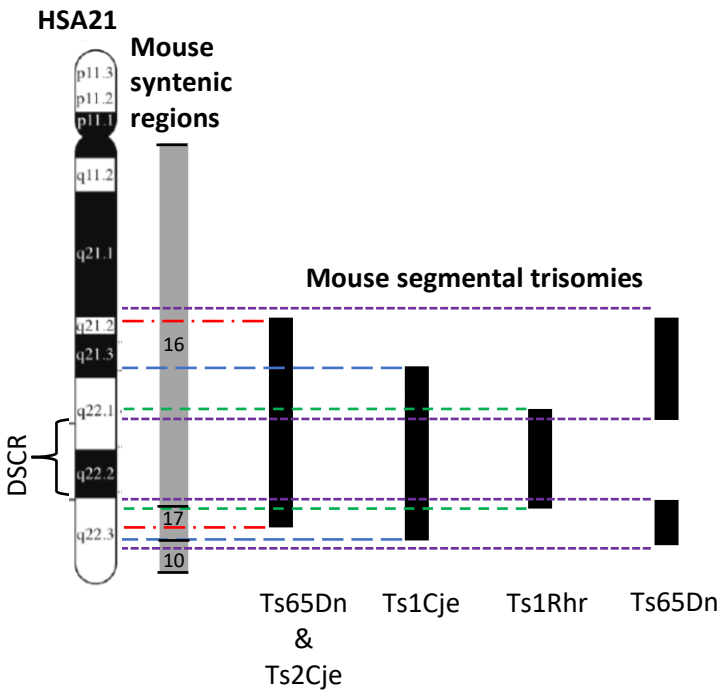


Figure 1.1: Representation of HSA21 and the syntenic regions within mouse chromosomes 16, 17 and 18 along with the different segmental trisomy21 mouse models highlighted in black (Adapted from Hewitt et

al,. 2007).

Induced pluripotent stem cells (iPSCs) have been more recently used to model DS phenotypes with knockdown of the epigenetic regulator DYRK1A, also located within the DSCR, rescuing neurodevelopmental defects and complete silencing of an extra copy of HSA21 proving successful and subsequently being described as the first step towards chromosomal therapy (Jiang *et al.*, 2013; Hibaoui *et al.*, 2014).

While there is no direct apparent regulator of B cell maturation present on chromosome 21 there are a number of key candidate regulators involved in lineage fate decisions, discussed below, which can be further explored and validated using the iPSC system.

1.1.4 Mouse Models

Mouse models and genome wide associated studies have to date been the approaches by which researchers have aimed to link genes or gene groups to Down's syndrome phenotypes and have proved particularly insightful with understanding neurocognitive and cardiac defects (Liu *et al.*, 2011; Baburamani *et al.*, 2019). The extent to which T21 genes are overexpressed relative to their copy number was described in the Ts65Dn DS mouse model which overexpresses 166 genes present on MMU16 that correspond to HSA21. Only 37% of genes were identified to be overexpressed to the 1.5 theoretical fold change value while 18% of genes were expressed at levels higher than the expected 1.5 fold change with the remaining expressed at significantly lower levels, revealing the phenotypes observed in DS, or maybe mouse models alone, may be caused by complex changes to the transcriptional network rather than by gene dosage (Lyle *et al.*, 2004). However, in the case of particular genotype-phenotype correlations knockdown of ERG from 3 to 2 copies in the adult TS65Dn mice highlighted its gene dosage effect in correcting myelo-megakaryocytic proliferation (Ng

et al., 2015). Building upon this Birger et al,. developed a double transgenic mouse model where overexpression of ERG induced expansion of megakaryocytes, as observed in human DS foetal liver, and synergised with overexpression of mutant truncated isoform of GATA - GATA1s - to induce the pre-leukaemic condition transient abnormal myelopoieisis (TAM) (Birger *et al.*, 2013). The ability of DS mouse models to accurately recapitulate foetal or adult DS haematopoiesis remains inconclusive however, as demonstrated by Malinge et al,. who crossed the Ts1RHr mouse with GATA1s knock in mice to demonstrate that overexpression of the oncogene thrombopoietin receptor^{W515L} (MPL) induced the transformation of TAM to ML-DS in a DYRK1A-dependent manner. However reports have also shown that MPL^{W515L} can induce TAM in the absence of GATA1s while DYRK1A is not dysregulated in foetal HSPC and therefore highlights the importance of model cellular context when identifying transformative genes (Ahmed *et al.*, 2004).

1.2 Haematopoiesis

1.2.1 Haematopoiesis

There are more than ten different cell lineages within the blood system with each lineage having varying functions – erythrocytes transport oxygen and carbon dioxide to and from tissues, leukocytes function as the innate and adaptive immune system and megakaryocytes produce platelets for blood clotting.

In mammals haematopoiesis arises in three different waves; the first wave occurs in the yolk sac and through an erythroid progenitor giving rise to erythrocytes and macrophages facilitating embryonic growth and development, a second wave in the aorta-gonad mesonephros gives rise to haematopoietic stem cells and a third wave of HSCs colonizing the foetal liver and giving rise to

lymphoid and Er-My progenitors occurs before HSCs migrate from the foetal liver to the bone marrow to produce the permanent adult haematopoietic system (Palis and Yoder, 2001; Ciau-Uitz, Patient and Medvinsky, 2016).

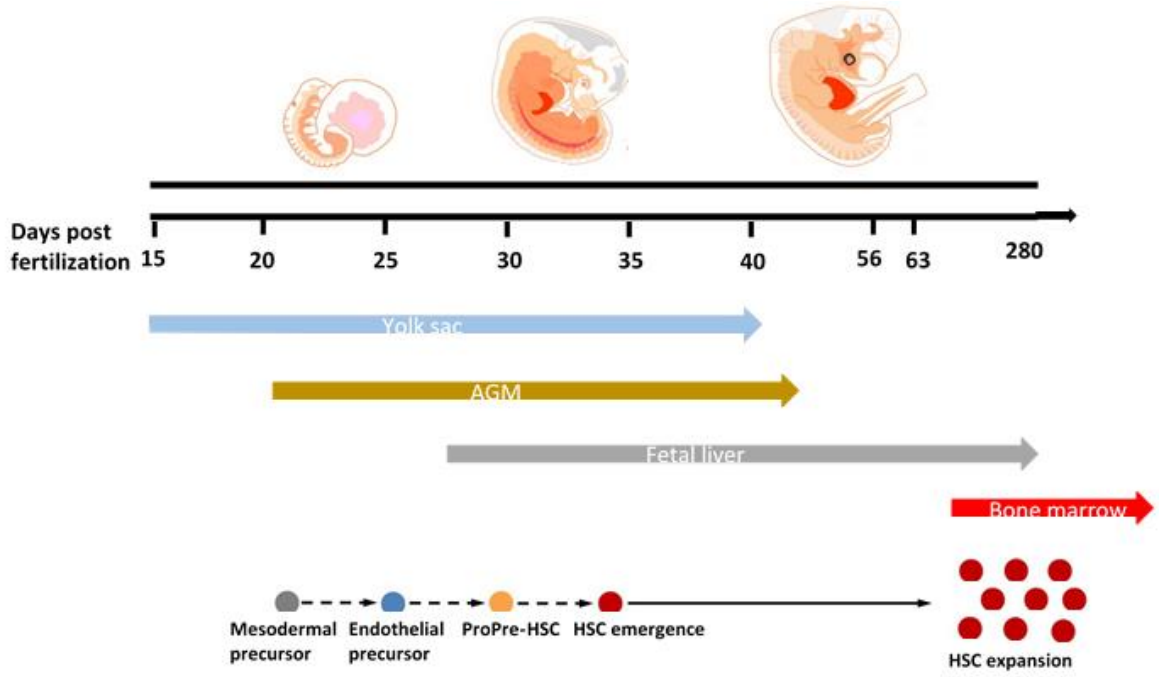


Figure 1. 1: Time scale showing the major human haematopoietic sites of development (Adapted from Tavian 2005).

The characterisation of the human haematopoietic hierarchy has gained much of its knowledge from the understanding and characterisation of the mouse system through the use of transplantation studies. Lorenz et al,. first described the presence of HSCs when the blood system of lethally irradiated mice could be reconstituted upon transplantation with the cells from the spleen or bone marrow of non-irradiated mice (Lorenz, Congdon and Uphoff, 1952). Upon this, Till and McCulloch used single cell clonal in vivo repopulation assays of irradiated mice to identify the presence of multipotential cells that give rise to colonies in the spleen, subsequently stimulating a wave in the development of in-vivo clonal assays, cell sorting and cell surface antibody recognition and development (McCulloch and Till, 1961). Lineage tracing of in-vivo studies and in-vitro

differentiations of embryonic stem cells (ESCs) have uncovered a heterogenous CD34+CD45- population, defined as hemogenic endothelium, that resides within the aortic endothelial layer highlighting that, along with endothelial progenitors, clonogenic haematopoietic progenitors expressing the endothelial marker VE-Cadherin emerge from this area (Oberlin *et al.*, 2010). Further informative studies in mice identified a lineage-negative (Lin^-), c-Kit^+, Sca-1^+ population, referred to as LSK, wherein a CD34lo/- fraction of cells have shown to possess the ability to reconstitute the lymphohaematopoietic system in 21% of mouse recipients (Masatake Osawa, Ken-ichi Hanada and Nakauchi, 1996). Comparison of gene expression profiles of purified HSCs and self-renewing multipotent progenitors (MPP) identified the signalling lymphocyte activation molecule (SLAM) receptor family as a strategy by which mouse HSCs could be further purified as CD90 ^{lo}(Thy-1)Sca-1^+Lineage$^-$c-kit^+ and using limiting dilution assays provided long-term multilineage reconstitution in irradiated mice (Kiel *et al.*, 2005). Isolation and transcriptional analysis of such HSC and progenitor populations resulted in the development of a detailed mouse hierarchy by which multipotent and unipotent progenitor states could be identified and characterised.

The physiological differences between mice and humans such as size, lifespan and environmental variation can subsequently result in differing rates of cell proliferation, cycle, differentiation and quiescence highlighting the need for further development of primary sample analysis and understanding of the human haematopoietic hierarchy. The key characteristics of HSCs – self-renewal and multi-lineage differentiation capabilities– facilitate the ability to produce daughter stem cells and maintain a pool of differentiating progenitor populations creating a ‘‘roadmap’’ of progenitor cells. However engraftable human haematopoietic stem cells are estimated to be at a frequency of 1 in 10,000 cells in human bone marrow and so isolating single HSCs to characterize the haematopoietic ‘‘roadmap’’ and understanding the biological properties and mechanisms of progenitor cells driving differentiation and proliferation remains a challenge.

Civin et al., first identified and described the purification of $CD34^+$ cells within human bone marrow that could separate haematopoietic progenitors from stromal cells and aid in sub-classifying leukaemias (Civin *et al.*, 1984). Post purification of the $CD34^+$ population, additional cell surface markers were identified and the hierarchy fractionated with Baum et al., identifying CD90 as a marker of HSCs and CD45Ra and CD38 further extrapolating differentiated progenitors resulting in the human HSC immunophenotype $Lin^-CD34^+CD38^-Thy1^+CD45Ra^-$ (Baum *et al.*, 1992). Transplantation of $CD34^+CD38l^o$ cells into NOD-*Scid* mice produced myelo-erythroid engraftment alone and facilitated immunophenotypic characterisation of short-term HSCs and MPPs subsequently identifying CD38 as a marker of mature haematopoietic progenitors as outlined in figure 1.3.

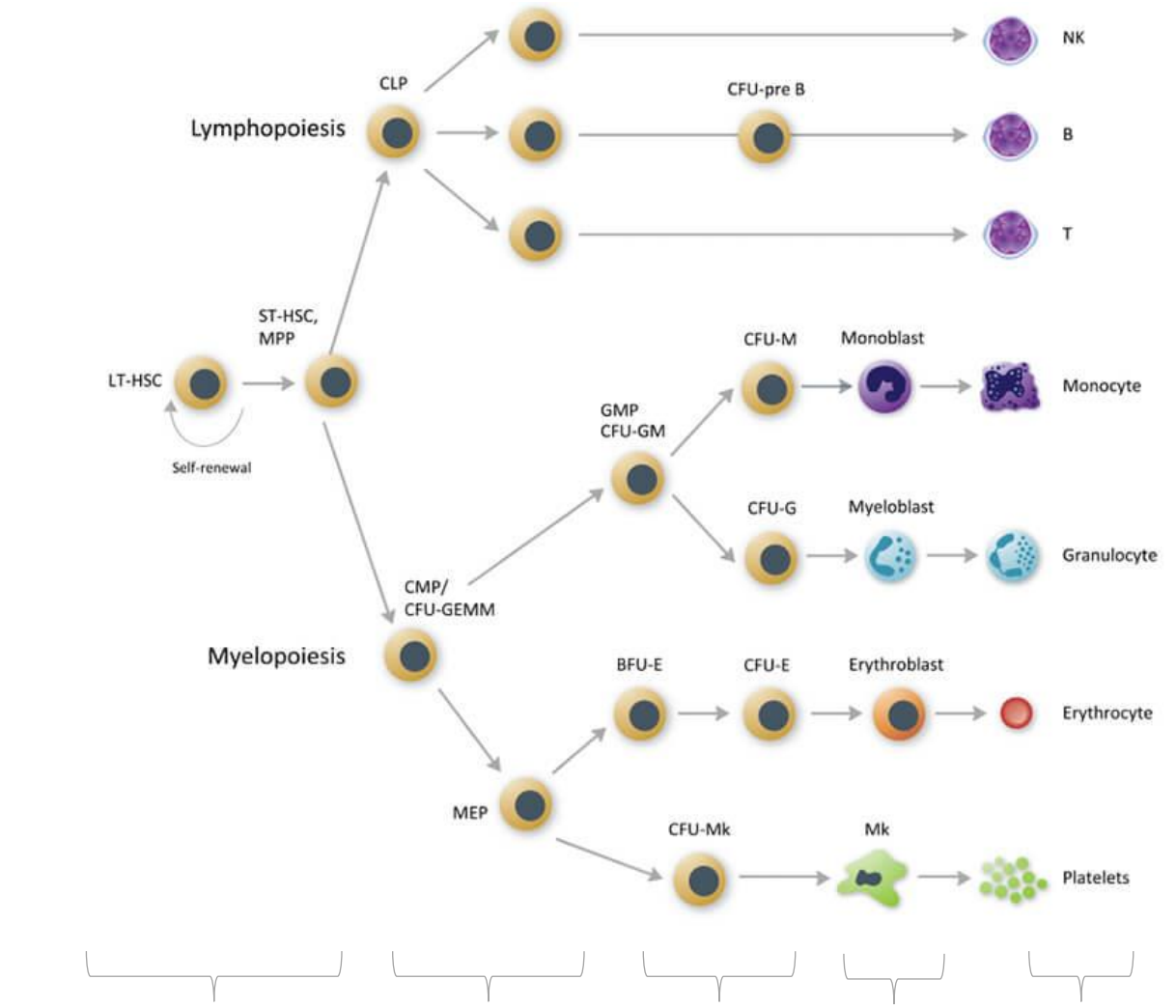


Figure 1. 2: Overview of mouse and human haematopoietic hierarchy highlighting cell surface markers used to identify HSPC and progenitor cell populations. Common lymphoid progenitor (CLP), common myeloid progenitor (CMP), megakaryocyte erythroid progenitor (MEP), granulocyte macrophage progenitor (GMP), megakaryocyte progenitor (MkP) erythroid progenitor (EP), granulocyte progenitor (GP), macrophage progenitor (MacP), dendritic cell (DC), natural killer (NK), lineage (Lin) (adapted from Stem cell technologies).

1.2.2 Haematopoietic Hierarchy

Haematopoiesis is initiated and maintained by haematopoietic stem cells that drive reconstitution of the blood system through a continuous production of differentiating multi-, oligo- and unipotent progenitors that no longer maintain the potential for self-renewal generating approximately 4-5 x 10^{11} haematopoietic cells per day (Kaushansky, 2006). The classical hierarchical model represents HSCs, that are immunophenotypically identified as $CD34^{+}CD38^{-}CD90^{+}CD45Ra^{-}$, as a homogenous population from which ST-HSCs produce multipotent progenitors (MPP) that no longer maintain CD90 expression. MPPs then bifurcate into committed myeloid and lymphoid lineages, with production of common myeloid and lymphoid progenitors (CMP $CD45Ra^{-}CD135^{+}CD123^{Low}$) and CLPs ($CD45Ra^{+}CD90^{-}CD10^{+}$). In this linear model, progeny of CMP and CLPs are restricted to their respective lineages with CMPs producing megakaryocyte erythroid and granulocyte monocyte progenitors (MEP and GMPs) that give rise to erythrocytes, megakaryocytes, monocytes, eosinophils, neutrophils and dendritic cells while CLPs produce B, T and natural killer (NK) cells. This hierarchical dogma was challenged using single cells derived from mouse foetal liver resulting in the proposal of a 'myeloid based' model with progenitor myeloid potential persisting in lymphoid branches (Kawamoto *et al.*, 2010). Adolfsson et al., first described an $LSKCD34^{+}Flt3^{hi}$ lymphoid primed multipotent progenitor in mouse adult bone marrow that has the potential to generate both mature lymphoid and myeloid cells but failed to produce erythroid and megakaryocyte cells showing that multipotent potential is maintained beyond HSCs and that erythroid and megakaryocyte potential arises from an independent progenitor (Adolfsson *et al.*, 2001). This model was similarly applied to the human hierarchy with a $CD34^{+}CD38^{-}CD90(^{neg-lo})CD45Ra^{+}$ population being identified within cord blood and adult bone marrow that could give rise to all lymphoid cell types along with macrophages, dendritic cells and monocytes highlighting that myeloid cells can arise from progenitors that have undergone lymphoid specification and that myeloid and lymphoid progenitors arise from a common progenitor

with bifurcation occurring between lympho-myeloid and megakaryocyte-erythrocyte branches (Doulatov *et al.*, 2010). More recently single cell transcriptomic, epigenetic and time-lapse analysis of adult bone marrow has provided snapshots of the hierarchical differentiation trajectory revealing a continuum of differentiation states that form a *hierarchical-like structure* leading to our current hierarchical tree that depict continuous states of differentiation that produce mature progenitors. While this has been depicted within the adult bone marrow and cord blood, human foetal haematopoiesis undergoes a different pattern of regulation and development (Laurenti and Göttgens 2018).

1.2.3 Haematopoietic Stem Cells

The cellular potential of HSCs has classically been defined using the transplantation of donor cells into lethally irradiated mice that are lacking a functional haematopoietic system and subsequent assays of the ability of the transplanted cell/s to reconstitute the haematopoietic system. Advances in antibody development facilitated the enrichment of HSCs from the bone marrow with Spangrude et al,. first identifying and isolating $Thy1^{Lo}Lin^{-}Sca\text{-}1^{+}$ HSCs from murine bone marrow (Spangrude, Heimfeld and Weissman, 1988).

HSCs occur at frequency of approximately 1 in a million cells within the bone marrow and so additional cell-surface markers have since been added to refine the immunophenotype of the HSC population, the most commonly used are the SLAM markers comprising of CD34, Sca-1 and c-Kit (Hideyuki Oguro, Lei Ding, 2008). The effect of developmental stage and age on stem cell function was first described by Landsorp et al,. who observed a decrease in the proliferative capacity of HSCs with age when comparing foetal liver, cord blood and adult bone marrow, similarly an increase in age

of donor stem cell transplant correlates with an increased incidence in mortality (Lansdorp, Dragowska and Mayani, 1993; Kollman *et al.*, 2001).

Identification of mouse HSCs enabled the characterisation of the changing characteristics of HSCs during development. During embryogenesis HSCs emerge through a diverse process involving four embryonic niches; foetal liver, aorta-gonad mesonophros, yolk-sac and the placenta where they develop the ability to self-renew. HSCs reside in the bone marrow after birth where they are regulated by their microenvironmental, transcriptional, self-renewal and differentiation states in order to give rise to the adult haematopoietic system (Wilson and Trumpp, 2006). The majority of the adult HSC pool enters senescence, during homeostasis, that is regulated by both internal and external factors such as cyclin-dependent kinase inhibitor p27 and osteoblasts that secrete TGF-ß amongst other growth factors to maintain the stem cell pool and reduce the probability of replicative induced mutations (Li, 2011). The frequency of HSCs is maintained by their asymmetric self-renewal whereby cell fate is distributed unequally amongst daughter cells or by environmental asymmetry in which one daughter cell maintains HSC potential and is lineage determined by the microenvironmental niche in which it resides (Wilson and Trumpp, 2006).

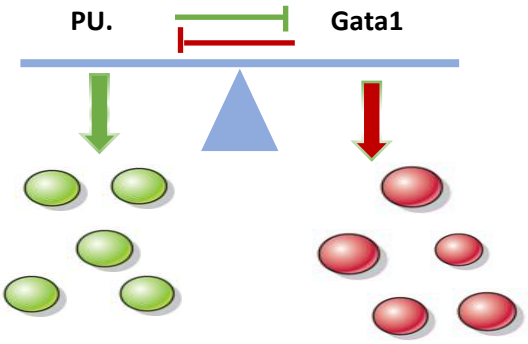
Mathematical modelling of the frequency at which $CD34^{+}38^{-}ALDH^{+}$ HSCs vs leukaemic blasts occur in the bone marrow of matched diagnostic and relapse samples of patients with AML has shown to be an indicator of successful response to and subsequent remission post chemotherapy. Moreover, a decrease in HSC number within these patients correlated with an increased affinity of LSCs for the bone marrow niche and subsequent relapse, again highlighting the importance of HSC frequency and the haematopoietic niche on development (Wang *et al.*, 2017).

1.3 Molecular Basis of Haematopoietic Specification

1.3.1 Haematopoietic Specification

The commitment of haematopoietic stem and progenitor cells to mature to functional progenitors requires the crosstalk of distinct molecular cues and developmental signalling pathways that are governed by the needs of the organism. The maturation from HSC to more committed progenitors can be viewed as a landscape of unstable and stable states that can be depicted as a mountain range of basins and ridges (Graf and Enver, 2009). Within the low-lying basins reside mature committed progenitors that no longer have the ability to de-differentiate back to a HSPC phenotype, while lying on the ridges are metastable uncommitted progenitor cells that are under the influence of competing cross-antagonistic transcription factors that impact the transition through development and lineage commitment. Depending upon the gene expression threshold of the cell, the lineage decision may enter a fully committed state or a partial transitory state where a second stimulus is necessary to drive lineage specification. There are two widely discussed models by which the relationship between cytokines and MPP commitment to a specific lineage occur; the 'instructive' model whereby cytokines 'instruct' MPP to a specific lineage or the 'selective' model where cell autonomous decisions are made and the cytokines that are present support the survival and proliferation of progenitors that have previously autonomously committed to a lineage (Enver and Jacobsen, 2009). Rieger et al,. addressed this question using single cell imaging of fluorescently labelled murine GMPs in the presence of M-CSF and G-CSF and observed that GMPs have a high cloning efficiency producing mature neutrophils and monocytes identifying that in this experimental scenario haematopoietic cytokines have the potential to instruct lineage choice (Rieger *et al.*, 2009). However, the physiological relevance of these findings is unclear and such single cell tracing experiments would need to be repeated in the mouse model to confirm findings (Enver and Jacobsen, 2009).

A)



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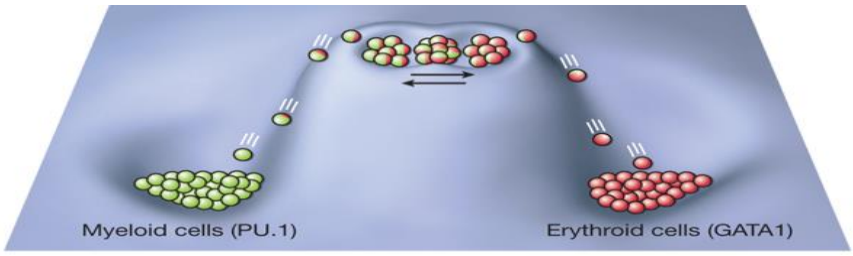


Figure 1. 3: Lineage commitment and transcription factor crosstalk scenarios between haematopoietic progenitor populations myeloid (green) and erythroid (red) progenitors highlighting the potential interactions that result in the commitment to either lineage. B) Synopsis of the transcriptional priming of erythro-myeloid cells present in the ridge with committed cells present in the basin (Adapted from Enver et al,. 2009).

Large-scale research focusing on tissue specific transcriptional regulation has shown that transcriptional networks are governed by a connectivity of transcription factors and co-factors along with proximal and distal binding sites that can compete to control gene expression and cellular phenotypes making the onset of gene expression a complex mechanism. Susumu Ohno first identified a regulator of gene expression that 'occupies the very top of a regulatory hierarchy' which 'by its very definition should not be under the regulatory influence of any other gene' when describing sex determination and it was later used to describe the hierarchy of cell specification in

yeast and plants (Ohno, 1979; Hamdi, Teller and Louis, 1987; Herskowitz, 1989). More recently this model has been applied to haematopoiesis whereby master regulators are expressed at the commencement of a developmental stage or lineage choice that regulates directly or indirectly downstream genes and upon misexpression can reprogramme cells to different lineages (Kin Chan, 2013). This has been applied to development by ectopic expression of HOXB4 previously shown to enhance HSC expansion in murine ESCs, regulate multiple haematopoietic transcription factors and chromatin modifying enzymes and induce the expression of Runx1 that is necessary for definitive HSC development in-utero (Fan *et* al., 2017). Similarly, the primitive erythro and myelomonocytic cell fate regulator, Gata1 has been shown to regulate downstream genes in a manner with or without it's coregulatory Friend of Gata1 (FOG-1) (Johnson *et al.*, 2007). The cross-antagonistic effect of transcription factors that maintain MPPs in a 'primed' balance of lineage affiliation and subsequent lineage commitment remains unclear. Mathematical modelling of the well-established interactions of the master regulators Gata1 and Pu.1, unveiled the intrinsic architecture and time dependent interactions that induce MPP cell fate choice underpinning the theories of 'program accessibility' as a definitive characteristic of multipotency and the lineage affiliated genes are promiscuously expressed on MPPs (Huang *et al.*, 2007). The effect of master regulators on trans-differentiation and lineage switching was conveyed by Laiosa et al,. 2006 with the overexpression of the master regulators Pu.1 and CEBPα on committed T-cells inducing myeloid dendritic cell like and macrophage reprogamming respectively (Laiosa *et al.*, 2006). Similarly, the influence of master regulators on B cell lymphopoiesis was also highlighted with the ectopic expression of the myeloid transcription factors CEBPα and/or CEBPβ that resulted in the downregulation of CD19 and the induction of macrophage-1 antigen (MAC-1) inducing trans-differentiation of mouse B220+ B cell precursors (Xie *et al.*, 2004).

1.3.2 Haematopoietic Stem Cell Niche

HSCs inhabit specific niches within the bone marrow that influence HSC and progenitor behaviour through secretion of cytokines and extracellular matrix proteins such as interleukin-6 (IL-6) and the Wnt antagonists Wifl and Dkk1, which result in HSC proliferation and depletion respectively (Morrison and Scadden, 2014; Tie *et al.*, 2019). The idea that the bone marrow microenvironment can influence features of cellular occupants was first proposed by Schofield in 1978. The initial proposed factors that would define a stem-cell niche were; (i) a defined site, (ii) an area that would inhibit differentiation, (iii) a site where stem cells could be maintained and reproduce but also have limited space to maintain the number of stem cells and (iv) an environment that would allow slightly more mature progenitors to revert to a stem cell phenotype (Papayannopoulou and Scadden, 2008). This site was identified and subsequently proven true by Xie and Spradling using Drosophila ovaries who uncovered that female germ stem cells reside next to a heterologous cell type that is essential for maintenance of the stem cell phenotype and also release bone morphogenic protein (BMP) necessary for stem cell differentiation and germ line stem cell self-renewal (Ting and Spradling, 1998). The discovery that CFU-S increase in concentration under examination of cells from the central to the periphery margin of mouse femoral bone marrow along with an increase in granular cells towards the central arteriole provided evidence that there is a spatial relationship between HSPCs and proliferative and differentiation capacity (Lord and Testa, 1975). Improvements in technologies such as FACS and single cell transcriptomics have provided a more in-depth understanding of HSC interactions, lineage commitment and niche signals that regulate function. Using primary osteoblasts Taichman et al,. observed a 3 fold expansion in the long-term culture potential of HSPCs and proposed that HSPCs require osteoblast-derived cytokines for maintenance and differentiation and therefore reside in close proximity to endosteal surfaces (Taichman, Reilly and Emerson, 1996). Further investigation into the role of the bone marrow in haematopoietic regulation and the

interplay with HSPCs identified additional bone marrow cell types and growth factors that regulate stem cell function, examples are the interaction between c-Kit^{+}Sca-1^{+}Lin^{-} present on the bone marrow surface and the receptor tyrosine kinase Tie2 with angipoietin-1 in stem cells that regulates stem-cell quiescence, the regulation of stem-cell number by the glycoprotein osteopontin that is present on osteoblasts within the bone marrow and a necessity for calcium-sensing receptors on stem-cells in order for them to localize and engraft to the endosteal surface in the bone marrow (Arai *et al.*, 2004; Nilsson *et al.*, 2005; Adams *et al.*, 2006). Interestingly in-vitro studies of ES cells cultured in the absence of fibroblasts supplemented with fibroblast growth factor could give rise to insulin growth factor and transforming growth factor beta providing the environment for maintenance of ES cells and their self-renewal, indicating that, at least in-vitro, ESCs can give rise to the niche that supports their maintenance (Wamaitha *et al.*, 2020).

There are numerous cytokines produced by the niche that are essential for the function of hHSCs, two of the most studied are stem cell factor (SCF) and chemokine ligand 12 (CXCL12). CXCL12 is abundantly expressed on perivascular stromal cells and regulates HSC proliferation, self-renewal and trafficking. Interestingly, conditional deletion of CXCL12 on osteoblasts in a mouse model showed no effect on HSC maintenance but did have impaired lymphocyte proliferation and differentiation in the bone marrow indicating that MSCs committing to the osteoblast lineage may be regulators of lymphopoiesis (Greenbaum *et al.*, 2013). Similarly, deletion of SCF from osteoblastic, perivascular and haematopoietic cells did not affect HSC frequency demonstrating synthesis of SCF by mensenchymal cells and endothelial cells for HSC maintenance (Morrison and Scadden, 2014).

Recent work suggests that the bone marrow niche has an imperative role in the development of haematological malignancies including leukaemia, myeloproliferative neoplasms (MPN) and myeloidysplastic syndromes. In murine models deletion of downstream Notch signalling molecules such as RBP-J_K and the retinoblastoma tumor suppressor in bone marrow stromal cells induced the

development of an MPN-like phenotype. Development of MPN has also been shown to reinforce the haematopoietic niche in BCR/ABL double transgenic mice through the expansion of endosteal osteoblast cell by mesenchymal stromal cells (MSCs) secretion of thrombopoietin and chemokine ligand 3 – that are implicated in stromal changes in leukaemias - myelofibrosis of the bone marrow and subsequent impairment of normal HSC function while there was no effect observed on leukaemic stem cells (LSCs) (Schepers, *et* al,. 2013)

1.3.3 Foetal Haematopoiesis

Due to the potential clinical significance of HSCs, recent research has aimed to decipher the different sites and developmental impact of these different origins on foetal haematopoiesis. Foetal haematopoiesis is a unique system whereby ontogeny initiates prior to the emergence of HSCs.

As mentioned above foetal haematopoiesis is a complex mechanism that involves a number of sites of haematopoietic initiation, yolk-sac, AGM, placenta and foetal liver, and so largely differs from the adult system where permanent haematopoiesis moves to the bone marrow postnatally. Dissecting foetal haematopoiesis has proved to be challenging for a number of reasons; i) movement of the heterogenous population of foetal stem and progenitor cells between these different anatomical sites both spatially and temporally and their interactions within these niches, ii) one definitive HSC (dHSC) – defined by the engraftment capacity - exists per haematopoietic organ in the mouse E11.5 embryo and the number of dHSCs contributing to the permanent adult system is unknown and iii) the lack of dHSC surface markers (Medvinsky, Rybtsov and Taoudi, 2011). The depth of our knowledge of HSC development will be largely informed by the growing number of in-vitro systems recapitulating embryonic development and thus providing an insight into the origins of dHSC and permanent haematopoietic development at immunophenotypic and molecular levels (Ivanovs *et al.*, 2017).

1.3.4 Sites of Foetal Haematopoiesis

The developing mammalian embryo requires the development of a functioning cardiovascular system in order to survive growth beyond the post implantation period and therefore vascular and cardiac organ systems are the first to emerge during embryogenesis. Foetal HSCs differ from adults in their cell-cycle and differentiation potential and so it is possible that the different sites of HSC origin may play a role in engraftment and self-renewal capability.

The onset of haematopoietic and endothelial cell development from the yolk sac 'blood islands' requires the cross-talk between the primitive endoderm layer and the mesoderm layer. The mesodermal layer eventually ingresses through the endoderm layer and into the yolk sac subsequently producing enucleated erythroblasts that express embryonic haemoglobin. A modern definition of the onset of mouse blood development is described as 'between E7 and E7.5 mesodermal cells in the visceral yolk sac proliferate and form mesodermal cell masses that are the precursor of blood islands. Central cells accumulate haemoglobin, and outer cells flatten and form endothelium' (Malcom A.S. Moore, 2004). Genetic targeting approaches in mice have identified the Indian hedgehog pathway and Wnt proteins as key players in the onset of vascular and haematopoietic cellular development within the mesoderm (Dyer *et al.*, 2001; Ruiz-Herguido *et al.*, 2012). In mice the extraembryonic yolk sac blood islands are the first source of blood cells and fetomaternal transport producing the first primitive large enucleated erythroid progenitors and definitive progenitors that eventually colonise the foetal liver (McGrath and Palis, 2005). Primitive haematopoiesis initiates during the second and third week of gestation and the onset of ontogeny within the yolk-sac produces both blood and endothelial cells from the yolk-sac mesoderm through a common bipotent progenitor called the haemangioblast (Sabin, 1917). Using a dual differentiation approach of a single cell in-vitro culturing system of $CD133^{+}$ cells enriched from peripheral blood, in the presence of vascular endothelial growth factor (VEGF), stem cell growth factor (SCGF),

granulocyte-colony stimulating factor (G-CSF) and Flt-3 ligand, Loges et al,. identified that 2% of single $CD133^{+}$ seeded cells had both endothelial and haematopoietic potential claiming identification of haemangioblasts within peripheral blood (Loges *et al.*, 2004). However in-vivo single cell evidence of bipotent vascular and haematopoietic potential remains to be proven.

FACS and CFU potential of cell clusters within the AGM and extraembryonic arteries of human and mouse have identified progenitor populations that express immunophenotypic and functional characteristics of HSCs that are believed to be the HSCs of definitive haematopoiesis, while although the HSCs that arise in the yolk sac do not contribute to definitive haematopoiesis transplantation assays have shown they do possess the ability to colonise the liver of newborn recipient mice (Müller *et al.*, 1994; Yoder, Hiatt and Mukherjee, 1997) . It has been controversial as to the originating site of HSCs that colonise the foetal liver, whether AGM-derived HSCs alone can support the quickly growing foetal liver or wether the yolk sac also provides assistance in HSC expansion later in development. It is believed the first seeding of the foetal liver initiates at E9.5-10.5 and is colonized by the myeloerythroid progenitors that migrate through vitelline vessels from the yolk sac producing a colony forming unit erythroid (CFU-E) rich environment (Mikkola and Orkin, 2006). The emergence of HSCs in the foetal liver occurs at E11.5 of which the majority are believed to be derived from the AGM with migration occurring through the umbilical vessels. At E12.5 the foetal liver becomes the primary site of dHSC expansion and becomes rich in single-lineage progenitors to produce sufficient differentiated progeny (Mikkola and Orkin, 2006). The HSCs within the foetal liver are largely in cell cycle in comparison to the bone marrow where most HSCs are quiescent. Using a tamoxifen inducible mouse system of the stem cell leukaemia (SCL) transcription factor Gothert et al,. addressed the lineage relationship between foetal and adult HSCs, identifying that midgestation foetal liver aged HSCs that were transplanted into adult mice could indeed reconstitute transplanted adult mouse

bone marrow and that there is a direct lineage relationship between foetal liver and adult mouse bone marrow, indicating that there was no further maturation of HSCs in late foetal and neonatal stages of development (Gothert, *et* al,. 2005). Single cell clonogenic potential of CMPs isolated from foetal liver revealed oligopotent potential with Erythroid-Megakaryocytic (Er-Mk) and Myeloid-Erythroid-Megakaryocytic (My-Er-Mk) output in comparison to adult bone marrow whose hierarchy is predominant with My or Er potential, thus highlighting the differences in foetal and adult ontogeny that may explain differences observed in childhood and adult leukaemic onset and progression (Notta *et al.*, 2016).

Recent findings support a model of haematopoietic development in which the primary maturing erythroblasts and progenitor cells produced by the yolk sac facilitate survival until the onset of AGM-derived haematopoietic stem cells that do not differentiate until exit from the AGM and go on to seed the foetal liver and then differentiate into mature blood cells (Godin *et al.*, 1999). The fundamental differences in programs between each of the haematopoietic sites, e.g. production of erythroblasts in the yolk sac and the lack of lymphoid precursors, indicate that the microenvironment plays a fundamental role in the output of each program and may influence the later haematopoietic output and fate choice.

1.3.5 Primitive and Definitive Haematopoiesis

The development of blood within the vertebrate system involves two waves, the first transitory primitive wave during early embryonic development that is succeeded by a second definitive wave that occurs later in development and involves a transitory erythro-myeloid progenitor and HSCs that provide the permanent adult haematopoietic system. Development of the mouse haematopoietic system begins at embryonic day 7.25 where the first emergence of blood occurs in the yolk sac where

extraembryonic mesoderm undergoes differentiation to blood islands that produce primitive erythro-myeloid precursors (Ferkowicz and Yoder, 2005). In comparison to the unique model of primitive ontogenesis, definitive haematopoiesis follows the classical model of haematopoietic development and is identified with the emergence of HSCs in the AGM. The characteristics that define definitive haematopoietic stem cells were first described using CFU-S that possessed the ability to produce multipotential HSPCs (multipotency) and daughter cells with the same potential as parent cells (self-renewal) (Siminovitch, McCulloch and Till, 1963).

Moore and Owen published in 1965 that adult haematopoiesis arises from extra embryonic tissues which was then further characterised by Dieter-Leueven in 1975 who upon transplantation of a quail embryo into a chick yolk-sac and tracing of HSCs identified that quail HSCs alone were present in the developing spleen and thymus of the engrafted chick, subsequently identifying that definitive haematopoiesis arises from a site other than the yolk-sac and so must be from an intra-embryonic site (Owen, 1965; Dieterlen Lievre, 1975). This led to a series of investigations into the origins of HSCs and their relationship with endothelium. Medvinsky et al,. identified that haematopoietic progenitors arise in the developing aorta and later appear in the foetal liver which was confirmed in-vitro with the identification of erythrocyte, neutrophil and macrophage precursors within embryoid bodies derived from ESCs (Medvinsky, Rybtsov and Taoudi, 2011). This led to the identification of the haemangioblast that has the potential to give rise to both haematopoietic and endothelial precursors (Müller *et al.*, 1994; Kennedy *et al.*, 1997).

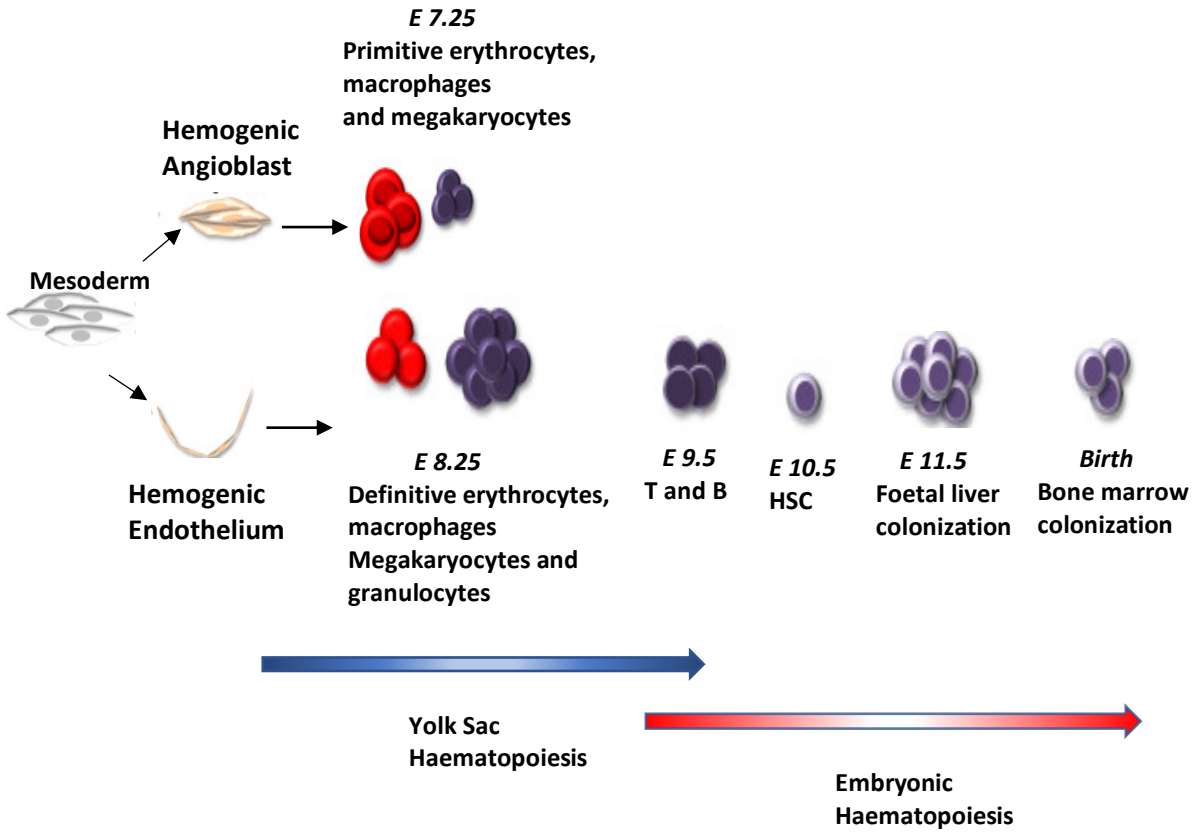


Figure 1. 4: Illustration of embryonic haematopoietic precursor development with primitive and definitive waves at E7.25 and E8.25 with the emergence of lymphocytes and HSCs at E9.5 and E10.5 respectively prior to the colonization of the foetal liver and bone marrow. (adapted from Lacaud and Kouskoff 2017)

Knockdown studies of zebrafish have identified Gata1 and Pu.1 as key regulators of primitive erythro-myeloid haematopoiesis. Gata1$^{-/-}$ mice die during gastrulation due to differentiation block in pro-erythrobalsts to mature erythrocytes, moreover knockdown of Gata1 in zebrafish models resulted in a switch from erythroid to myeloid lineage with an upregulation in the expression of myeloid-specific genes such as myeloperoxidase and l-plastin and therefore identified Gata1 as a suppressor of myelopoiesis. Similarly, knockdown of Pu.1 in zebrafish resulted in an increase in Gata1 expression in the posterior lateral mesoderm – which is equivalent to the human blood islands – indicative of an erythroid switch in progenitor cell-fate and that it may be a competition between Gata1 and Pu.1 that regulates the development and fate of primitive erythro-myeloid progenitors (Rhodes *et al.*,

2005). Another member of the GATA transcription factor family, GATA2, which through its co-localisation and interaction with SCL regulates the transcription of Pu.1, induces a lineage switch in mouse ESCs from macrophage to megakaryocyte and erythroid differentiation (Kitajima *et al.*, 2006). Moreover, the role of GATA2 in HSC specification and both primitive and definitive haematopoiesis was highlighted with knockdown of GATA2 proving embryonically lethal in mice at E10.5 (Gao *et al.*, 2013). Using a GATA2 piggyback tetracycline inducible overexpression system in hESCs GATA2 was found to regulate definitive haematopoiesis in a cell cycle dependent manner (Zhou *et al.*, 2019).

1.4 B-cell Development

1.4.1 B-cell development

B-cells and their antibodies are the central players of the humoral immune response that protect against a diverse range of pathogens. The diversity of antibody specificities due to the complimentary-determining regions that are generated through somatic hypermutation of the immunoglobulin heavy and light chains enable such a response. Due to such diverse repertoire of immune responses being mounted B cells undergo checkpoints to ensure there is no autoreactivity and that a correct specificity against pathogens has been mounted. The affinity of the B-cell receptor-antigen (BCR) interaction determines which developing B-cells will undergo deletion or editing (Kouskoff *et al.*, 1998).

ln humans and mice B-cell development begins in the primary lymphoid tissue - foetal liver or adult bone marrow (Jankovic *et al.*, 2004). During maturation recombination activating gene (RAG) mediates rearrangement of gene segments termed variable (V), diversity (D) and joining (J) to generate unique antigen receptors, firstly on the immunoglobulin heavy chain locus followed by

recombination of the V and J regions on the light chain loci, thus producing a functional pre B-cell antigen receptor. The pre-BCR has two roles; to terminate RAG activity ensuring that rearranged B cells express 2 heavy chains with the same antigen specificity and to initiate rearrangement of the light chains genes (Boekel, Melchers and Rolink, 1998). If these rearrangements are successful and produce a structurally diverse repertoire of functional antibodies these lymphocytes, now encoding the BCR, can exit the bone marrow and migrate to the secondary lymphoid tissues – spleen and lymph nodes – where they mature to long-lived follicular B-cells through basal BCR signalling or differentiate to marginal B-cells in the spleen (Cariappa *et al.*, 2007). Follicular B cells undergo further mutation of the Ig locus upon antigen exposure and T-cell stimulation by the enzyme activation induced cytidine deaminase (AID) that creates a further specific repertoire of antibodies - terminal deoxynucleotidyl transferase (TdT) in the adult bone marrow. Upon exposure to foreign pathogens these cells either further differentiate into antibody secreting plasma cells or memory B-cell.

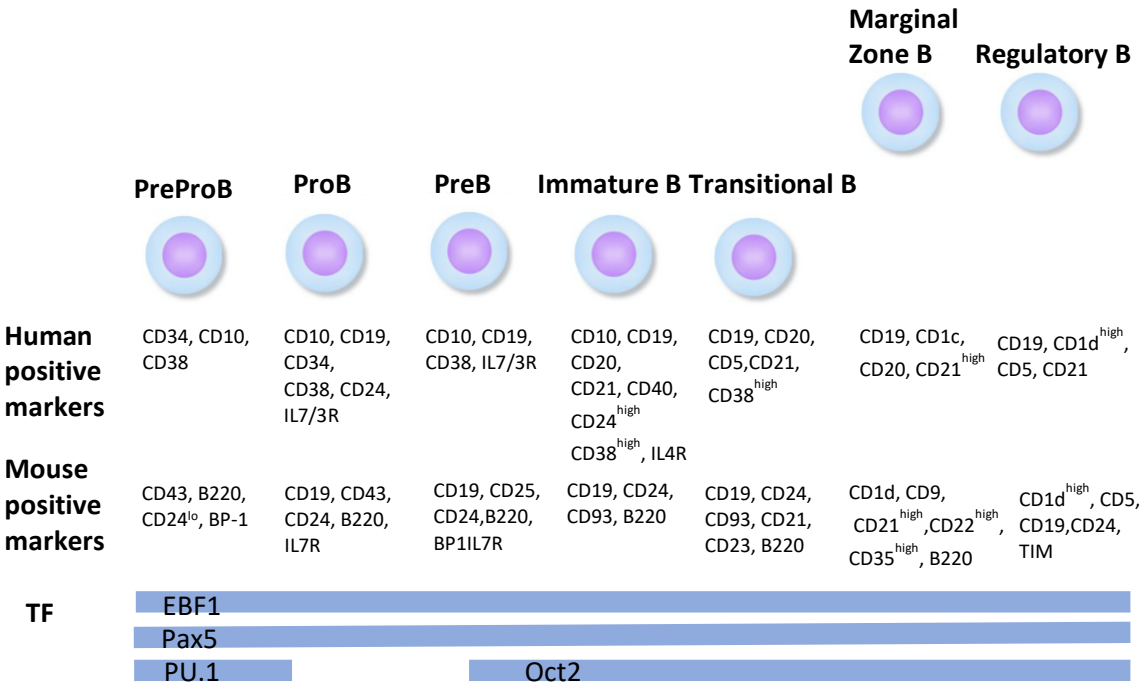


Figure 1. 5: Positive and negative selection markers for human and mouse B-cell development along with their regulatory transcription factors (TF).

Characterisation of the lymphoid hierarchy was first completed in mouse with the stratification of $B220^+$ IgM^- B-lineage cells into four separate sections identified by the cell surface expression of CD43, BP-1 and 30F1/CD24 with subsets being able to be functionally divided based on Ig gene rearrangement status (Richard R. Hardy, Condie E. Carmack and John D. Kemp, 1991). Human and mouse haematopoietic cell surface markers overlap in cases and so the characterisation by Hardy facilitated identification of human cell surface markers.

The first B cell – termed preproB cell – is positive for cluster of differentiation markers $CD43^+$, $B220^+$, $CD24^{lo}$ and BP-1 in mouse while humans are $CD34^+CD10^-CD19^+$ (Richard R. Hardy, Condie E. Carmack and John D. Kemp, 1991). Co-culture of human CB $CD34^+$ cells on S17 stroma and immunophenotypic characterisation of derived progenitors identified that multipotent progenitors $CD34^+CD45Ra^+CD19^-CD10^-$ give rise to a wave of $Pax5^+TdT^-$ unilineage pre/proB $CD34^+CD10^-CD19^+$ progenitor cells and common lymphoid progenitors $CD34^+CD10^+CD19^-$ that can give rise to ProB $CD34^+CD19^+CD10^+$ cells through two independent pathways (Sanz *et al.*, 2010). It is at the proB stage of development where both mouse and human systems show similarities in surface expression, one being CD19 which is continuously expressed throughout B cell maturation. CD19 is a 95-kDa B cell specific transmembrane protein that precedes expression of the pre-BCR. Upon BCR cross-linking CD19 is rapidly phosphorylated and functions as an essential downstream component of BCR survival signalling (Otero, Anzelon and Rickert, 2003). The maturation of pro to preB cells occurs with the loss of CD34 and downregulation of Rag1 and Rag2 upon expression of the preB cell receptor and upregulated again in rearranging preB-II cells (Grawunder *et al.*, 1995). Pro to preB differentiation is dependent on successful VDJH rearrangement and transcription of the Igμ heavy chain that couples with the non-covalently linked lambda-5 and VpreB proteins resulting in the transient expression of the preB cell receptor on large cycling preB cells (Mårtensson and Ceredig, 2000). After 3-5 rounds of cell division large preB cells differentiate into non-dividing small preB cells and upon rearrangement of the

immunoglobulin lamda (IgL) chain and surface immunoglobulin M (IgM) expression immature B lymphocytes are formed. After comprehensive selection against autoreactivity functional B cells are formed, exit the bone and migrate to the spleen for further maturation into mature B lymphocytes. ProB cell survival and proliferation is dependent on interleukin 7 receptor (IL7R) signalling and activation of the STAT5 pathway through upregulation of the pro-survival gene Mcl1 and ordering of immunoglobulin (Ig) rearrangment (Malin, McManus and Busslinger, 2010; Clark *et al.*, 2014).

1.4.2 B-cell commitment and differentiation

Lymphocyte development requires the synchronisation of a network of cytokines and transcription factors that positively and negatively regulate gene expression and ensure activation of the lymphoid program and silencing of other cell fates. There are at least ten transcription factors whose functional role in early B lineage commitment and differentiation has been described of particular importance, Ikaros, E2A, EBF1 and Pax5 amongst them and have found to be aberrantly regulated in leukaemia inducing the expansion of immature progenitor subsets (Nutt and Kee, 2007).

Ikaros zinc family transcription factors (IZKF) are regulators of immune development including B cells and $CD4^+$ T cells. Ikaros is pivotal for super enhancer activity that is necessary for BCR signalling and differentiation along with the repression of 'extra lineage' transcription factors such as YAP1 and TEAD1/2 that are involved in epithelial and mesenchymal development (Hu, Yoshida and Georgopoulos, 2017). In conjunction with repressing such transcription factors, Ikaros regulates their interaction with B-cell master regulators such as EBF1 and Pax5 resulting in a lymphocyte super-enhancer network. Ikaros associates with enhancer regions of genes involved in cellular adhesion and self-renewal. Loss of Ikaros has been shown to result in co-operation of such targets with B-cell

specific regulators and the onset of aberrant B-cell development and function (Hu, Yoshida and Georgopoulos, 2017).

Pax5 is exclusively expressed in the B-cell lineage. It binds to promoter sequences in the Ig loci regulating Vh-DJh rearrangement and governs over 170 genes and has been described as one of the most multifunctional transcription factors for B-cells. The role of Pax5 in B-cell development differs between foetal and adult development with PAX5-null embryos differentiation inhibited prior to the emergence of $B220^{+}$ progenitors in comparison to the bone marrow of $Pax5^{-/-}$ adult mice that contained mature c-$Kit^{+}B220^{+}$ HSPCs and also express E2A and EBF1 transcription factors indicating that PAX5 acts downstream of E2A and EBF1 in the B cell transcriptional hierarchy. Moreover, Nutt et al,. described that mice proB cells lacking PAX5 are incapable of B-cell differentiation, exhibit multilineage gene expression patterns and can differentiate into functional macrophages, osteoclasts and dendritic cells with restoration of Pax5 supressing the lineage promiscuous transcription signature and restoring B lineage commitment (Nutt *et al.*, 1999).

EBF1 is a zinc finger transcription factor that regulates the SLAM family of co-receptors in B-cells that induce differentiation by modulating BCR activity. EBF1 also regulates PAX5 promoter regions in pre-preB cells (Somasundaram *et al.*, 2015). E2A and EBF1 null uncommitted progenitors similarly possess the potential to differentiate into both myeloid and lymphoid lineages (Ikawa *et al.*, 2004; Pongubala *et al.*, 2008). Genome wide CHIP analysis revealed that of the 565 genes that are transcriptionally regulated by EBF1 are also targets of PAX5 suggesting that both EBF1 and PAX5 synergise during B-cell commitment (Medvedovic *et al.*, 2011).

E2A is a transcription factor that upon heterodimerisation with tissue specific basic helix-loop-helix proteins play a pivotal role in embryogenesis. In contrast to the pivotal roles played by EBF1 and PAX5, PAX5 overexpression could rescue commitment to the proB stage in E2A deficient haematopoietic progenitors, suggesting that E2A may play a lesser role in underlying B-cell

commitment (Medvedovic *et al.*, 2011). In conjunction with this, early lymphoid commitment is dependent on SCF and fms-like tyrosine kinase (FLT3) ligand while interleukin-7 (IL-7) and interleukin-3 (IL-3) are necessary for B-cell proliferation and survival of primitive progenitors with lymphoid potential (Nishihara *et al.*, 1998; Cho *et al.*, 1999; Carpenter *et al.*, 2011). Roy et al, has shown that in T21 human foetal liver B-cell maturation is defective with a decrease in lymphoid primed HSCs and preproB cells (Roy *et al.*, 2012). This indicates that childhood DS leukaemogenesis may be induced by the proliferation of multipotential immature progenitors within the HSC compartment prior to branching into myeloid and lymphoid lineages, due to the overexpression of the haematopoietic HSA21 genes RUNX1, C-KIT and Pu.1 (De Vita *et al.*, 2010).

1.4.3 Foetal B-cell development

There is increasing evidence that the lymphoid potential of HSCs from embryo, foetus, neonate and adult development is distinct during each course of development. For example TdT is present in the adult bone marrow alone while foetal liver proB cells are unique as they are responsive to TSLP-IL7R signalling to support cell proliferation while foetal and adult preB cells share a responsiveness to TSLP (Voßhenrich *et al.*, 2003; Dias *et al.*, 2005). Studies in mice have shown that transfer of foetal and adult ProB cells into recipient mice gave rise to two independent B cell populations with $IgM^{high}IgD^{low}CD5^{+}$ cells arising from foetal ProB cells while adults produce $Igm^{low}IgD^{high}CD5^{-}$ progenitors, thus indicating that B-cell development undergoes a developmental switch between foetal and adult life (Richard R. Hardy and Kyoko Hayakawa, 2015). Two populations of B lymphocytes exist, B-1 and B-2 cells that emerge from $Lin\text{-}CD34^{+}CD38^{lo}$ HSPCs whose 'switch' during ontogeny is regulated by the Lin28b-Let-7 signalling axis (Zhou *et al.*, 2015; Quách *et al.*, 2016). One model that has been proposed to describe this is that there is a 'layered immune system' whereby 'conventional (B-2) and Ly-1 (B-1) cells belong to separate lineages deriving from distinct progenitors

emerging at different times during development' (Herzenberg and Herzenberg, 1989). In the developing foetus B-1 cells are part of the innate immune system and produce novel VDJ rearrangements that recognise self-antigens including those with repetitive epitopes resulting in positive selection of newly formed B cells. B-1 cells can be divided into B-1a and B-1b classes depending on their function in mounting an immune response. Meanwhile, B-2 cells are generated throughout adult life and comprise of a majority of follicular and a minority of marginal zone B cells and reside in the secondary lymphoid organs. B-2 cells can undergo Ig class switching and are a part of the adaptive immune response (Baumgarth, 2017). The emergence of differing populations from foetal and adult tissue and that of B-1 and B-2 cells was described by Herzenbergs as the 'layered immune system' rather than the 'selection model', the latter proposed that B-1 or B-2 commitment was dependent on the exposure to particular antigens (Montecino-Rodriguez and Dorshkind, 2012).

Montecino-Rodriguez et al,. first identified and characterised a B-1 B-cell progenitor, $Lin^{-}CD93^{+}CD45R^{lo/neg}CD19^{+}$, present in the foetal bone marrow and at a decreased frequency in the postnatal bone marrow that could reconstitute B-1 $CD5^{+}$ B-cells more efficiently in-vivo than $Lin^{-}CD93^{+}CD45RI^{o/neg}CD19^{+}$ adult BM (Montecino-Rodriguez, Leathers and Dorshkind, 2006). B-1 B-cell precursors arise simultaneously in the yolk sac and PSp autonomous from each other. The presence of B-1 progenitors within the adult bone marrow, although with a declining frequency with time, could be reinitiated with the overexpression of Lin28b. This may imply a three tier model in which B-1 cell development occurs during foetal, postnatal and adult development with a reversible HSC state within the adult bone marrow influenced by Lin28b (Kristiansen *et al.*, 2016).

B lymphopoiesis in the foetal liver has been closely linked with myeloid lineage. Boiers et al,. first identified a lympho-myeloid progenitor that emerged at E11.5 that expresses IL7Rα. Single cell transcriptional and clonogenic analysis revealed that this progenitor had both lymphoid and GM lineage priming but lacked MkE potential . Using a Rag1-GFP knock-in mouse model emergence of

this progenitor was traced to emerge at E9.5 and preceded dHSC emergence and colonization of the FL that could contribute to the innate and adaptive immune systems, subsequently identifying the earliest immune restricted progenitor and lympho-myeloid progenitor (Böiers *et al.*, 2013). Examination of E13.5 FL $CD19^{+}$ proB cells identified that the macrophage colony stimulating factor receptor (CSF1-R) is expressed on foetal proB cells and not on proB cells in the adult BM. Expression of CSF1-R was critical for development of B lymphopoiesis that exhibited residual myeloid priming underlining the biphenotypic nature of proB cells and the potential implications of CSF1R expressing proB cells in childhood preleukaemic initiation where CSF1R rearrangements have been reported (Roberts and Izraeli, 2014; Zriwil *et al.*, 2016). Engineered human iPSCs derived proB cells expressing the oncogene ETV6-RUNX1 displayed increased myeloid potential in comparison to wild type controls again reiterating the influence of myeloid potential observed in foetal and pre-leukaemic development (Böiers *et al.*, 2018).

1.4.4 Lineage plasticity of B-cell development

The plasticity of stem cells has been demonstrated with the ability of stem cells from a tissue differentiating into cells of a new host tissue in response to microenvironmental stimulus, thus questioning the stability of lineage commitment. This plasticity is dependent on a number factors such as cell cycle stage and the expression of cell adhesion integrins (Quesenberry *et al.*, 2001). The understanding of the more defined mouse lymphopoietic hierarchy has provided much of the insight into the human B-cell lineage. Expression of the immature mouse B-cell surface marker B220, a spliced form of CD45, with co-expression of other surface markers such as CD24 and CD93 have enabled the detection and isolation of uncommitted lymphoid $CD19^{-}$ subsets. Functional and single cell analysis of $B220^{+}$ $CD19^{-}$ uncommitted haematopoietic progenitors have revealed the ability of these cells to have both myeloid and lymphoid differentiation potential (Alberti-Servera *et al.*, 2017).

Similarly transduction of CLP with the GM-CSF receptor and exposure to GM-CSF induced lineage conversion of CLPs into macrophages and granulocytes (Kondo *et al.*, 2000). Interestingly exposure to IL-7 inhibited the transdifferentiation effect of GM-CSF, which may indicate the presence of a window in which committed progenitors may undergo lineage switching upon external stimulus of lineage associated cytokines (Heyworth *et al.*, 2002). Borzillo et al,. similarly showed that overexpression of CSF-1R on early pre-B cells induced transformation to MAC-1+ macrophages with phagocytic potential and upon addition of CSF-1 conferred a proliferative advantage and this was similarly inhibited with a hCSF-1R antibody (Borzillo, Ashmun and Sherr, 1990).

These data along with the reprogramming potential of GM-committed progenitors to transdifferentiate into erythroid, eosinophil and basophil like-states in response to exposure to GATA-1 highlight that the plasticity of the haematopoietic system is conserved onto a stage of more committed and lineage restricted progenitor populations. Moreover, the plasticity observed within foetal liver hHSPCs and the expression of myeloid markers on B-cell progenitors may reflect the ontogeny of infant and childhood BCP-ALL (Hrušák and Porwit-MacDonald, 2002).

1.4.5 Initiation of childhood leukaemia

Childhood acute lymphoblastic leukaemia has through history been clinically silent before diagnosis. In recent decades there has been a large amount of evidence highlighting the in-utero origins of childhood ALL, with the ETV6-RUNX1 fusion subtype being particularly informative for studying the onset and transformation to overt leukaemia. The onset of cALL can be described as two-step process with the first step occurring whereby a gene fusion or hyperdiploidy generates a concealed pre-leukaemic clone and the second step with a small subset of these acquiring secondary aberrations, such RAG and AID mediated recombination that result in copy number changes of

tumour suppressors and oncogenes such as ETV6 and RUNX1. To backtrack the clonal origins of cALL Greaves and colleagues utilised DNA sequencing of the uniquely variable breakpoints that occurs between the two fusion genes in ETV6-RUNX1, MLL-AF4 and BCR-ABL1 subtypes to develop sensitive clonal specific markers for childhood pre-leukaemic initiation (Gale *et al.*, 1997).

Use of long range inverse PCR method to amplify genomic breakpoints of the ETV6-RUNX1 of cALL samples Wiemels et al,. identified breakpoints within introns I and 2 of RUNX1 and intron 5 of ETV6 identifying unique microcluster breakpoint regions the can be used to track the clonal origins of disease (Wiemels *et al.*, 2000). The most informative studies of cALL clonal initiation have developed from studies using Gutherie spots identifying the presence of leukaemic fusion partners within the neonatal blood spots (Maia *et al.*, 2001). The hypothesis that the in-utero initiation occurred in one twin and through vascular anastomoses facilitating blood cell chimerism and pre-leukaemic clones arising the second twin was first proposed when observing parallel responses to chemotherapy in identical twins (Wolman, 1962). This was confirmed more recently with the study of samples from monozygotic leukaemic twins that shared the same breakpoint denoting the single cell in-utero origins of the leukaemias (Ford *et al.*, 1993). Using samples from monozygotic twins, one twin with the TEL-AML1 lesion but had not developed leukaemia and the other with TEL-AML1 and frank leukaemia, it was shown that TEL-AML1 is indeed a pre-leukaemic initiating event in-utero, with the identification of $CD34^{+}38^{-/low}CD19^{+}CD10^{-}$ B-cells with DJ recombination, and no VDJ, observed in the healthy twin while there was highly related DJ recombination sites as seen in the healthy twin along with VDJ rearrangement in the leukemic twin, indicative of the TEL-AML1 clone arising in a common cell that has previously undergone DJ rearrangement (Hong *et al.*, 2008). This evolutionary trajectory of leukaemic onset has similarly been applied to DS myeloid leukaemias and was first described in monozygotic twins in which the only cytogenetic aberration was T21 with one twin developing

leukaemia 9 months prior to the other with differing cytogenetic changes in each twin (Stark *et al.*, 2002). 80% of DS children enter spontaneous remission from TMD postnatally indicating that T21 alone has moderately weak oncogenic potential (Polacov *et al.*, 2018). Although T21 induces foetal haematopoietic skewing, the transformation of immature lymphoid and myeloid cells to leukaemia requires the addition of secondary oncogenic hits.

1.5 Down Syndrome Haematopoiesis

1.5.1 DS Foetal Haematopoiesis

The most haematologically clinically significant effect of constitutional T21 with 1:10 newborns presenting is the pre-leukaemic condition termed transient myeloproliferative disorder (TMD) which arises due to mutations in the GATA binding protein1 (GATA1) resulting in a premature stop codon and the synthesis of a truncated version of GATA1 lacking an N-terminal domain (Wechsler *et al.*, 2002). While a large majority of neonates with this condition spontaneously enter remission, 10% will subsequently develop AMKL (Bhatnagar *et al.*, 2016). Along with an increased leukaemic predisposition DS children have a range of other haematological disorders, listed in table 1.1.

Condition	*Lineage*	*Reference*
	Neonates	
Transient abnormal myelopoiesis	Leucocytes	Bhatnagar et al,. 2016 Bombery et al,. 2014
Polycythemia	Erythrocytes	Choi 2017 James et al,. 2009
Macrocytosis	Erythrocytes	Webb et al,. 2007
Thrombocytopenia	Platelets	Choi 2017
Thrombocytosis	Platelets	Nakamura et al,. 2015
Leukemoid reaction	Leucocytes	Weinberg et al,. 1982
Neutrophilia (neonates)	Neutrophils	Choi 2017
	Children	
Myelodysplastic syndrome	Leucocytes Erythrocytes	Choi 2017
Leukaemia	Leucocytes	Malone et al,. 2017

Table 1. 1: Haematological disorders observed in DS patients during neonatal and childhood periods highlighting the specific lineage that undergoes transformation

The extent to which there are a significant number of disorders being presented in DS neonates is indicative of the function of T21 during foetal development.

The initiation and evolution of DS haematological abnormalities can be understood by a three step model of foetal haematopoiesis; (i) T21 is the sole initiating event in-utero, (ii) mutations in biologically relevant genes are required to collaborate with T21 or (iii) primary and secondary mutations along with T21 are needed to induce the disease phenotype. FACS and clonogenic assays

of second trimester T21 foetal liver mononuclear myeloid progenitors, lacking GATA1 mutations, had a lineage bias towards MEP 55.9% vs 17.1% of $CD34^{+}CD38^{+}$ cells while CMP and GMP progenitors were markedly decreased 19.6% vs 44% and 15.8% vs 34.5% respectively, in comparison to D21 controls, further highlighting the role of T21 in skewing DS foetal haematopoiesis resulting in an increased predisposition to leukaemic development. Interestingly there were no differences observed in the frequency of myeloid progenitors observed within normal and T21 foetal bone marrow proposing that DS lineage skewing initially occurs in the foetal liver and is independent of GATA1 mutational status or may imply that the impact of T21 differs in a developmental context between primary sites of haematopoiesis during development (Tunstall-Pedoe *et al.*, 2008).

Klusman et al,. similarly proposed a triad of factors influencing DS foetal haematopoiesis and subsequent development of TAM; (i) foetal microenvironment, (ii) presence of T21 and (iii) truncation of GATA1 on the basis that constitutional T21 exists in all embryonic cells and so the interaction between DS FL haematopoietic cells and the microenvironment will result in a complex interaction of deregulated gene expression signatures (Tunstall-Pedoe *et al.*, 2008; Klusmann *et al.*, 2010). To understand the role of T21 in the development of lymphopoietic impairment and MkE bias, clonogenic functional assays and gene expression of matched DS and euploid second trimester FL samples revealed a reduced frequency of B-cells emerging from HSCs and LMPPs along with a decrease in the expression of transcription factors involved in B cell commitment in LMPP and ELP HSPCs (IL7Rα, CRLF2, MEF2C and EBF). Moreover, T21 FL displayed an increase in Mk-erythroid genes within the HSC compartment along with a concomitant 10-fold reduction in the ability of prepro and ELP to gain CD19 expression and commit to the B-cell lineage (Roy *et al.*, 2012).

The role of the foetal liver microenvironment in HSC function and proliferation has previously been described by Zhang et al,. who identified that insulin-like growth factor 2 facilitates HSC proliferation and is also a regulator of embryonic and foetal apoptosis, cellular metabolism and growth but not in

adults who may experience different levels of sensitivity to IGF signalling (Zhang and Lodish, 2004). Using patient GATA1s^{+} TAM and DS-AMKL blasts Klusman uncovered that IGF/IGF1 receptor signalling pathway facilitates proliferation of foetal but not adult megakaryocytic progenitors through the E2F signalling pathway where under normal physiological conditions is restricted by full length GATA1 which induces cell cycle exit (Klusmann *et al.*, 2010). Similarly mutant CEBPα failed to regulate E2F transcriptional function and to inhibit proliferation and differentiation of adipocytes highlighting the key role of E2F in lineage instruction and differentiation (Porse *et al.*, 2001).

1.5.2 Trisomy 21 in DS Foetal Haematopoiesis

Chromosome 21 likely effects haematopoietic and foetal development through complex signalling mechanisms that are cell context dependent and specific to stages of development (Liu *et al.*, 2015). Sequencing of Hsa21 revealed a number of candidate genes that may be involved in DS phenotypes such as RUNX1, DYRK1A, ETS2 and ERG, however the mechanisms by which T21 gene imbalance induces these phenotypes i.e. gene dosage effects of T21 or a network of complex interactions between trisomic, disomic and microenvironmental cells is not known. To address this, engineered mouse models overexpressing segments of Hsa21 have been adopted however they fail to develop all features of TAM (Bhatnagar *et al.*, 2016). Although the molecular implications of T21 on stem cells is unknown the pathophysiological effects on stem cell, progenitor cell and tissue function include increased and impaired proliferation and differentiation along with premature ageing resulting in pre-leukaemic initiation, testicular germ cell tumour, craniofacial and congenital heart defects and premature ageing in adults (Liu *et al.*, 2015).

Interestingly second trimester DS foetal liver did not show the expected 1.5 fold increase in expression of the chromosome 21 haematopoietic genes across HSC, LMPP, ELP, CLP, MEP, GMP and preproB when compared to matched normal controls, however modest differences between

aneuploid samples are difficult to interpret from interindividual differences (Birchler, 2010; Roy *et al.*, 2012).

More recently hESCs and iPSCs have been used to recapitulate DS development and to investigate HSA21 protein coding genes in DS. There have been a number of candidate genes arising from these studies including the key haematopoietic regulators located within the DSCR; DYRK1A, SON and GABPA (Chou *et al.*, 2012) .

RUNX1 is a member of the RUNX family and encodes the transcription factor runt-related transcription factor-1 that is involved in cellular proliferation, differentiation and is expressed on all sites of haematopoietic development and necessary for the development of all haematopoietic cells with the exception of mature erythrocytes (North, *et* al., 2004). QPCR of c-Kit^{+}CD41^{-} haematopoietic progenitors derived from transchromosomic trisomic mouse ESCs have increased mesodermal colony formation vs normal matched mouse ESC line. siRNA targeting of RUNX1 revealed that increased mesodermal formation was due to overexpression of RUNX1 and a concomitant increase in signalling through c-Kit and GATA-2 (Tarleton and Lemischka, 2010). Interestingly RUNX1 is not overexpressed in DS iPSC myeloid derived progenitors, T21 foetal liver or in ML-DS vs non-DS AMKL patient samples, leaving to question it's role in the development of DS haematopoietic disorders (Bourquin *et al.*, 2006).

DYRK1A (Dual specificity tyrosine phosphorylation regulated kinase 1A) is located within the DSCR and is a regulator of the NFAT pathway that controls cellular proliferation, chromatin remodelling, mitochondrial function and coordinates the transition from proliferation to quiescence during lymphoid development (Thompson *et al.*, 2015). CRISPR screen of the paediatric cell line, KMT2A-R, identified DYRK1a as a regulator of G2-M phase and reduction in cell proliferation (Hurtz *et al.*, 2019). Prenatal administration of DYRK1A inhibitors that cross the placenta and blood brain barrier have shown to improve DS defects involved in craniofacial abnormalities in Ts65Dn pups (McElyea *et al.*,

2016). Overexpression of DYRK1A in murine bone marrow further confirmed by shRNA knockdown and molecular inhibition showed that DYRK1A alone is necessary for the expansion of megakaryoblasts and inhibits tumour promoter 12-Otetradecanoylphorbol13-acetate megakaryocyte differentiation in non-DS and DS-AMKL cell lines (Malinge *et al.*, 2012).

Further validation and characterisation of such candidate targets using ESC, IPSCs and primary foetal tissue is necessary in order to understand the initiation of DS haematopoietic abnormalities and improve screening and therapeutic interventions.

1.5.3 DS Haematopoietic Niche

As described above the haematopoietic niche plays a vital role in the maintenance of HSPCs under both normal and pathological conditions. It is well documented that cALL initiates in-utero underpinning the influence of the foetal microenvironment on leukaemic progression (Gale *et al.*, 1997; Maia *et al.*, 2001; Hong *et al.*, 2008; Cazzaniga *et al.*, 2011). Moreover the development of TMD in the foetal liver and subsequent cessation of TMD once haematopoiesis shifts to the bone marrow, approximately 3 months after birth, further highlights the unique dependence of TMD on foetal haematopoiesis and more specifically the role of the foetal liver microenvironment on the development of pre-leukaemia (Roy *et al.*, 2009). To understand the mechanism of TMD development, Saida et al,. used NOD/Shi-*scid*, inteleukin2Ry^{null} mice to develop a xenograft model of TMD from a patient who went onto develop ML-DS. Genomic analysis of bone marrow derived TMD cells identified a heterogenous pool of subclones containing copy number alterations with a chromosome 6q deletion and chromosome 1q gain along with differing GATA1s mutations than those identified in the diagnostic sample providing a potential insight into the relationship between the haematopoietic niche and the evolution and development of pre-leukaemia (Saida *et al.*, 2013). Co-culturing of patient derived TMD blasts along with bone marrow and foetal liver derived stromal cells

from human foetuses Myauchi et al,. identified that the secretion of granulocyte-macrophage colony stimulating factor (GM-CSF) by foetal liver stromal cells, that was nearly undetectable in the foetal bone marrow, is necessary for the proliferation of TMD blasts within the foetal liver versus the more mature bone marrow microenvironment (Miyauchi and Kawaguchi, 2014). Moreover, trisomic human and mouse cells have decreased proliferative capacity, similarly cells harbouring mutations that promote chromosomal instability have reduced or at best equal proliferative potential to diploid cells (V. Roschke and R. Kirsch, 2012). As autosomal aneuploidy is largely embryonically lethal in mice to address the fitness of aneuploid foetal HSPCs, FL-HSCs isolated from trisomic 16, homologous to a large portion of human chromosome 21, and trisomic 19 E12.5 and E15.5 mice foetuses that were injected into lethally irradiated mice showed decreased area in colony forming units within the spleen, indicative of decreased proliferative fitness versus wild type littermates (Pfau *et al.*, 2016).

Sullivan et al,. completed comparative genomic and transcriptomic analysis of age and gender matched individuals with and without DS identifying constitutive activation of the interferon signalling pathway and increased surface expression of IFN receptors on B-cells derived from T21 individuals (Sullivan *et al.*, 2016). Moreover, co-culture of T21 HSC, MPP and LMPP with T21 foetal bone marrow MSCs showed impaired B-cell differentiation and increased expression of interferonα (IFNα) receptors IFNαR1 and IFNαR2, while IFNα was not detected in the supernatant of normal HSPCs co-cultured with normal foetal bone marrow. In addition RNA-seq revealed an increase in pro-inflammatory signatures within the supernatant of T21 co-cultures conveying that T21 pro-inflammatory driven signals as a result of T21 HSPCs interaction with the T21 BM microenvironment may be responsible for the initiation of DS-ALL (O'Byrne *et al.*, 2019).

1.5.4 Transient Myeloproliferative Disorder

Children with constitutional trisomy 21 have a 20-fold increased risk of developing preB cell ALL and 150-fold increased risk of developing AMKL while very few cases of mature B-cell and T cell ALL have been reported (Xavier, Ge and Taub, 2009). Childhood ALL initiates from a single clone that arises in utero (Bateman 2010). 10-15% of children with DS present with the pre-leukaemic condition TMD classically diagnosed by mutations in the N-terminal of the GATA1 gene that arises during foetal development with >10% blasts during the first few days of life. DS patients with TMD present with a variety of clinical features such as hepatosplenomegaly, jaundice and renal dysfunction, with 20% of neonates with TMD being asymptomatic. The majority of infants with TMD enter remission within 3-6 months of age and do not require chemotherapy while 10-20% of children develop myeloid leukaemia of Down syndrome (ML-DS) before the age of 3 (Bombery and Vergilio, 2014). While the majority of patients presenting with TMD enter remission 10% of those relapse with AMKL (Bhatnagar *et al.*, 2016). Mutational analysis of GATA1 identified identical patient specific mutations in both TMD and ML-DS samples indicating that the same clone originating in utero gives rise to ML-DS (Ahmed *et al.*, 2004). The Oxford-Imperial Cohort Down Syndrome Study (OICDS) retrospective study employed next generation sequencing and blood counts of newborns with DS identifying 20% of cases in which GATA1 mutants were undetectable by conventional screening methods such as denaturing high performance liquid chromatography but were identified using next generation sequencing and so adopting the term silent TMD for such a finding, further indicative of the underlying prenatal origins of DS myeloid leukaemias (Roberts *et al.*, 2013).

Mouse models overexpressing ERG and a truncated GATA1 mutant have an expansion of MEPs, develop postnatal thrombocytosis and anaemia in the Ts65Dn mouse but fail to develop full blown leukaemia (Birger *et al.*, 2013). Moreover, correction of ERG from trisomy to disomy in the Ts65Dn

mouse model corrected haematological and pathological myeloproliferation further implying the gene dosage effect of ERG on predisposing DS patients to haematological disorders (Ng *et al.*, 2010). Interestingly endogenous expression of GATA1s in foetal HSPCs resulting in an expansion of erythroid progenitors with no effect on neonatal or adult HSPC differentiation (Gialesaki *et al.*, 2018).

DNA sequencing of 11 DS-TMD and DS-AMKL patients revealed that transition to a leukaemic state occurs with the sequential acquisition of activating mutations in Flt3, JAK3 and JAK2 genes (Malinge *et al.*, 2008). Moreover, analysis of DS neurons in-vitro has proposed overexpression of HSA21 genes superoxide dismutase and cystathionine beta synthetase, involved in oxidative metabolism, perturb folate metabolism resulting in the accumulation of uracil and incorporation into DNA subsequently proposed to induce mutations in GATA1 (Busciglio and Yankner, 1995; Cabelof *et al.*, 2009) . The foetal microenvironment has been shown to facilitate expansion of HSCs by secreting IGF-1 in turn activating IGF and E2F signalling inducing increased proliferation of foetal HSPCs, previously demonstrated to be upregulated in DS-AMKL, TMD blasts and a DS-AMKL mouse model exposing a perturbed foetal specific regulatory network (Klusmann *et al.*, 2010).

1.5.5 Incidence of malignancy in DS

The first diagnosed case of leukaemia in a DS patient occurred in 1930 and was followed up by the first systematic study in 1957. Analysis of data from the Danish Cancer Registry showed that patients with DS have a cumulative risk of leukaemic development of 2% by the age of 5 and 2.7% by the age of 30 with no cases of leukaemia reported in cases older than 30. Both child and adult DS patients also have a decreased risk of developing solid tumours indicating the tumour suppressive properties of chromosome 21 (Hasle, Haunstrup Clemmensen and Mikkelsen, 2000). It has been reported that an increase incidence of germ cell tumours occur within DS patients with a decrease in risk of developing T-ALL, this may reflect the impact of T21 on differing progenitor stem cell compartments

during embryogenesis (Satgé *et al.*, 1997). In addition to having an increased incidence of leukaemias, DS patients also exhibit different clinical features, response to therapy and timing of occurrence (Rabin and Whitlock, 2009). The outcome of DS-ALL is worse than that of sporadic B-cell precursor ALL due to intrinsic resistance to therapy and treatment related mortality (Izraeli *et al.*, 2014).

1.5.6 Down syndrome leukaemia

DS-ALL childhood leukaemic patients range in the ages of 1-5 years with an immunophenotype typical of childhood B cell precursor ALL ($CD10^{+}19^{+}79a^{+}$) however only up to $1/10^{th}$ have the common cytogenetic subtypes of TEL-AML1 and hyperdiploid that are present in non-DS ALL (Maloney *et al.*, 2010). DS-AMKL patients present on average at 2 years of age and is preceded by prenatal TAM, as discussed above (Malinge, Izraeli and Crispino, 2009).

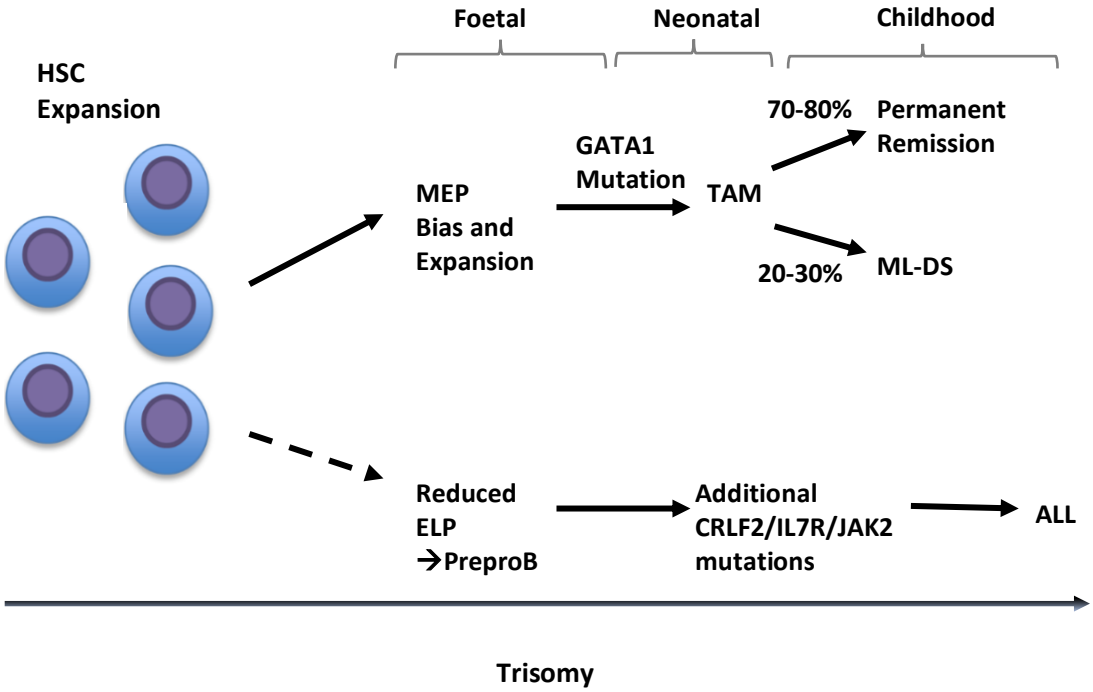


Figure 1. 6: Model of perturbed haematopoiesis that occurs during DS foetal, neonatal and childhood development resulting in an increased predisposition to leukaemic development. (adapted from Bhatnagar et al,. 2016)

80% of DS-ALL have either JAK2 and/or CRLF2 aberrations leading to downstream JAK/STAT signalling in comparison to 5% observed in non-DS BCP-ALL where they are a poor prognostic indicator (Vesely *et al.*, 2017). CRLF2 signalling has essential roles in B-cell proliferation, T and dendritic cell development and inflammation (Parrish *et al.*, 2009). In contrast to myeloproliferative disorders that have homozygous JAK2 V617 point mutations, DS-ALL JAK2 mutations are heterozygous and occur at R683S (Zhou *et al.*, 2005). Schwartzman et al., performed whole genome analysis, RNA sequencing and variant calling on 25 matched diagnostic and relapsed samples of DS-ALL and could subsequently stratify patients based on $CRLF2^{Lo}$ and $CRLF2^{Hi}$ subtypes that have varying cooperating mutations (Schwartzman *et al.*, 2017). Moreover the mutational landscape differs between $CRLF2^{+}$ and $CRLF2^{-}$ DS-ALLs such that $CRLF2^{-}$ DS-ALLs are characterised by oncogenic RAS signalling and chromatin remodelling genes in comparison to $CRLF2^{+}$ that have high levels of proliferative signalling due to activating JAK/STAT mutations and in cases accompanying RAS activation. The dynamics of mutational signatures and the emergence of subclones upon relapse for both $CRLF2^{-}$ and $CRLF2^{+}$ DS-ALLs was shown to be mutated in RAS sublcones that were undetected at diagnosis (Schwartzman *et al.*, 2017). Such a higher prevalence of these cooperating events in DS-ALL suggests that constitutional T21 may increase the risk of foetal B-cell transformation or possibly influence the development of stromal cells within the foetal liver and bone marrow subsequently effecting haematopoiesis (Roberts *et al.*, 2013).

Activating $JAK2^{R683S}$ mutations occur in 21% of DS-ALL cases with 83% of these having cytokine receptor like factor 2 (CRLF2) gene rearrangements such as IGH-CRLF2 translocations or a P2RY8-CRLF2 fusion which forms as a result of interstitial deletion within the PAR1 region of the X chromosome resulting in CRLF2 overexpression (Harvey et al,. 2010; Mullighan *et al.*, 2009). Longtemplate PCR of genomic DNA isolated from leukaemic cells identified that these breakpoints are highly conserved and lie upstream of CRLF2 exon 1 and are distal to P2RY8 exon1. The presence

of recombination signal sequences adjacent to the breakpoints and non-consensus aligning sequences suggests that PAR1 deletion may arise due to the aberrant activity of the RAG genes, which has also been implicated in other deletions in ALL and chromosomal rearrangements such as c-Myc and BCL-6 genes (Mullighan *et al.*, 2009; Lieber, 2016). Other cytogenetic abnormalities identified in DS-ALL, and common to non DS-ALL, are extra copies of chromosomes X, 4, 6, 10, 14, 17 and 18 while DS-AML and non DS-AML do not have any overlapping chromosomal aberrations suggesting that DS-ALL and normal B-cell precursor ALL may share a common genetic mechanism that induces leukaemic transformation (Forestier *et al.*, 2008).

Under normal physiological conditions CRLF2 can form a monomer, homodimer or a heterodimer with the IL7Rα. The CRLF2-IL7Rα dimer forms the receptor for the epithelial derived TSLP ligand, that activates the JAK/STAT pathway and facilitates regulatory T cell production and early B cell proliferation and differentiation (Shochat *et al.*, 2011).

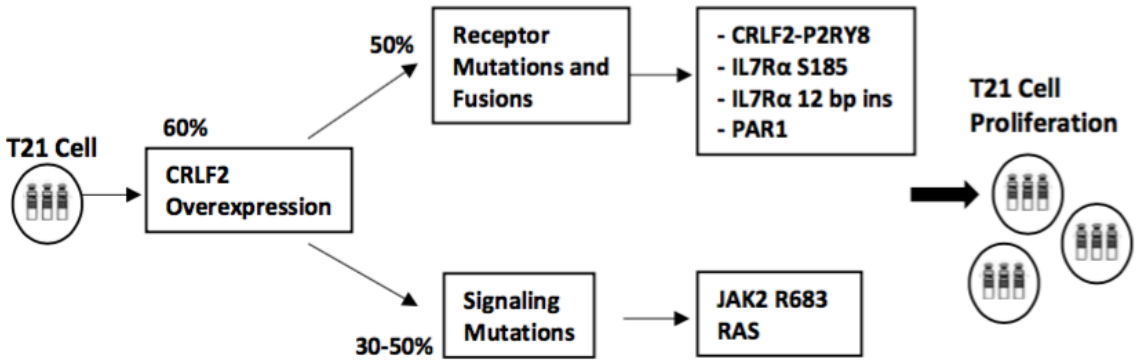


Figure 1. 7: Aberrations within the IL7Rα and CRLF2 pathways resulting in constitutive signalling and subsequent proliferation and survival of B and T cells (Adapted from Hertzberg et al,. 2010).

While CRLF2 overexpression correlates with the $JAK2^{R683S}$ point mutation, IL7Rα expression has shown to be independent of JAK2 and CRLF2 (Hertzberg *et al.*, 2010). Moreover, the transformation of murine Baf3 proB cells that lack IL7Rα was, along with the immortalisation of primary mouse bone marrow cells, dependent on $JAK2^{R683S}$ and CRLF2 overexpression thus indicating that JAK2 mutants and CRLF2 overexpression may act as a secondary hit in lymphoid committed progenitors and induce a growth advantage independent of IL7Rα (Bercovich *et al.*, 2008; Hertzberg *et al.*, 2010). Similarly, the S185C point mutation within the extracellular domain (IL7Rα S185C) and a 12 base pair insertion in the transmembrane domain of IL7Rα (IL7Rα12bp ins) facilitates JAK/STAT signalling independent of TSLP binding or can homodimerize and provide a CRLF2 independent survival advantage (Shochat *et al.*, 2011). Geron et al., displayed that lentiviral overexpression of IL7Rα12bp ins under the control of the B-cell specific Emu/B29 enhancer/promoter in human CD34+ enriched cord blood resulted in enhanced self-renewal of a $CD34^{+}10^{+}19^{+}$ B-cell population in transplanted mice. Gene expression analysis of the $CD34^{+}10^{+}19^{+}$ compartment and a candidate pre-leukaemic population identified as $CD10^{hi}19^{lo}$ revealed enrichment of the Philadelphia like B-ALL gene expression signature concluding that overexpression of activated IL7Rα12bp ins alone has the potential to perturb human B-cell development and induce leukaemic transformation (Geron et al., 2021).

1.6 Pluripotency

1.6.1 Induced Pluripotent Stem Cells

After the isolation of ESCs there was the emergence of a number of 2D differentiation protocols that aimed to recapitulate the early stages of embryogenesis and the derivation of a desired germ layer prior to commitment to a specific lineage. The discovery that the four embryonic stem cell regulators Oct4, Sox2, Klf4 and c-myc (OSKM) when retrovirally transduced into human fibroblasts were

sufficient to reprogram to a pluripotent state now provides a novel approach to understand developmental disease states and to advance our current approaches to drug discovery (Takahashi and Yamanaka, 2006). The methods by which the pluripotent state can be achieved and the cell types that can successfully undergo the reprogramming has been well documented, with scar free gene delivery systems and small molecule compounds being successfully used to reprogram multiple cell sources (Junying *et al.*, 2009; Hou *et al.*, 2013). The derivation of patient specific iPSCs using such techniques facilitates application of iPSCs to disease modelling via the commitment of a target cell lineage.

The reprogramming of somatic cells to a pluripotent state induces a transition through distinct intermediate states governed by gene expression and epigenetic signatures (Aranda *et al.*, 2009). Kim et al,. 2010 described that while there is a reset in genomic methylation, transcription factor derived iPSCs maintain a residual DNA methylation signature that is characteristic of their tissue of origin, while iPSCs derived by somatic cell nuclear transfer more closely resemble ESCs (Kim *et al.*, 2010). This has in turn highlighted an epigenetic memory and a potential biased differentiation potential of iPSCs derived from different tissues which must be overcome if iPSCs are to have potential in the clinic.

The use of iPSCs to model developmental disorders has gained much attention since their development in 2006. In the context of Down's syndrome a large focus has been to model the neurodevelopmental abnormalities DS patients experience (Hibaoui *et al.*, 2014; Xu *et al.*, 2019). However a caveat remains in obtaining an IPSC isogenic control for which to accurately model the DS phenotypes.

While this needs to be overcome, the in-vitro use of iPSCs to model disease has become a valuable tool.

1.6.2 In-vitro Differentiation of Pluripotent Stem Cells

The potential of iPSCs to model and treat disease has shown much potential in the more characterised mouse system, with genomically engineered iPSCs derived from a mouse model of sickle cell anaemia and differentiated into haematopoietic progenitors being able to successfully used to treat sickle cell mice (Hanna *et al.*, 2007).

The derivation of haematopoietic progenitors was first described using human embryonic stem cells and led to the development of multiple protocols that utilised both embryoid body and feeder free systems (Kaufman *et al.*, 2001). To recapitulate haematopoiesis iPSCs must undergo two waves of haematopoietic development; the first wave producing a multi-lineage haemato-endothelial progenitor, which can be achieved through embryoid body structures or feeder free systems, and a second definitive wave developing more committed progenitors, with lineage commitment depending on the culture system and cytokines used, discussed below (Lim *et al.*, 2013). A key development in the use of iPSCs to recapitulate early haematopoietic development has been the description of the waves of haematopoiesis and the identification of cell surface markers CD73, CD43 and CD235a that could discriminate hemogenic from non-hemogenic endothelium (Choi *et al.*, 2012).

Although there have been a number of protocols describing the production of haematopoietic progenitors from iPS cells there have been limited descriptions of the successful production of B cell progenitors. The use of the OP9 mouse stromal co-culture system to derive CD34+ cells with phenotypic and functional haematopoietic properties from human ESCs was first described by Vodyanik et al,. (Vodyanik *et al.*, 2005). Subsequently, this system and the potential to derive haemogenic endothelium precursors capable of producing definitive HSCs from hPSCs was described in detail (Choi *et al.*, 2012).

Carpenter et al,. described the derivation of B cells from iPSCs using the OP9/MS5 co-culture system (Carpenter *et al.*, 2011). This system was similarly used by Boiers and Richardson et al,. and identified an expansion of the bi lineage $CD34^{+}CD45^{+}CD19^{-}IL\text{-}7R^{+}$ progenitor, a phenotype similarly shown to be present in the human foetal liver showing that iPSCs recapitulate foetal development, and subsequent partial B cell block in ETV6:RUNX1 genomically engineered iPSCs highlighting the ability of the system to recapitulate early foetal haematopoiesis and model pre-leukaemic initiation (Böiers *et al.*, 2018).

Abbreviation	Phenotype	Day of isolation	Definition
$V^+73^-235^-$ HEP	VE-cadherin$^+$CD73$^-$ CD235a/CD43$^-$ CD117^{intermediate}	5	Hemogenic endothelial (HE) progenitors that, although are have primary endothelial traits, have the potential to generate blood and endothelial cells upon co-culture with stroma
V^+73^+ Non-HEP	VE-cadherin$^+$CD73$^+$ CD235a/CD43$^-$CD117^{high}	5	Non-HE progenitors that form only endothelial colonies on OP9 stroma
$V^+235^+41^-$ AHP	VE-cadherin$^+$CD73$^-$CD43^{low} CD235a$^+$CD41a$^-$CD117$^-$	5	Angiogenic haematopoietic progenitors that have hematopoietic potential in response to FGF2 and are also capable of generating endothelial cells
$V^+235^+41^+$ EMkP	VE-cadherin$^+$CD73$^-$CD43$^+$ CD235a$^+$CD41a$^+$	5	E-Mk enriched Haematopoietic progenitors
MHP	lin$^-$CD34$^+$CD43$^+$CD45$^+$CD38$^-$	8	Lineage negative MPPs that can produce all myeloid progenitors in semi-solid medium supplemented with haematopoietic cytokines
$K^{br}A^+P^-$ HVMP	EMHlin$^-$KDR^{bright}APLNR$^+$ PDGFRα$^{low/-}$ *	4	Hematovascular mesodermal progenitors that facilitate extraembryonic mesoderm and angiohaematopoietic commitment and can form haematoendothelial precursors on OP9 stroma
A^+P^+ PM	^{EMH}lin$^-$APLNR$^+$PDGFRα$^+$ *	3	Primitive mesoderm cells expressing primitive genes with the potential to form haemangioblast colonies

Table 1. 2: Characteristics of progenitors with endothelial and haematopoietic potential derived from the iPSC OP9 co-culture system. (Adapted from Choi et al,. 2012)

1.6.3 Mesoderm Initiation and Early Haematopoietic Development

Mesodermal induction in-vitro, as in-vivo, is regulated by the combinatorial roles of canonical WNT signalling, FGF, BMP4 and activin and can be characterised by the cell surface expression marker KDR.

It's induction is promoted by signalling through the FGF2 and ALPN receptors along with the expression of the primitive streak transcription factors MIXL1 and FOXF1 and gives rise to cells with the potential for blast-CFC generation (Slukvin, 2013). Upregulation of KDR and sensitisation to VEGF-A, which is produced by the visceral mesoderm, subsequently facilitates blood and endothelial development (Carmeliet *et al.*, 1996).

Derivation of the most primitive mesodermal precursors occurs at day 3 of the embryoid body or OP9 co-culture system. They have the potential to form haemangioblast colonies in the presence of VEGF and FGF2 and lack endothelial (VE-Cadherin, CD31), haematopoietic (CD45, CD43) and mesenchymal (CD105, CD73) markers (Slukvin, 2013). Mesodermal commitment towards haematopoietic and endothelial lineages is induced by the downregulation of PDGFRα and upregulation of the angiohaematopoietic transcription factors GATA2, TAL1 and KDR generating lin^- $KDR^{bright}APLNR^+PDGFR^+$ hematovascular mesodermal progenitors (HVMPs). It has been suggested that there are two forms of mesoderm that exist during hESC differentiation to haematopoietic and endothelial lineages with both having haematopoietic and endothelial potential – posterior mesoderm cells that produce primitive haematopoietic precursors and HVMPs generating more definitive progenitors (Slukvin, 2013). To distinguish the emergence of definitive and primitive haematopoietic progenitors from hESCs and hiPSCs in-vitro differentiation of HPSCs, Kennedy et al,. used serum free differentiation conditions to derive T-cell subsets from the HE-like compartment expressing CD34, VE-cadherin, LMO2, RUNX1 and GATA2 in the presence or absence of the morphogens activin and nodal. The emergence of a $CD34^{low/-}CD43^+$ $CD45^+$ on day 12 of hPSCs co-culture with the OP9-DL4 stromal cell line give rise to erythroid and myeloid colonies along with T cells independent of activin and nodal signalling while primitive $CD34^{low/-}CD43^+CD235a^+CD41^+$ progenitors, necessary for the derivation of erythroid and myeloid only colonies, are dependent on activin and nodal signalling (Kennedy *et al.*, 2012).

The first haematopoietic precursors negative for the expression of the foetal haematopoietic marker CD41 and positive for glycophorin and the pan-haematopoietic marker CD43 derived from in-vitro differentiation of hPSC OP9 co-cultures arose from VE-cadherin^{+} cells at day 4 of culture. These CD43^{+}CD235a^{+}CD41a^{-}VE-Cadherin^{+} cells primarily produced haematopoietic progenitors and although they lacked endothelial specific markers CLDN5 and CAL1 they maintained endothelial potential and so are termed angiogenic haematopoietic precursors (Choi *et al.*, 2012). Initiation of hPSC haematopoietic commitment is indicated by the acquisition of CD41 and occurs within the CD43^{+}CD235a^{+}VE-Cadherin^{+} HSPCs population that is enriched for megakaryocyte-erythroid progenitors while also having an underlying myeloid potential in serum-free differentiation conditions (Vodyanik, Thomson and Slukvin, 2006; Klimchenko *et al.*, 2009). It was also observed that the CD45^{+}CD43^{+}CD235a$^{/}$CD41a^{-} population expanded to day 10 of OP9 stage of differentiation and there was a concomitant increase in the expression of the myelomonocytic markers CD15, CD14 and CD11b (Choi *et al.*, 2008).

The first progenitors with potential for lymphomyeloid output, lin^{-}CD34^{+}CD43^{+}CD45^{-} with a Flt3^{low}RUNX1^{low}GATA3^{high} gene expression profile, emerge at day 8 of OP9 co-culture and display immunophenotypic traits of HSCs such as CD90 and c-kit expression while remaining negative for CD45Ra and CD38 and have the same potential as cord blood to generate all haematopoietic colonies with increasing myeloid commitment upon the gain of CD45 expression (Vodyanik, *et* al,. 2008). The successful derivation of lymphoid progenitors from hiPSCs has great clinical potential. However, the development of protocols that produce reasonable quantities of B-cells has proven difficult.

The most efficient system to produce immature B cells from hESCs or hiPSCs comprises of derivation of Lin^{-}CD34^{+} cells from OP9-hiPSC co-culture and subsequent culture on MS-5 stromal cells in the presence of human recombinant (hr) IL3, hrSCF, hrIL7 and hrFlt3l. Using this system French et al,.

reported the derivation of $CD10^{+}CD19^{+}$ B cells that express surface $sIgM^{+}$ from a $CD144^{+}CD73^{-}$ $CD43/235a^{-}$ population identified as HE (French *et al.*, 2015).

The transcriptional profile of the hESC and hiPSC derived $sIgM^{+}$ B cells differed only by 45 transcripts in comparison to the differentiation of UCB HPSCs into B-cells. Interestingly the addition of the B cell cytokine hrIL7 inhibited the ability of B cells to mature beyond the preB stage, as determined by expression of sIgM, but did not affect total B cell numbers generated, indicating that IL7 may inhibit RAG activity and light chain rearrangement (Johnson *et al.*, 2008; French *et al.*, 2015). The OP9 and MS-5 co-culture systems have been demonstrated to faithfully recapitulate B-cell foetal lymphopoiesis (Böiers *et al.*, 2018). Moreover, this system has been more recently utilised to model childhood B-cell lymphopoiesis, pre-leukaemic initiation and to identify the cell of origin in which known oncogenes induce cellular transformation (Böiers *et al.*, 2018). The above highlights the potential of the iPSC-OP9-MS5 co-culture system to model DS pre-leukaemic initiation and to identify and model candidate progenitors that are susceptible to secondary mutations.

1.6.4 Engraftment of Definitive HPSC

A caveat that remains of human ESCs and iPSCs is the inability to derive bona fide haematopoietic progenitors with long-term engraftment potential in host mice. Overexpression of master regulators in hESCs has not proven fruitful in dHSC production leading to the hypothesis that genomic engineering of single gene products will not facilitate HSC production and that overexpression of a combination of factors may be necessary to induce dHSC production from hiPSCs/ESCs (Wang *et al.*, 2005). The primary reason for the inability to derive functional HSCs from mouse or human iPSCs is that the cell types within the foetal and adult haematopoietic niche are not known or fully understood and so in-vitro recapitulation of the microenvironment is not accurate. Suzuki et al,. using a scaffold of OP9 stroma and supplementation with SCF and TPO have shown success in deriving

engraftable HSCs, although at very low level, from hiPSCs derived teratomas that yielded both lymphoid and myeloid precursors in host mice upon engraftment and therefore may potentially be recapitulating primitive haematopoiesis as seen in the yolk-sac (Suzuki *et al.*, 2013). Sugimura et al,. used a doxycycline inducible system overexpressing a cocktail of 26 key haematopoietic transcription factors in haemogenic endothelium derived from iPSCs and could produce definitive haematopoietic progenitors that could reconstitute lymphoid and myeloid lineages upon serial transplantation. Although promising, there were differences in engraftment and reconstitution capabilities between bona-fide HSCs and the engineered HSPCs derived from the haemogenic endothelium (Sugimura *et al.*, 2017). The inability to derive dHSCs with the potential for long term engraftment is believed to be due to hiPSCs differentiating into a haematopoietic foetal like state, exemplified by the expression of embryonic globulins on erythrocytes and has similarly been shown when modelling cardiomyocytes or intestinal organoids, hence the inability to recapitulate adult haematopoiesis (Finkbeiner *et al.*, 2015; Ronaldson *et al.*, 2018; Rowe and Daley, 2019). In an effort to derive dHSCs there have been a number of approaches adopted such as small molecules to expand HSC populations, overexpression of HSC homing molecules and adaptation of cell culture conditions to mimic haematopoietic niche environments (Uenishi *et al.*, 2014; Lee *et* al,. 2015).

1.6.5 Cancer Stem Cell Hypothesis

The hierarchical and clonal organisation of cancer that occurs in a sequential manner with somatic mutations selecting the emergence of sublcones was first described by Peter Nowell in 1976 (Nowell, 1976). This theory was then applied to haematopoietic malignancies by Bonnet and Dick 1997 who, through serial transplantation of human AML primary samples into primary and secondary mouse recipients, identified a <2% leukaemia initiating cell (LIC) population retaining a primitive $34^{+}38^{-}$ immunophenotype. The $CD34^{+}38^{-}$ but not more mature $CD34^{+}38^{+}$ could generate AML-colony forming

unit (AML-CFU) and leukemic blasts that maintained the cell surface antigen pattern as seen in the patient, highlighting the primitive origins of AML thus deriving the theory of the primitive leukaemic stem cell as a key target for therapeutic intervention and eradication of malignancy (Bonnet and Dick, 1997). However, the precise cell of origin i.e. stem cells or non-stem cells, remains to be identified along with their immunophenotypic and functional traits. Pisco and Huang proposed that the acquisition of stem cell like properties and exploitation of their plasticity occurs in response to external stress such as chemotherapeutic agents inducing latency and resilience (Pisco and Huang, 2015). Interestingly, real-time PCR of breast cancer stem cells, defined by their drug resistant properties and ability to propagate tumour formation after transplantation in SCID mice, identified increased expression of stem cell genes Oct4, Notch1, Sox1 and Aldh1 (Wright *et al.*, 2008). Overexpression of the infantile oncogene MLL-AF9 in GMP cells and subsequent isolation of leukaemic GMP cells that arose from the transduced MLL-AF9 GMP cells and further transplantation into mice revealed the GMP cells propagating leukaemia maintained the global identity of the GMP progenitor but also adopted a limited stem-cell programme (Krivtsov *et al.*, 2006). This finding highlights that LSCs can arise from a committed progenitor and while the first oncogenic hit may not render leukaemic transformation, normal haematopoietic hierarchical systems may remain. This model is similarly applicable to the initiation and development of Down syndrome leukaemias. The expansion of the HSC compartment with an erythroid-megakaryocyte gene expression profile as described by Roy et al,. highlights the influence of human T21 on foetal haematopoiesis in a primitive progenitor compartment with a $CD34^{+}CD38^{-}$ immunophenotype similarly described to that of leukaemic stem cells as described by Bonnet and Dick (Bonnet and Dick, 1997; Roy *et al.*, 2012).

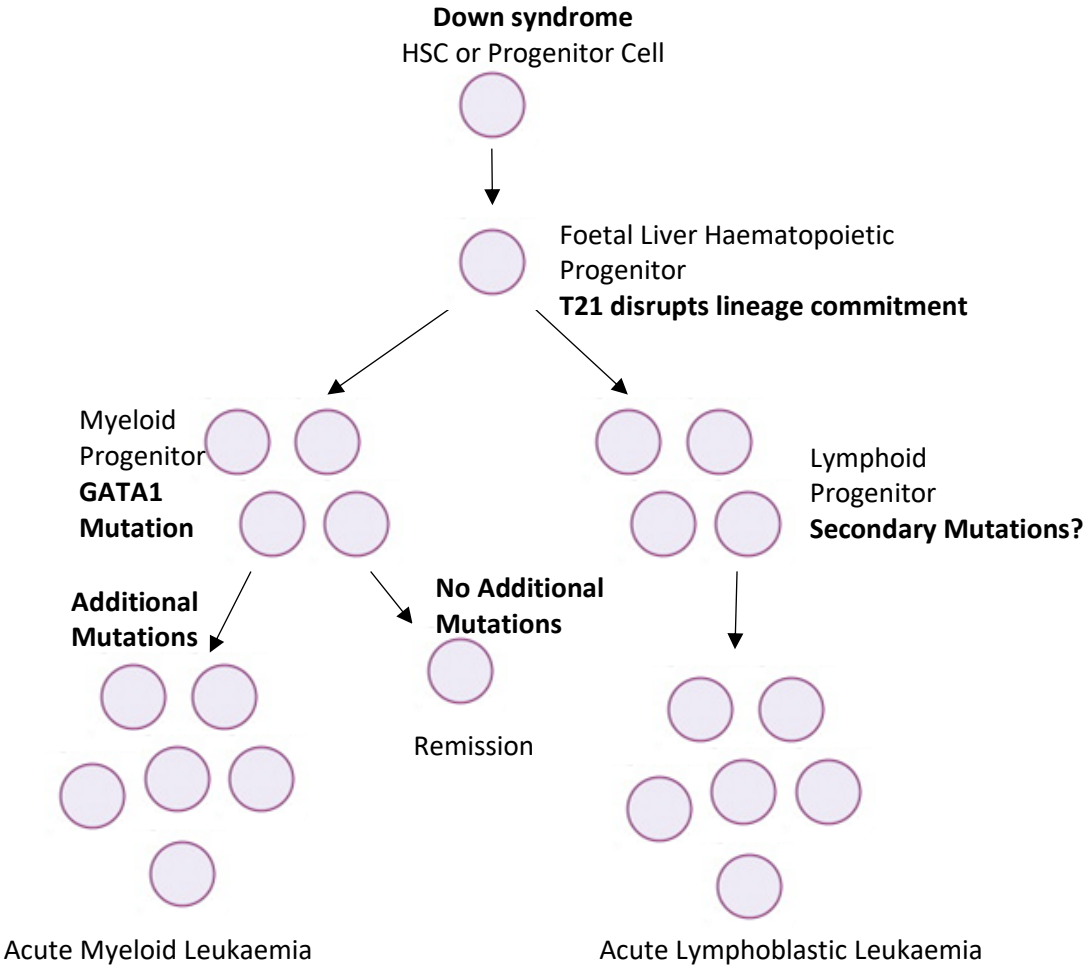


Figure 1. 8: The proposed cancer stem cell hierarchy observed in DS leukaemias, highlighting the primitive multipotent progenitor compartment in which T21 disrupts lineage commitment (Adapted from Mulligan 2008)

The development of TMD in-utero suggests impairment of lineage resolution in a foetal multipotent haematopoietic progenitor compartment results in the increased predisposition to leukaemic onset that is observed in children with DS (Tunstall-Pedoe et al., 2008). The potential of iPSCs to recapitulate early foetal haematopoiesis and model the effect of primary oncogenic mutations on early B-cell development uniquely positions the iPSC system as a tool to dissect the role of T21 on the MkE bias and impaired B-cell potential observed in DS foetal liver (Boeirs et al., 2018).

Chapter 2

Materials and Methods

2 Chapter 2: Materials and Methods

2.1 Tissue Culture Protocols

2.1.1 Mouse Embryonic Fibroblasts

Irradiated mouse embryonic fibroblasts (MEFs) were purchased from ThermoFisher Scientific (4×10^6 cells per vial).

T175 flasks were coated overnight with 0.1% bovine gelatin, diluted in PBS, in preparation for MEF plating. Thawed MEFS were washed in 5ml MEF medium (DMEM, 10% FBS, 2mM L-Glutamine, 1X penicillin-streptomycin) and 1.3×10^6 MEFS were plated into individual T175 flasks and expanded to 90% confluency. 9.6×10^5 MEFS are required per 6-well plate and so 1.08×10^6 MEFs were frozen in 10% DMSO + 90% FBS in order to account for 10% loss of viable cells upon thaw.

2.1.2 Human Induced Pluripotent Stem Cells

Down's syndrome derived induced pluripotent stem cell lines (named DS1 and DS2) were kindly provided by Professor Mitchell Weiss, Children's Hospital of Philadelphia. DS2 was generated from non-malignant T21 peripheral blood mononuclear cells from an 8 day old female with transient myeloproliferative disorder and DS1 from T21 male fibroblasts. Both iPS cell lines were generated using a single polycistronic lentivirus encoding the four reprogramming factors OCT4, SOX2, MYC and KLF4.

Miff3 iPS cell line was derived from foreskin fibroblasts and the reprogramming factors transiently expressed as mRNA.

Isogenic Down syndrome derived human induced pluripotent stem cells used were Boston Children Hospital Stem Cell Line #1469, 1470 (trisomic clones) and #1474 (disomic clone). These iPSC lines were retorvirally derived from fibroblasts in the lab of Professor George Q Daley (Boston Children's Hospital Stem Cell Program, Coriell Institute for Medical Research and NIGMS Human Genetic Cell Repository). See appendices for karyotype reports.

2.1.3 Human Pluripotent Stem Cell Culture

2.1.3.1 Thawing of human induced pluripotent stem cells on MEF

Single vials of hIPSCs for each of #1469, #1470 and #1474 were removed from liquid nitrogen and thawed in a 37°C water bath until a small frozen aliquot of cells remained. 1ml of human embryonic stem cell medium (hESC) (outlined in table 2.1 below) supplemented with the 10µM Rho-associated kinase inhibitor (ROCK) Y27632 (Cambridge Biotechnologies) was carefully added to the thawed cells, gently resuspended and transferred to a corresponding labelled 15ml falcon tube. 7ml of hESC medium supplemented with ROCK inhibitor was added to each sample to dilute the DMSO and samples were centrifuged at 300g for 5 minutes at room temperature. All media was removed, cells resuspended in 1ml of hESC supplemented with ROCK inhibitor medium and transferred to individual wells in a 6-well plate. The plate was gently agitated to achieve an even distribution of cells and transferred to the tissue culture incubator.

Human ESC Media	
80%	**Knockout DMEM**
20%	**GIBCO knockout SR**
1%	**Non-essential amino acid solution**
1mM	**L-glutamine**
0.1mM	**2-mercaptoethanol**
4ng/ml	**human bFGF.**

Table 2.1: Constituents of human ESC media for the thawing and maintenance of iPSC on MEFs.

2.1.3.2 Culturing of hIPSC on MEFs

Due to the low viability of iPS cells upon thaw, 15-20 hours post plating overnight media was removed and transferred to a new 6-well plate containing MEFs and fresh hESC media added to the thawed samples.

Upon identification of large distinguishable iPSC colonies all media was aspirated and 0.5ml of collagenase 1mg/ml added and samples incubated for 5-10 minutes until iPSC colony edges appeared to detach along the edges. Colonies were gently scraped using a cell scraper and all media transferred to a 15ml falcon, 5ml hES media added to each sample and centrifuged at 300g for 5 minutes. Samples were gently resuspended in 1ml hES medium and transferred onto newly thawed MEFs and subsequently expanded and passaged onto matrigel/mTeSR.

2.1.3.3 Culturing of hIPSC on matrigel/StemFit or mTeSR

hIPSCs were cultured on matrigel (Corning) coated 6-well plates with the feeder-free medium mTeSR (Stem Cell Technologies).

Matrigel was prepared for 2 x 6-well plates by resuspending matrigel stock aliquots (~0.15ml – dilution factor as per manufactures guidelines) in 12.5ml in chilled DMEM/F-12. 6-well tissue culture plates were coated with 1ml of resuspended matrigel per well and were brought to 37°C by 1 hour incubation, or secured with parafilm and stored at 4°C for 5 days, DMEM/F-12 media was aspirated prior to plating cells. mTeSR was prepared as per manufactures instructions and 45ml aliquots stored in -80°C prior to the addition of 100X penicillin/streptomycin (Gibco).

To passage iPS cells media was aspirated and incubated for 10 seconds in 0.5ml of the enzyme free dissociation reagent ReLEsR and further incubated at 37°C for 5 minutes until colonies began to slightly dissociate. Cells were resuspended in 1ml of mTeSR in the presence of 10μM Y-27632 and diluted 1:10 – 1:50 into a 6-well plate containing 1.5ml mTeSR supplemented with ROCK inhibitor. 15-20 hours post passage media was aspirated and 1.5ml mTeSR was added. mTeSR media was replenished on a daily basis due to the instability of b-FGF and TGFβ.

2.1.3.4 Single Cell Dissociation of hIPSC

To increase viability of iPSC single cell dissociation, iPS cells were incubated with mTeSR supplemented with ROCK inhibitor for two hours. 0.5ml of ReLEsr was added to individual wells for 15-20 seconds and cells incubated at 37°C for 10 minutes or until colonies were largely dissociated. 1ml of mTeSR supplemented with ROCK inhibitor was used to vigorously resuspend cells and cells diluted 1:10 – 1:100 to corresponding matrigel coated culture plates.

2.1.3.5 Karyotyping

To induce mitotic arrest 2 wells containing cells 3-5 days post passage were cultured in mTeSR supplemented with 0.1ml colcemide 2μg/ml overnight. Media and suspension cells were collected in

a labelled 15ml falcon and 0.25% trypsin/EDTA added to the adherent cells and incubated at 37°C for 8 minutes to ensure complete dissociation. Samples were resuspended and transferred to the above 15ml falcon, 8ml of 37°C 0.0375M potassium chloride/distilled H_2O slowly added and centrifuged at 300g for 5 minutes. Supernatant was removed and the cell pellet vigorously resuspended prior to fixation in 4ml 3:1 methanol-to-acetic acid while agitating cells. Samples were centrifuged at 300g for 8 minutes, supernatant removed, cells resuspended in 0.5ml of supernatant and the fixative procedure repeated twice more. Samples were stored at -20°C. G-banding was completed by Duncan Baker (Sheffield Diagnostic Centre) and by Dr. Thorsten Schlaeger (Boston Children's Hospital).

2.1.3.6 hiPSC Colony Picking and Freezing

Single cell clones were grown until day 8 of culture or sizeable colonies were formed. Colonies were picked under microscopic guidance, plated into individual wells of a 24-well plate containing StemFit or mTeSR supplemented with ROCK inhibitor and these clones further expanded into 2 wells of 6 well plates. One well of each clone was expanded for screening by DNA FISH and the other well frozen in 90% knock out serum replacement (Invitrogen) and 10% DMSO (Sigma) supplemented with ROCK inhibitor.

2.1.4 In-vitro B-cell Differentiation of hiPSC

In-vitro B-cell differentiation of human iPSCs was completed as described by Richardson et al., 2020.

2.1.4.1 OP9 Stromal Cell Maintenance

OP9 stroma is a macrophage colony stimulating factor-deficient bone marrow stromal cell line derived from mouse that facilitates the generation of CD34+/43+ haematopoietic progenitors. OP9 stroma were cultured 1 : 6 – 1 : 8 in OP9-M (αMEM powder (Invitrogen), 20% Defined FBS batch

tested (Gibco) , 100μM monothioglycerol [MTG] (Sigma), 2.5ml 1X penicillin streptomycin) on 10cm tissue culture plates (BD) that were coated overnight at 4°C with 0.1% bovine gelatin (Sigma) dilute in PBS. During routine culture OP9 cells were passaged every 4 days to a level of 70% confluency and adiposity < 20%. To passage OP9 cells were washed twice with dPBS and 5ml 0.05% trypsin (diluted in dPBS and 0.5mM EDTA) added and cells incubated at 37°C for 5 minutes or until cells detach upon gentle agitation. Trypsin was neutralised and detached cells were resuspended in OP9- maintenance media and transferred to a 50ml falcon tube and centrifuged at 300g for 5 minutes. Cells were resuspended in OP9 maintenance media and evenly distributed 1:6 onto pre gelatinised 10cm plates. OP9 stroma were overgrown to days 7-10 in OP9 maintenance media prior to co-culture with iPSCs.

2.1.4.2 Harvesting hiPSC for OP9 Culture

Approximately 1 x 10^6 iPSCs were detached using 1mg/ml collagenase diluted in DMEM/F-12 for 10-90 minutes at 37°C and gently resuspended in 4ml OP9-D media (αMEM liquid (Invitrogen), 10% Defined FBS batch tested (Gibco), 1000X MTG and 1X penicillin streptomycin) using a 10ml stripette. Samples were carefully transferred to a 50ml falcon tube, washed twice in 10ml OP9-D media, cells allowed to sit by gravity and carefully plated onto an overgrown OP9 10cm dish containing 10ml OP9-D media. A complete media change with 20ml OP9-D media was carefully completed on day 1 not to disturb any colonies that have attached. On days 4, 6 and 8 of the OP9 co-culture, 10 ml of media was carefully removed and a fresh 10ml OP9-D media added.

2.1.4.3 OP9-iPSC CD34+ Enrichment

hiPSC OP9 co-culture colonies were gown to day 10 of culture. On day 10 all media was removed and stored in 50ml falcon tubes to capture cells in suspension. The OP9-iPSC layers digested with 7ml collagenase 1mg/ml in DMEM/F-12 media for 25 minutes and removed and 0.05% trypsin/0.5M EDTA

for a further 7 minutes until breaking up of the monolayer upon gentle agitation. Trypsin was neutralised with 7ml of OP9-D media and cells gently swirled in the 10cm dish and triturated first using a 10ml stripette and then a 1ml pipette. Once the OP9 layer was digested cells were strained through a pre-wetted 40μM filter (BD Falcon) into the 50ml falcon tube containing the previously removed suspension media mentioned above. The culture plates are then washed in 5ml of MACS buffer (dPBS, 10% FBS and 0.5M EDTA) and filtered into a corresponding sample tube and the filter similarly washed with 2x1ml of MACS buffer to remove residual sample. Samples are centrifuged at 300g for 5 minutes at 4°C.

2.1.4.4 Magnetic Enrichment of CD34+ haematopoietic progenitors

After centrifugation the cell pellet was resuspended in 300μl of MACS buffer, 100μl Fc block and 100μl of CD34+ beads (Miltenyi Biotech) and incubated at 4°C for 30 minutes. The MACS stand is assembled by fitting the LS MACS column to the magnet and a 30μM filter placed on top of the column, wash through sample can be collected in 15 ml falcon tubes fixed below the LS column. Samples were washed in 5ml MACS buffer and centrifuged at 300g for 5 minutes before resuspension in 500μl of MACS buffer and passed through a pre-wetted 30μM filter (Falcon) into a CD34+ MACS LS enrichment column (Miltenyi Biotech). Sample tubes were washed 3 times in in 3ml of MACS buffer and passed through the 30μM filter after each wash. The 30μM filter and LS column were also washed 3 times independently. 5ml of MACS buffer was added to the LS column and the column removed and the sample rapidly plunged into a 15ml falcon and centrifuged at 300g for 5 minutes. The cell pellet was resuspended in 1ml OP9-D media and 10μl of sample diluted 1:2 with trypan blue and CD34+ cells identified as small, bright and round cells, counted using a haemacytometer, large dark stromal cells were ignored.

2.1.4.5 MS-5 Stromal Cell Maintenance

MS5 stroma were cultured 1:5 - 1:10 using MS5 maintenance media (αMEM liquid (Invitrogen), 10% Defined FBS batch tested (Gibco), 1X penicillin/streptomycin) in T75 flasks pre-gelatinised with porcine gelatin (Sigma) (0.5g porcine gelatin and 500ml dPBS autoclaved twice) for >2 hours at 37 °C. 3 days prior to seeding of hiPSC derived CD34+ enriched cells, 2 x 10^4 MS5 cells were seeded onto each well of a pregelatinized 6 well plate. 3 - 5 x 10^4 CD34+ hiPSC derived cells were seeded onto individual wells using OP9-D 1X cytokine media (OP9-D media containing IL3 10ng/ml, IL7 20ng/ml, Flt3L 50ng/ml and SCF 50ng/ml) (Peprotech). On day 7, 2ml of 1X cytokine media minus IL3 was added and on day 14 2ml of OP9-D 1X cytokine media without IL3 was removed and replenished onto each well. Cell culture media was removed between days 17-21 of the MS-5 co-culture and MS5 stromal layer digested by tituration with 2ml of MACS buffer and passed through 40μm filter into a corresponding 50ml falcon tube.

2.1.4.6 Flow Cytometric Analysis

Samples were spun at 300g 4°C for 5 minutes, supernatant was removed. The cell pellet was resuspended to single cell suspension with monoclonal antibodies mix diluted in MACS buffer (outlined table 2.2) and Fc receptor block diluted 1:5 with MACS buffer added to the sample-antibody mix. Samples were incubated at 4°C for 30 minutes then washed in 5ml of MACS buffer and centrifuged at 300g for 5 minutes before addition of near-infrared viability dye. Samples were stored on ice and FACS analysed.

The LM Progenitor was identified as $CD45^+$ $CD34^+$ $CD38^-$ $CD33^{hi/+}$ $CD45Ra^{hi/+}$, ProB cells $CD45^+$ $CD34^+$ $CD19^+$, PreB $CD45^+$ $CD34^-$ $CD19^+$, IL7R progenitor $CD45^+$ $CD34^+$ $CD19^-$ $CD45Ra^+$ $IL7R^+$. Cell sorting was completed with the assistance of the flow cytometry core using the BD FACSAria IIIB cell sorter and the Diva software.

Antibody	Fluorophor	Clone	Assay
CD19	APC	HIB19	iPS Modelling and FL Analysis
CD34	PeCy7	581	iPS Modelling and FL Analysis
CD45	AF700	HI30	iPS Modelling and FL Analysis
CD45Ra	FITC	HI100	iPS Modelling and FL Analysis
CD127	BV421	HIL-7R-M21 / AO19D5	iPS Modelling and FL Analysis
CD38	BV785	HIT2	iPS Modelling and FL Analysis
CD90	PE	5E10	iPS Modelling and FL Analysis
CD33	PeCy5	WM53	iPS Modelling and FL Analysis
CRLF2	PE	1B4	iPS Modelling and FL Analysis
Near-Infrared (Viability)		Ref L10119	iPS Modelling and FL Analysis

Table 2. 2: Antibodies with their corresponding clone ID used for characterisation of B-cell differentiations and foetal liver analysis.

2.1.5 In-vitro Myeloid Differentiations

2.1.5.1 Semi-Solid Methylcellulose-based differentiation of iPSCs

$CD34^+$ $CD19^-$, LM, pro and preB cells derived from DS-iPSCs through the above described B-cell differentiation protocol were sorted into 1.5ml eppendorf tube containing 1ml of methocult H4435 (Stem Cell Technologies) which had been previously brought to room temperature. Samples were briefly vortexed and allowed to sit until all air bubbles had risen. 1ml of methocult-cell solution was gently plated onto each well of a 24 well tissue culture plate (Corning) . Samples were incubated for 7-14 days colony forming potential evaluated.

2.1.5.2 Extended Liquid Culture on Terasaki Plates

The myeloid potential of progenitor populations sorted on day 29 of the B-cell differentiation was tested using 60-well non adherent Terasaki plates. $CD34^+$ $CD45^+$ $CD19^-$, LM, pro and preB populations were sorted into 1ml Eppendorf tubes containing IMDM (Invitrogen) + 20% batch tested FBS (Gibco) + 1% penicillin/streptomycin + 1% L-Glutamine supplemented with M-CSF, GM-CSF, G-CSF, IL-3, TPO and SCF (Peprotech) as outlined in table 2.3. Samples were centrifuged at 300g for 3 minutes and resuspended in 20µl IMDM media supplemented with cytokines listed in table 2.3. Half feeds of the described media were completed on day 7 and 14 of culture and colonies were scored and harvested for cytospin and morphological evaluation on day 18 of myeloid culture.

Cytokine	Final Concentration
M-CSF	25ng/ml
GM-CSF	20ng/ml
G-CSF	50ng/ml
IL-3	50ng/ml
TPO	50ng/ml
SCF	50ng/ml

Table 2. 3: Cytokines and the corresponding concentration used for extended terasaki liquid culture

2.1.5.3 Fixing Cells for Cytospin Evaluation

Samples were centrifuged at 300g for 5 minutes and resuspended in PBS supplemented with 20% FBS. Slides were labelled accordingly and assembled with the slide holder, funnel and filter. The filter was pre-wetted with PBS and the slide allowed to air dry. Samples were added to a corresponding funnel and centrifuged at 500g for 5 minutes with minimum acceleration setting selected. Slides were air dried overnight. Slides were immersed in May-Grunwald stain (VWR International) for 5 minutes,

washed twice with PBS, allowed to air dry and further stained with newly made 1 : 3 Giemsa stain diluted in sterile water (VWR International) for 15 minutes. Slides were washed in water for 5 minutes and allowed to air dry for one hour. Coverslips were then mounted and sealed.

2.2 Lentivirus procedures

2.2.1.1 Lentiviral construct generation

IL7Rα12bp ins and CRLF2 cDNA were originally cloned into the pRRL plasmid kindly gifted by Shai Izraeli. IL7Rα12bp ins and CRLF2 cDNA were PCR amplified using Q5 High-Fidelity DNA polymerase (NEB) and PCR primers containing BglII and SpeI recognition sites at 5' and 3' ends respectively. Reaction setup was completed on ice prior to denaturation on a thermocycler preheated to 98°C. Reaction setup and conditions used are outlined in table 2.4 and 2.5 below.

Input DNA	1µl
Nuclease free Water	22.5µl
Forward primer @ 10M (20X)	2.5µl
Reverse primer @ 10µM (20X)	2.5µl
dNTP 10mM (50X)	1µl
5x Q5 Buffer	10µl
5x Q5 high GC enhancer	10µl
NEB Q5 hot-start high fidelity Taq polymerase	0.5µl

Table 2.4; PCR reaction setup to amplify CRLF2 and IL7Rα12bp ins cDNA from the pRRL plasmid.

Thermocycling conditions used were;

Step	Temperature	Time
Denaturation	98°C	30 seconds
Cycled 34 times	98°C	10 seconds
	65°C	30 seconds
	72°C	2 minutes
Extension	72°C	2 minutes
Hold	4°C	

Table 2.5; PCR reaction conditions used to amplify CRLF2 and IL7Rα12bp ins cDNA from the pRRL plasmid.

IL7Rα12bp ins and CRLF2 PCR products were purified using the QIAquick PCR purification kit (Qiagen), run on a 0.8% agarose gel and the gel product excised and cleaned up using the GFX PCR DNA gel band purification kit (Cytvia). CRLF2 and IL7Rα12bp ins inserts were prepared by digestion with BglII and SpeI to remove additional overhangs, run on a 0.8% agarose gel and gel excised and purified as above. The CEI vector was prepared by digestion with BglII and SpeI restriction enzymes and dephosphorylated using calf intestinal phosphatase (New England Biolabs). Digested CEI vector was run on a 0.8% agarose gel and purified with the GFX PCR DNA gel band purification kit. Ligation reaction using T4 DNA liagase (New England Biolabs) for prepared CEI vector and CRLF2 and IL7Rα12bp ins was completed using an insert : vector ratio 10 : 1 and the reaction setup completed as described in table 2.6 below. Samples were gently mixed by pipetting, incubated at room temperature for 10 minutes and heat inactivated at 65°C for 10 minutes. Ligation reactions were transformed in competent bacterial cells as described in section 2.2.1.2 below.

DNA Vector (12ng)	4.5
DNA Insert	0.5
Nuclease free water	3.5
10X T4 DNA ligase	1
T4 DNA ligase	0.5
Final volume	10

Table 2.6; T4 DNA ligase reaction set up for CEI vector and CRLF2 and IL7Rα12bp ins inserts.

Transformed clones were digested with SpeI and BglII to ensure successful ligation and underwent sanger sequencing at Source Bioscience to ensure integrity and correct insert orientation of the constructs.

2.2.1.2 Bacterial transformation

C2987H competent bacterial cells (New England Biolabs) were thawed on ice for 10 minutes and gently mixed by pipetting. 20ng of plasmid DNA was added to 5µl of competent cells, samples gently mixed by flicking and placed on ice for 30 minutes. Samples were incubated at 42°C for 30 seconds and placed on ice for 5 minutes. 950µl SOC media was added to the mixture and incubated on a thermomixer at 37°C for 60 minutes at 250rpm. Samples were mixed by inversion and 10-fold serial dilutions added and spread onto pre-warmed luria broth (LB) selection plates containing 100µg ampicillin. Selection plates were incubated overnight at 37°C. Individual colonies were picked and incubated in 5ml LB broth for 2 hours at 37°C shaking at 220rpm prior to incubation for 18 hours at 37°C 220rpm in 200ml LB broth supplemented with 100µg ampicillin. Bacterial cultures were

centrifuged at 3000g for 20 minutes at 4°C and DNA harvested using the Qiagen Plasmid Maxi Kit. DNA was quantified using a nanodrop.

2.2.1.3 Lentiviral preparation

Lentivirus was prepared in a CAT 3 containment tissue culture hood.

Plasmid mix was prepared consisting of 3µg CEI lentiviral plasmid, 2µg psPAX2 and 1µg pMD2G packaging plasmids in OPTI-MEM to a volume of 105µl per T175 flask. 70µl Fugene transfection reagent was added to 460µl Opti-MEM and plasmid mix added to incubate for 30 minutes at room temperature.

HEK293T cells were grown to 80-90% confluency in T175 flasks. Media was changed with 20ml fresh media (DMEM, 10% FBS, penicillin/streptomycin) and added 580µl of lentiviral reagent mix was added to each T175 flask containing HEK293T. 18-24 hours later media was removed and 20ml fresh media added. 72 hours post transfection media was harvested and transferred into 50ml falcon tubes and centrifuged at 4000g for 5 minutes to remove cell debris. Supernatant was passed through a 0.45um filter into ultracentrifugation tubes (Themo-Nalgene) and centrifuged at 50,000g for 150 minutes at 4°C in an Avanti J26-XPI centrifuge. Supernatant was carefully discarded and pellets resuspended 150ul of mTeSR media and aliquoted in 30ul aliquots. Viral preparations were stored at -80°C.

2.2.1.4 Lentivirus titre

Lentiviral preparations were titred on HEK293T. 2 x 10^5 HEK293T cells were plated in 24-well plate format and incubated overnight. The next morning 6 viral dilutions were prepared (2ul, 1ul, 1:10, 1:100, 1:1,000 and 10,000 fold dilution) and added to individual wells. 72 hours later cells were

harvested and GFP positivity determined by FACS analysis on live cells. Viral titre was calculated using the following formula; viral particles/ml = (N x P)/(V x D), N = cell number per well, P = percentage GFP+ cells, V = virus volume used per well, D = Dilution fold of virus. Lentiviral plasmids used in this study will be referred to from here on as CEI for control empty plasmid and CEI_IL7Rα12 base pair insert within exon 6 of the transmembrane domain and CEI_CRLF2_IL7Ra12 base pair insert will be referred to as IL7Ra12bp ins and CLRF2_IL7Ra12bp ins respectively.

2.2.1.5 Lentiviral transduction of human DS-iPSCs

Human DS-iPSCs were transduced overnight in 750ul mTeSR media supplemented with 2µl of virus. 48 hours post infection all media was removed and 1.5ml fresh media added. Cells were maintained in culture as described in section 2.1.3.3.

2.2.1.6 Lentiviral transduction of haemato-endothelial CD34+ progenitor cells

DS-iPSCs were differentiated on OP9 stroma for 10 days to derived CD34+ haemato-endothelial progenitors as described in methods section 2.1.4.2. Samples were MACS enriched for CD34+ progenitor cells as outlined in section 2.1.4.3 and 2.1.4.4. 50,000 CD34+ cells were plated in 24-well tissue culture plates (Corning) and infected with CEI control, CRLF2_IL7Rα12 bp ins or IL7Rα12 bp ins lentivirus at an MOI of 50-100 with 4ug/ml polybrene in 2ml of OP9D media for 3 hours. Cells were then harvested and washed three times with OP9D media and plated onto MS-5 stroma as described in section 2.1.4.5 for downstream B-cell analysis.

2.2.1.7 Fixation and permeabilsation for intracellular flow cytometry

CEI and CRLF2_IL7Rα12bp ins transduced DS-iPSCs were dissociated to single cell using ReLeSR (stem cell technologies) as described in section 2.1.3.4, washed in MACS buffer and centrifuged

at 300g at 4°C for 5 minutes. Samples were stained with antibody cocktail listed in table 2.7 below for 30 minutes at 4°C and washed in MACS buffer by centrifugation at 300g at 4°C. DS-iPSCs were permeabilised and fixed using the ThermoFisher Scientific eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set, described herein. Samples were resuspended in intracellular fixation buffer, vortexed briefly and incubated at 4°C for 30 minutes. To permeabilise the cell membrane cells were washed in 1X permeabilization buffer and centrifuged at 300g at 4°C for 5 minutes prior to staining with intracellular antibody mix described in table 2.7 at 4°C for 30 minutes. Samples were washed twice in permeabilization buffer by centrifugation at 300g at 4°C for 5 minutes and subsequently stained in near infrared viability dye (ThermoFisher Scientific) prior to analysis by flow cytometry.

Antibody	Fluorophor	Clone	Assay
CD127	BV421	HIL-7R-M21 / AO19D5	iPS Modelling and FL Analysis
CRLF2	PE	1B4	iPS Modelling and FL Analysis
Near-Infrared (Viability)		Ref L10119	iPS Modelling and FL Analysis

Table 2.7: Antibodies with corresponding fluorophore and clone ID used for intracellular detection of IL7Rα and CRLF2.

2.3 Molecular Biology

2.3.1.1 DS-iPSC Isogenic Subclone Screening

1) qPCR

qPCR was performed in triplicate wells using 20ng of genomic DNA. The PCR recipe and the sequences for forward and reverse primers for Down syndrome critical region3 (DSCR3) and GAPDH along with the Taqman probes designed to span within the DSCR3 and GAPDH genes were taken from *Hu et al., 2004*. The PCR conditions used were; 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

The PCR recipe used is listed below all volumes are in μl.

Taqman Master Mix	12.5
Forward Primer (900nM)	2.5
Reverse Primer (900nM)	2.5
Taqman Probe (200nM)	2.5

The sequences of forward and reverse primers;

DSCR3; *Forward* 5'-TAGCAAGTCAGAGGTTTCCTT-3'

Reverse 5'-AACATT GACTGTGGCTCTTGC-3'

Probe 6-FAM-5'-CACTCTCCAGCAAC GTCAAACTGCTGTCACA-3'-TAMRA

GAPDH; *Forward* 5'-CTGTCCAGTTAATTTCTGACC-3'

Reverse 5'-CTTTGTACATGGTATTCACCAC-3'

Probe VIC-5'- GTGCAG CTGAGCTAGG CAGCAGCA GCAAGCA- TT-3'-TAMRA

2) DNA FISH

DS-iPSCs were harvested by scraping and centrifuged at 0.4g for 7 minutes. The AML1 probe (Cytocell) and sample slides were pre-heated to 37°C Samples were washed in cold methanol acetic acid (1:3), centrifuged at 0.4g for 7 minutes. Residual methanol acetic acid was removed and the sample gently agitated. A small aliquot of sample was added dropwise from a height onto a slide. Slides were washed twice in saline-sodium citrate buffer (SSC) for 5 minutes and then dehydrated through room temperature ethanol series of 70%, 85% and 90% and allowed to air dry between each step. The prewarmed slide was placed on the hybrite at 37°C and the AML1 probe added to the sample surface area. A 22 x 22 cover slip was carefully placed on the sample area and gently sealed with fixogum.

Both sample and probe were denatured simultaneously by heating to 75°C for precisely 2 minutes. Sample slides were cooled to room temperature by removal from the hybrite and placing on the lab bench for 15-30 seconds. To hybridise the sample and probe, samples were maintained overnight at 37°C in a humid environment. The coverslip was then removed and the slides washed in 0.4 X SSC at 72°C for 2 minutes. Sample slides were drained and further washed in 2 X SSC/0.1% IGEPAL (Sigma) for 30 seconds. Slides were drained, 10µl of DAPI (Cytocell) applied and a 22 x 50 coverslip applied and fixed.

2.3.1.2 q-RT-PCR Taqman Gene Expression Assay

CRLF2 and IL7Rα transduced iPS cells were harvested and centrifuged at 300g for 5 minutes, washed twice in dPBS and 800µl of Trizol added. RNA was extracted as described in section 2.2.1.4. RNA was reverse transcribed into cDNA using superscript III reverse transcriptase (Invitrogen) as described in section 2.2.1.3.

qPCR was performed on Applied Biosystems 7500 FAST Real-Time PCR System using a standard run and a 96-well fast reaction plate. qPCR was performed in triplicate on 100ng of cDNA using the Taqman gene expression assay. Reaction mix was set up as described in table 2.7 below. Cycling conditions used are listed in table 2.8 below.

Taqman probes used:

IL7Rα ID; Hs00233682_m1, Exon boundary 4-5

CRLF2 ID; Hs00845692_m1, Exon boundary 2-3

GAPDH ID; Hs99999905_m1, Exon boundary 2-3

HPRT1 ID; Hs99999909_m1, Exon boundary 6-7

qPCR Component	**Single reaction**	**Three replicates**
cDNA template (100ng)	1µl	3µl
Nuclease free water	5µl	15µ’
20X Taqman gene expression assay	1µl	3µl
2X Taqman gene expression mastermix	10µl	30µl

Table 2.7; TaqMan gene expression components used for detection of CRLF2 and IL7Rα expression in transduced isogenic DS-iPSCs.

Step	Temperature	Time
Hold	50°C	2 minutes
Hold	95°C	20 seconds
Hold (42 cycles)	95°C	3 seconds
	50°C	30 seconds

Table 2.8; qPCR reaction conditions used for detection of CRLF2 and IL7Rα expression in transduced isogenic DS-iPSCs.

2.3.1.3 cDNA Preparation

cDNA was prepared from 100ng of RNA for each sample. 50ng of random primers and 1µl of 10mM dNTPmix was added to 100ng of RNA. Samples were incubated at 65°C for 5 minutes and then incubated on ice for 1 minute. Samples were collected by brief centrifugation and 4µl 5X First-Strand Buffer, 1µl 0.1M DTT, 1µl of RNaseOUT and 1µl of SuperScript III RT added to each sample. The samples were gently mixed by pipetting and incubated at 25°C for 5 minutes. The reaction temperature was increased to 50°C for 60 minute. The reaction was terminated by incubation at 70°C for 15 minutes.

2.3.1.4 RNA Isolation

RNA isolation for RT-qPCR was performed using an RNeasy kit (Qiagen) as described in manufactures protocol.

RNA isolation from FACS sorted populations was performed as follows.

50-150 pro and preB and 5,000 – 10,000 LM cells were sorted into 800µl of Trizol, quickly vortexed and immediately stored at -80°C. After thawing 200µl of chloform was added and total RNA extracted

from the aqueous phase and precipitated by mixing an equal volume of isopropanol supplemented with 5µg of linear polyacrylamide (Sigma). RNA was pelleted by centrifugation at 16g for 15 minutes and then washed twice with 80% ethanol. The RNA pellet was resuspended in 5µl of sterile water by shaking at 42°C for 5 minutes. 1µl RNA was quantified on an Agilent Bioanalyser RNA 6000 Pico chip. 100pg of RNA was lysed in reaction buffer (0.2% Triton, RNaseOUT) that was supplemented with 12µM oligo-dt30VN at 72°C minutes. RNA was reverse transcribed into cDNA using the SMARTScribe RT kit (Takara).

2.3.1.5 cDNA preparation from low cell number

cDNA was prepared using the SeqAMp polymerase kit (Takara) with 16 cycles at thermal conditions used as per manufactures recommendations. cDNA was purified using Agencourt AMPreXP beads (0.8-1 beads to cDNA ratio used). cDNA was washed twice with 80% fresh ethanol and eluted in 15µl of elution buffer. 1µl of cDNA was quality checked using an Agilent Bioanalyser high sensitivity DNA chip. Sequencing libraries were produced using the Nextera XT DNA preparation kit (Illumina) using a modified version of the protocol with a 20-fold final volume reduction. 50picogram of cDNA was tagmented followed by 13 samples of amplification. Sequencing libraries were pooled using equimolar amounts using the Echo 525 liquid handler (Labcyte). Library pools were purified using 0.8X Agencourt AMPureXP magnetic beads and eluted in 25µl of elution buffer. cDNA was quantified on a Agilent Bioanalyser high sensitivity DNA chip. The cDNA pool was diluted to a final concentration of 2pm and sequenced using the Illumina NextSeq 500 platform using the NExtSeq500/500 High Output Kit v2.5 incorporating 2 x 76 paired end cycles. RNA sequencing was kindly performed by Elitza Deltcheva.

2.3.1.6 Bioinformatic Analysis

Sequence libraries were aligned on the genome assembly GRCh38 and assessed for quality control using the nf-core RNAseq pipeline (Ewels *et al.*, 2019). Samples were assessed using quality control metrics and those that failed to pass were excluded from further analysis. For downstream analysis the featureCounts software package was used to generate counts using the Ensemble reference genes. Differential gene expression analysis was performed using the DeSeq2 package (Love, Huber and Anders, 2014). Differential genes in each of the populations that underwent sequencing were termed trisomy 21 vs disomy 21 using a design model termed ''~cell_population+chr21_status''. To generate ranked lists for gene set enrichment analysis (GSEA) genes were ordered according to the 'STAT' output of DeSeq2 and each value was assigned an Entrez ID. Gene lists for the GSEA analysis were derived from the molecular signature database (MsigDB) for hallmark and C1 positional analysis, published genetic signatures (Laurenti et al,. 2013), or from manually formed lists (chromosome lists) (Subramanian *et al.*, 2005). GSEA analysis was performed using the fgsea package in R (Korotkevich, Sukhov and Sergushichev, 2016). clusterProfiler and ggplot2 were used to generate all plot (Hadley Wickham, et al,. 2016; Yu *et al.*, 2012). Bioinformatic analysis was kindly performed by Jason Wray.

2.3.1.7 Statistical analysis

Statistical analysis was performed using a non-parametric Kruskall-Wallis test for multiple comparisons. All data is displayed as mean values with standard deviation unless stated otherwise.

Chapter 3

In vitro B-cell Differentiation of Down syndrome Induced Pluripotent Stem Cells

3 Chapter 3

3.1 In vitro B cell Differentiation of Down syndrome Induced Pluripotent Stem Cells

3.1.1 Introduction

The role of trisomy 21 in embryonic, childhood, and adult development and haematopoiesis is well documented, however the precise mechanism by which T21 perturbs foetal haematopoiesis resulting in an increased predisposition to pre-leukaemic and leukaemic development is unknown. Although current mouse models of Down syndrome do recapitulate some of the phenotypes observed within DS patients, such as craniofacial abnormalities and cardiac septation and have also provided insights into genotype-phenotype relationships along with identifying candidate markers for leukaemic transformation they do not recapitulate the haematopoietic skewing observed in DS patients (Webb *et al.*, 1999; Olson *et al.*, 2007). This warrants the need to develop a human system that accurately mimics DS human haematopoiesis, with physiologically relevant chromosome 21 copy numbers and which facilitates immunophenotypic identification of immature progenitor populations suspected to be leukaemic precursors.

Examination of T21 foetal liver vs normal euploid controls identified a decrease in the frequency of prepro and proB cells in conjunction with HSC and MEP expansion (Roy et al,. 2012). Clonal analysis of monochorionic twins in which one twin developed B-ALL and the other expressed the pre-leukaemic lesion ETV6-RUNX1, but did not develop full blown leukaemia, revealed that the initiating ETV6-RUNX1 hit occurred in-utero with postnatal acquisition of secondary hits, suspected to be induced by RAG activity, resulting in leukaemic transformation (Hong et al,. 2008). The decreased incidence of haematological malignancies in adult DS patients and an increased incidence of

leukaemia in DS children vs non-DS children led me to hypothesise that leukaemic predisposition in DS children similarly initiates in-utero due to a genomic imbalance caused by T21 and that this could be recaptured using a human DS-iPSC model.

Derivation of immature B cells from human ESCs was first described by Vodyanik et al,. using a co-culture system of OP9 and MS5 stromal cells and the potential of iPSCs to produce lymphopoiesis subsequently demonstrated (Vodyanik *et al.*, 2005; Carpenter *et al.*, 2011). Further characterisation of the system has also shown that iPSCs do indeed recapitulate foetal haematopoiesis displaying the immunophenotype of primitive haematopoietic progenitors: CD90, CD117, CD164, and negative for CD38 expression with concomitant expression of the foetal-specific markers aldehyde dehydrogenase and Lin28B (Vodyanik *et al.*, 2005; Böiers *et al.*, 2018).

Development of an iPSC model genomically engineered to express the childhood B-ALL oncogene ETV6-RUNX1 identified an immature HSPC termed the 'IL7R progenitor' (IL7RP) ($CD45^+$ CD34+ $CD19^-$ $CD45Ra^+$ $IL7R^+$) that has increased myeloid potential relative to normal cells at the same differentiation stage, lies upstream of proB cells and is unique to foetal development. Overexpression of the ETV6-RUNX1 oncogene resulted in the persistence of a myeloid expression signature from the IL7R progenitor to the proB compartment and altered proB potential inducing a perturbed lympho-myeloid signature. This resulted in transition from proB cells into functional macrophages when genomically engineered proB cells were cultured in myeloid conditions (Böiers *et al.*, 2018).

Building on this, I aimed to establish an in-vitro system by which DS foetal lymphopoiesis could be recapitulated and immunophenotypically and functionally characterised. The objectives of this project were to; i) develop a human induced pluripotent stem cell model of DS haematopoiesis that recapitulates human B-cell development, ii) to characterise the effect of T21 on foetal lymphopoiesis

and iii) to immunophenotypically identify and characterise candidate pre-leukaemic populations. Subsequent molecular characterisation of the identified populations is described in chapter 4.

3.1.2 Establishment of in-vitro lymphopoiesis from DS iPSCs

To derive haematopoietic progenitors from DS-iPSCs, iPS cells were co-cultured on OP9 and MS5 stroma [kindly provided by Igor Slukvin, University of Wisconsin-Madison, and Lee Carpenter, University of Oxford, respectively] (see methods section 2.1.4). This method was previously described using human ESCs to derive lymphoid progenitors by Vodyanik et al, and further characterised by Carpenter using human iPSCs (Vodyanik *et al.*, 2005; Carpenter *et al.*, 2011). A schematic of the protocol is illustrated below (Figure 3.1).

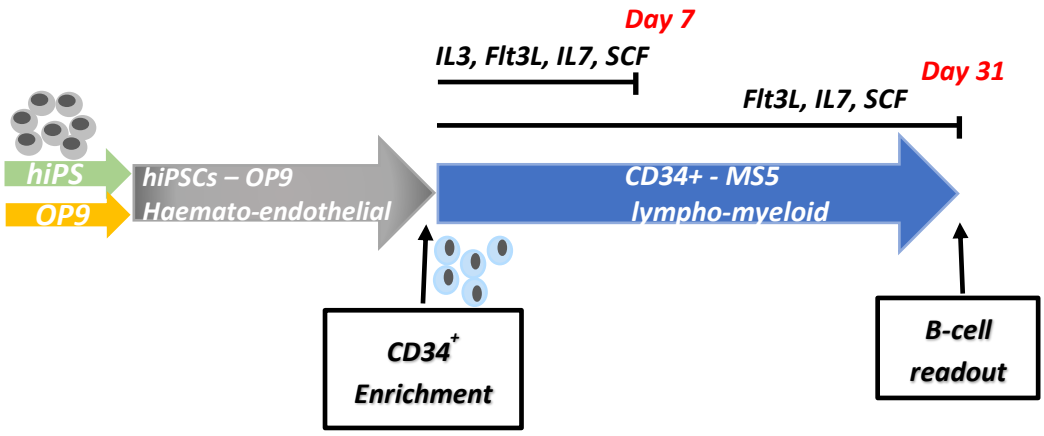


Figure 3. 1: Schematic representation of B-cell differentiation protocol. Human iPSC colonies are harvested and plated onto a layer of overgrown OP9 stroma. After 10 days of co-culture cells are harvested and approximately 25,000 – 50,000 CD34+ haemato-endothelial precursor cells are plated onto 6-well plates containing sub-confluent MS-5 stroma in the presence of cytokines necessary for lymphoid commitment, proliferation and differentiation – IL3, Flt3L, IL7 and SCF. 21 days post culture on MS-5 stroma cells are harvested and analysed immunophenotypically (adapted from Boiers and Richardson et al,. 2018).

The differentiation protocol of karyotypically normal hiPSCs into immature B-cell progenitors was previously established in the Enver laboratory by Dr. Simon Richardson. Derivation of B cell progenitors from hiPSCs is a technically challenging process that is subject to variability in output. The system has a number of key undefined components such as OP9 and MS5 stromal cell lines that require maintenance at a consistent confluency during routine culture. Additionally, to consistently derive CD19+ B cells from iPSCs I found the following to be critical; 1) the use of high performance FBS previously tested for optimal B-cell production, 2) overnight bovine gelatinisation of 10cm plates at 4°C used for culturing of OP9 stroma, 3) consistent maintenance of OP9 and MS5 stromal cells < passage 35 at a confluency between 50-80% and an undifferentiated state of greater than 90% and 4) preservation of iPSCs at an undifferentiated state of greater than 80%. A detailed protocol is described in section 2.1.4.

To address my primary aims of developing an in-vitro system of human DS lymphopoiesis and to characterise B-cell development from DS iPSCs versus disomic normal iPSCs I first used the human T21 human iPSC lines DS1 and DS2 (provided by George Daley, Harvard University) and the disomic human Miff3 iPS cell line. DS iPS cell lines were derived from fibroblasts and peripheral blood mononuclear cells of different DS patients through lentiviral introduction of the reprogramming factors Sox2, Klf4, c-myc and Oct4.

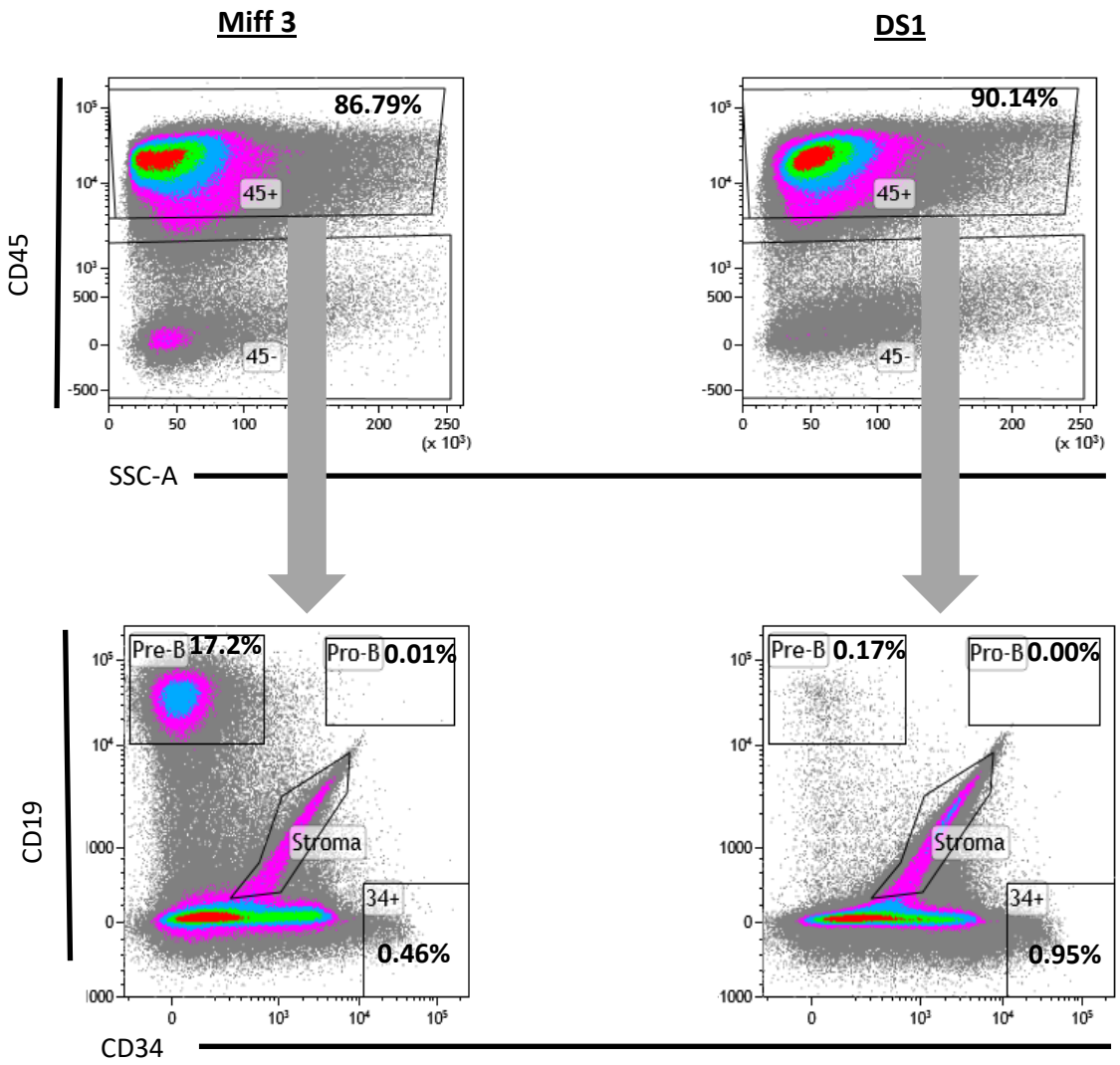
The Miff3 cell line was derived from fibroblasts of a healthy donor by transient mRNA expression of the same reprogramming factors

To determine, whether like Miff3, DS1 and DS2 iPSC lines can undergo B-cell lymphopoiesis in-vitro I terminated parallel B-cell differentiations of Miff3, DS1 and DS2 at day 31 of the differentiation protocol. HSPCs derived from DS1, DS2 and Miff3 iPSCs were harvested at day 31 of the differentiation protocol and immunophenotypically characterised using the antibodies outlined in

table 2.2 above and the below gating strategy (figure 3.2). HSPCs were quantified as a percentage of the pan haematopoietic marker $CD45^+$ and B-cells identified through the expression of cell surface markers CD45, CD34 and CD19 ($CD45^+$ $CD34^+$ $CD19^+$ proB or $CD45^+$ $CD34^-$ $CD19^+$ preB cells).

DS-iPSCs displayed a significant decrease in the frequency of preB cell production in comparison to that of normal Miff3 – DS1 0.17% vs Miff3 17% (100 fold) and DS2 3.76% vs 30.9% (8 fold). These experiments recapitulate the decreased B-cell frequency observed in T21 second trimester foetal liver versus normal euploid controls previously described by Roy et al,. 2012 with a concomitant minor expansion of the $CD34^+$ $CD19^-$ compartment (Roy *et al.*, 2012). This suggests that T21 induces a partial block in B-cell development that occurs in a $CD34^+$ $CD19^-$ progenitor compartment upstream of preB cells. It is possible that the difference in B-cell frequency observed between DS1 and DS2 may be due to genetic variation from the donors. Using iPSCs derived from PBMCs or fibroblasts from the same donors and analysing their ability to differentiate through haematopoietic lineages, Kyttälä et al,. described that it is the donor-based variability rather than the cell type of origin that influence the differentiation potential of iPSCs (Kyttälä *et al.*, 2016).

A)


Miff 3
DS1
86.79%
90.14%
45+
45-
CD45
SSC-A
Pre-B 17.2%
Pro-B 0.01%
Pre-B 0.17%
Pro-B 0.00%
Stroma
34+
0.46%
0.95%
CD19
CD34

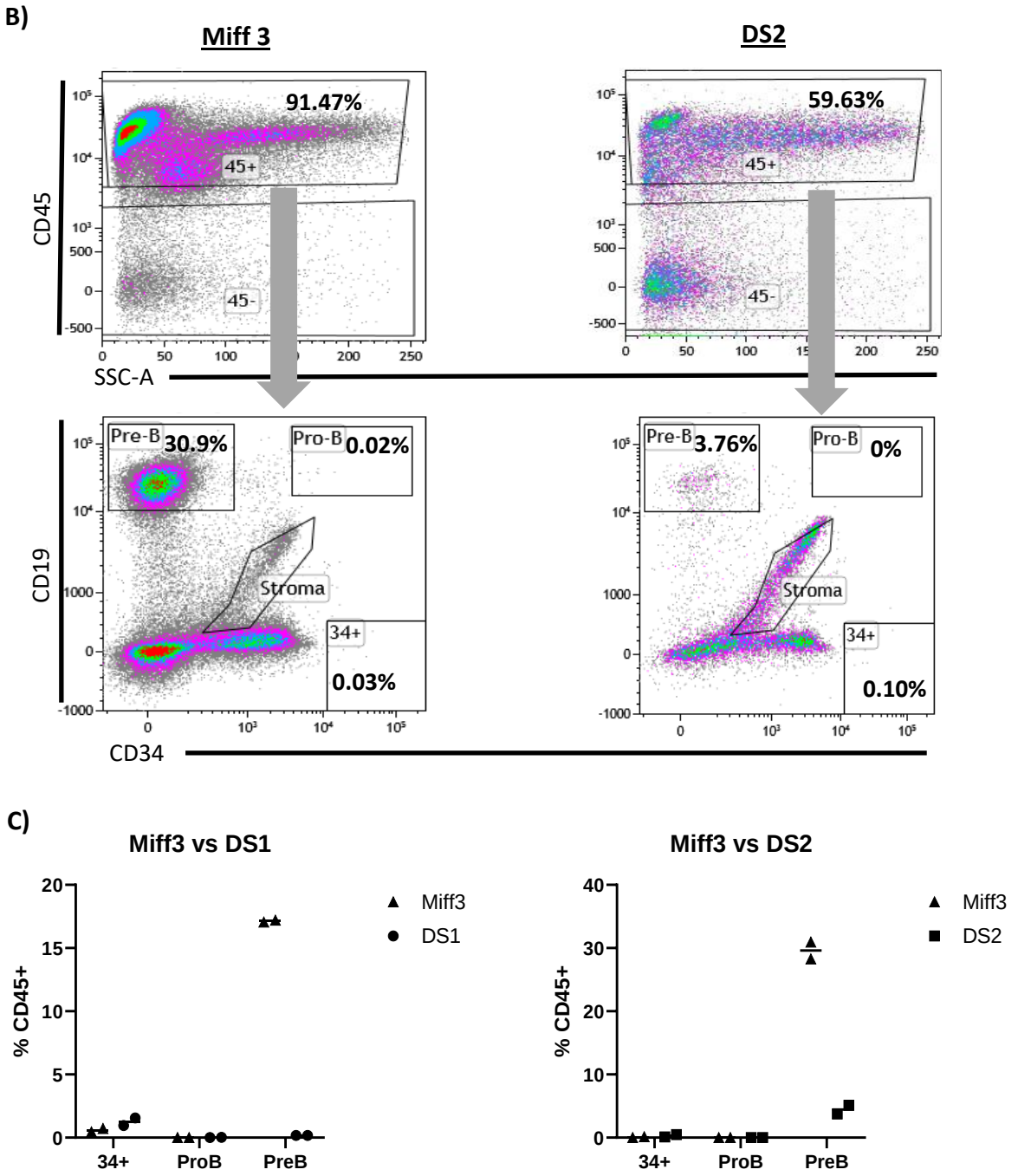


Figure 3. 2: Immunophenotypic analysis of Miff3, DS1 and DS2 iPS cell lines after a complete 31 day B-cell differentiation. CD45+ cells are gated on single and viable cells prior to analysing CD34+ and B-cell populations. Representative FACS plots of Miff3 and A) DS1 highlighting a 100X and B) DS2 ~8X decrease in the percentage of preB cells (34-19+) for both DS1 and DS2 in comparison to Miff3. C) Frequencies of CD34+ CD19-, preB and proB progenitor cells derived from Miff3, DS1 and DS2 analysed at day 31 of the B-cell differentiation protocol. Samples are expressed as a percentage of CD45+.

Of note, ProB cells were not detected upon completion of the 31 day B-cell differentiation (Figure 3.2 above). I speculate that this was most likely due to the low frequency of B-cell production and the quick transition to a more mature preB immunophenotype that occurs at an earlier timepoint. To identify the timepoint at which DS-iPSCs have optimal production of immunophenotypically identifiable proB cell during the differentiation protocol I analysed earlier timepoints.

I terminated parallel differentiations for Miff3 and DS1 cell lines at days 27-31 of the OP9/MS5 co-culture. Progenitor cells were harvested as described in methods section 2.1.4. ProB cells derived from DS1 and Miff3 iPSCs were detected as early as day 27 of the differentiation protocol with a peak of preB and proB cells of 5.78% and 0.13% on day 29 (figure 3.3 below). There did remain a significant increase in DS1 $CD34^{+}$ progenitors in comparison to Miff3 at each day of the time course as previously observed in 31 day B-cell differentiations (Miff3 data not shown).

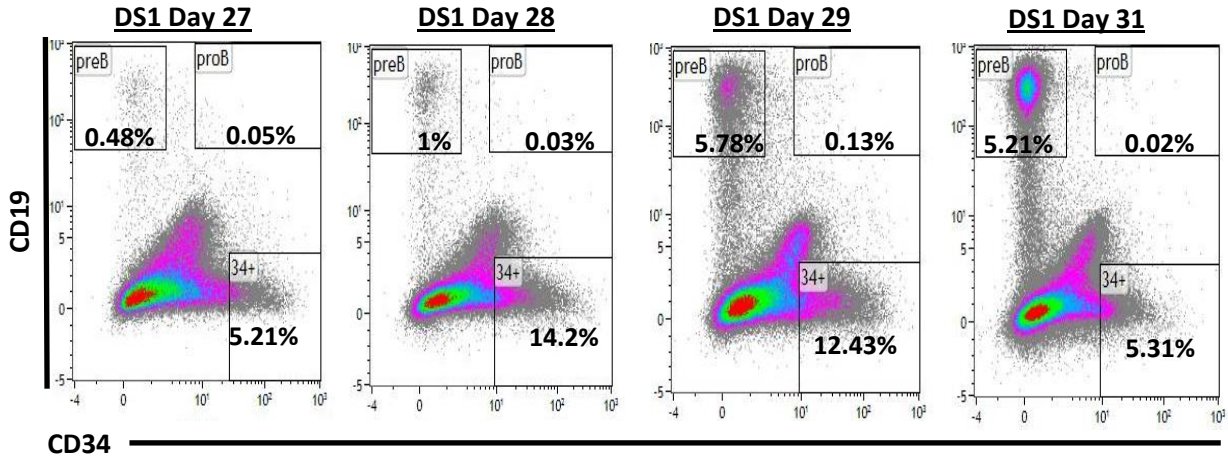


Figure 3. 3: Time-course and characterisation of B-cell emergence during in-vitro differentiation of DS-iPSCs. Identification of highest proB emergence from DS-iPSCs at day 29 of B-cell differentiation. Samples gated on CD45+ cells. Numbers demonstrate the percentage of cells gated.

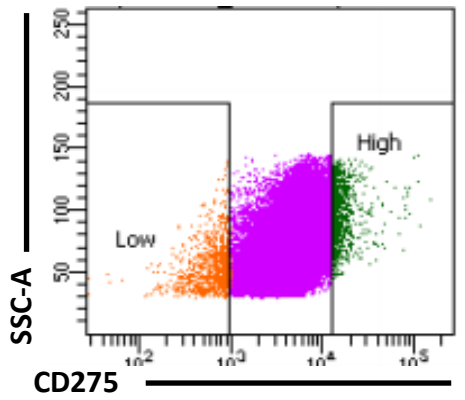
3.1.3 Derivation of DS isogenic iPS cell line

While impaired B-cell lymphopoiesis was observed within the DS iPSC lines vs Miff3, Miff3 is not an isogenic control for modelling DS haematopoiesis, I therefore aimed to obtain a true isogenic control for the DS-iPSCs by subcloning a disomic clone from each of DS1 and DS2 iPS cell lines. Similar to cases of Down's syndrome bearing various degrees of mosaicism, iPSCs have also been described to be karyotypically unstable for chromosome 21. The AML1 gene is encoded on the long arm of chr 21, 21.q22.1 and may therefore be used as a surrogate marker of the number chr 21 copies present within a cell. Hence I used an AML1 probe (Cytocell) for DNA FISH of DS1 and DS2 iPS cell lines (Figure 3.4B). DNA FISH displayed mosaicism for chromosome 21; 33% and 20% respectively of DS1 and DS2 parental cell were disomic (Table 3.1).

CD275 is a gene located on chr 21 encoding for a transmembrane protein. I hypothesised that enriching for the lower expressing CD275 population within DS iPSCs I may enrich for chr 21 disomy. I FACS sorted cells with low expression of the chr 21 encoded cell surface marker CD275 (Figure 3.4A). iPSCs are unstable when dissociated into single cells. To improve the stability prior to cell sorting I pre-incubated DS1 and DS2 with ROCK inhibitor 10μM for one hour which has previously been shown to reduce cell death resulting from anoikis (Wang *et al.*, 2009).

Disappointingly, sorted CD275 low expressing cells and parental DS iPSCs displayed a similar proportion of disomic and trisomic cells (DS1 parental 70% vs 30% DS1 CD275 sorted population and DS2 76% vs 24% DS2 CD275 sorted population) (Table 3.1 below). This may indicate that the presence of an extra copy of chromosome 21 does not result in a significantly higher expression of protein receptor, at least in iPSCs.

A)



B)

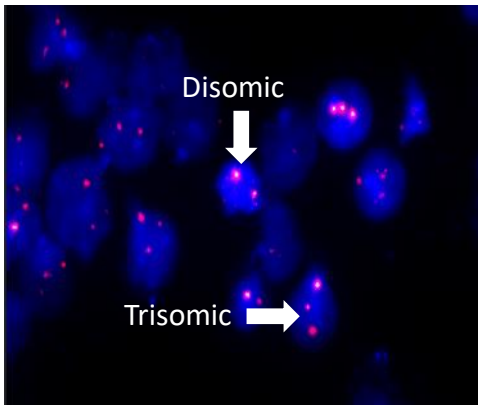


Figure 3. 4: DS parental iPSCs underwent FACS for lower expressing CD275 populations. Based on CD275 expression level within each of the DS-iPSCs and to acquire a sufficient number of viable cells DS1 were sorted for the lower 5% CD275 expressing cells and DS2 for the lower 8%. B) Identification by DNA FISH of both trisomic and disomic populations within the DS1 parental cell line, chromosome 21 disomic clones were similarly observed within the DS2 iPS cell line.

% FISH Counts for DS Subclones Enriched for Lower Expressing CD275				
	DS1 Parental	**CD275 Low Expressing**	**DS2 Parental**	**CD275 Low Expressing**
Trisomic	66	70	80	76
Disomic	33	30	20	24

Table 3. 1: Enrichment for the lower expressing CD275 population within the parental DS-iPSC lines did not produce a subclonal population with increased chromosome 21 disomy, 50 cells were scored per sample.

To further attempt to isolate a pure disomic clone I dissociated DS-iPSC colonies into single cells and either seeded cells via limiting dilution into 96 well plates or seeded 2,000 dissociated single cells cells into a 10cm dish to obtain single cell derived clones. Cells that underwent a limiting dilution in

96-well plates did not remain viable while those that were dissociated into single cells and seeded onto a 10cm plate remained viable and continued to expand when further cultured. Due to variation in viable clones obtained from each cell line 5 DS1 and 15 DS2 subclones were expanded and screened by qPCR and FISH.

Hu et al,. have previously established a high throughput real-time PCR screening method to prenatally diagnose DS. This was achieved by determining the ratio of the DSCR3 gene to GAPDH relative to non-DS samples (Hu *et al.*, 2004). To determine if an enrichment for disomy of chromosome 21 had occurred upon subcloning, I adopted this PCR approach using the Taqman probes described in methods section 2.2. The qPCR approach did not identify any subclone to have a DSCR3 expression level significantly lower or higher than that of Miff3 (data not shown), while DNA FISH using an AML1 probe, as described above, identified one clone from each DS cell line to be enriched to 50% disomy – DS1 #3 and DS2 #7 (Table 3.2). A possible explanation for not achieving the 100% disomy for chr 21 within the DS1 #3 and DS2 #7 subclones may be due to chromosomal instability and acquisition of trisomic copy of chr 21 or due to migration of cells between clonal populations. Genomic instability has previously been shown to occur during routine cell culture (Mayshar *et al.*, 2010).

Attempts to obtain pure disomic clones were hampered by a change in media from StemFit (Stem Cell Technologies) to a homemade essential 8 media in which single cell clones no longer remained viable. In an attempt to increase the viability of single cell clones I incorporated CloneR (Stem Cell Technologies), a single cell cloning supplement, into the media however the pluripotency and viability of clones did not improve and I was forced to proceed with the analysis of the DS poloyclonal cell lines.

FISH Counts for DS Subclones	
DS1 #3	
Trisomic	44.7%
Disomic	55.2%
DS2 #7	
Trisomic	50.7%
Disomic	49.3%

Table 3. 2: DS1 #3 and DS2 #7 subclones enriched for disomy of chromosome 21. Cell counts completed using DNA FISH. 50 cells scored per clone.

3.1.4 B-cell differentiation of Miff3 vs DS1 #3 and DS2 #7

If the presence of an extra copy of chromosome 21 induces a partial block in B-cell development, I hypothesised that an enriched disomic DS-iPSC line may have an increase in the proportion of DS B cells maturing to the preB stage and partially rescue the observed B-cell defect. To test this I completed a full 31 day B cell differentiation on both disomic enriched DS subclones and Miff 3 (results outlined in table 3.3 below).

Cell Line	% Gated 34^+ $CD19^-$	% Gated PreB
Miff3	0.92	1.16
DS1 #3	1.87	0.43
DS2 #7	3.51	0.77

Table 3. 3: Percentage of CD34+ and preB cells derived from, DS1 #3 and DS2 #7 enriched disomic subclones n = 1.

DS1 #3 and DS2 #7 subclones enriched for 55% and 49% disomy 21 displayed a partial rescue of the observed B-cell defect with a decrease fold change from 100 and 8 to an approximately 2.7 and 1.5 fold respectively in comparison to Miff3.

The $CD34^+$ $CD19^-$ population maintained a similar proportional difference to that observed when analysing parental DS1 and DS2 to Miff3 (2-fold and 3-fold increase in $CD34^+$ $CD19^-$ cells derived from DS-iPSCS). This finding suggests that DS $CD34^+$ $CD19^-$ HSPCs may be incurring a partial block in B lymphopoiesis in an immature progenitor compartment prior to B-cell commitment.

As the proportion of $CD34^+$ $CD19^-$ HSPCs produced by all iPS cell lines remained similar to previous experiments, the partial rescue in B-cell development observed is not due to a defective differentiation of Miff3 but suggests that it is due to increased chr 21 disomy.

To investigate the hypothesis that T21 disrupts B-lymphopoiesis within the $CD34^+$ $CD19^-$ compartment and that $CD34^+$ $CD19^-$ disomic clones would mature to committed B-cells more readily than trisomic, I attempted to sort the DS preB population for DNA FISH and compare the proportion of disomic to trisomic preB cells to those within the $CD34^+CD19^-$ progenitor population. I repeated this experiment 3 times however due to the limited number of viable preB cells derived from DS-iPSC B-cell differentiations and time constraints I was unsuccessful in completing FISH on a sufficient number of cells.

While attempts to derive isogenic DS iPSC lines were ongoing I obtained isogenic DS IPSC lines 1469 [trisomy 21], 1470 [trisomy 21] and 1474 [disomy 21] (kindly provided by Prof Stuart Orkin Harvard Stem Cell Institute). These IPSC lines were previously derived by lentiviral infection of a parental fibroblast cell line isolated from a patient. Pure disomic and trisomic populations were derived by

subcloning and karyotypically characterised using DNA FISH and DNA microsatellite analysis (MacLean, *et* al,. 2012)

To determine the hematoendothelial potential of the obtained DS-iPSCs I differentiated 1474 and 1469 cell lines on OP9 stroma for 10 days and completed FACS analysis on derived progenitors (figure 3.5 below). Using the cell surface markers CD45, CD34, CD38, CD45Ra, CD90 and CD19 I immunophenotypically identified HSC ($CD45^+$ $CD34^+$ $CD38^-$ $CD90^+$ $CDCD45Ra^-$), MPP ($CD45^+$ $CD34^+$ $CD38^-$ CD90- $CD45Ra^-$), LMPP ($CD45^+$ $CD34^+$ $CD38^-$ $CD45Ra^+$ $CD90^-$). As expected, due to termination of the differentiation protocol at day 10 prior to culturing HSPCs in conditions that facilitate B-cell production no B-cells ($CD45^+$ $CD34^{+/-}$ $CD19^+$) were observed. This confirmed that T21 1469 and D21 1474 iPSCs are capable of haematopoietic commitment and appear to produce immunophenotypically defined HSC, MPP and LMPP progenitors at a similar frequency (Figure 3.5).

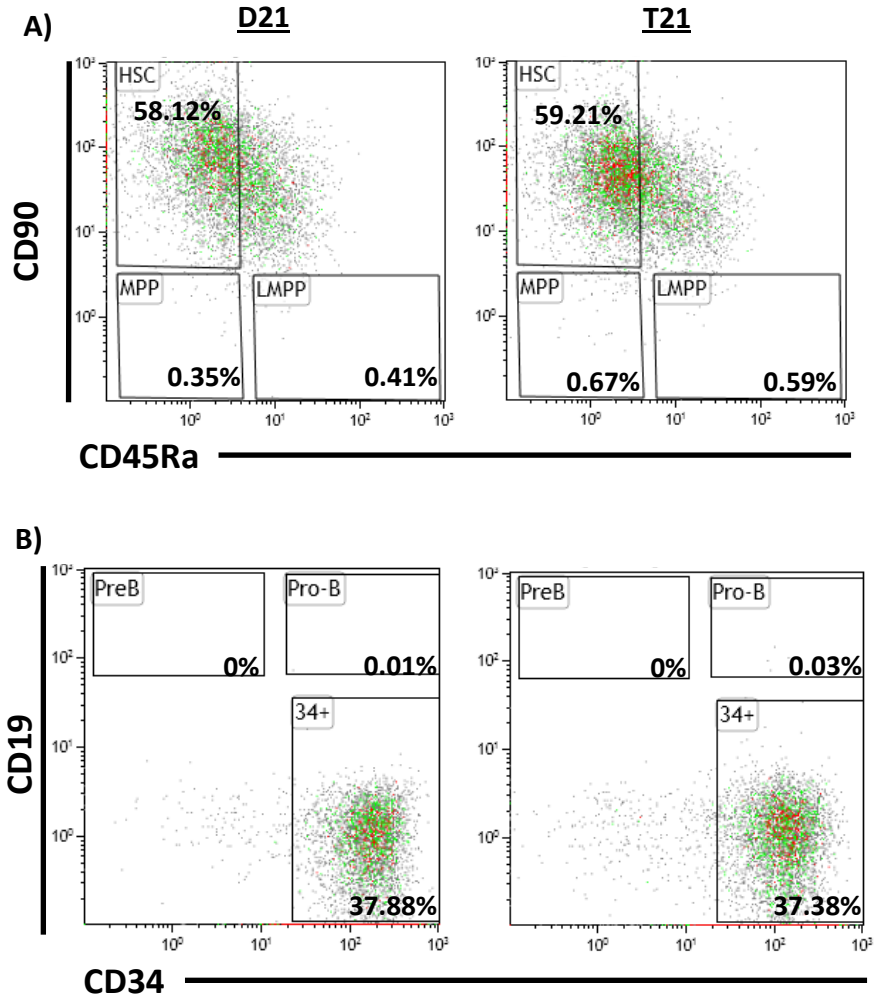
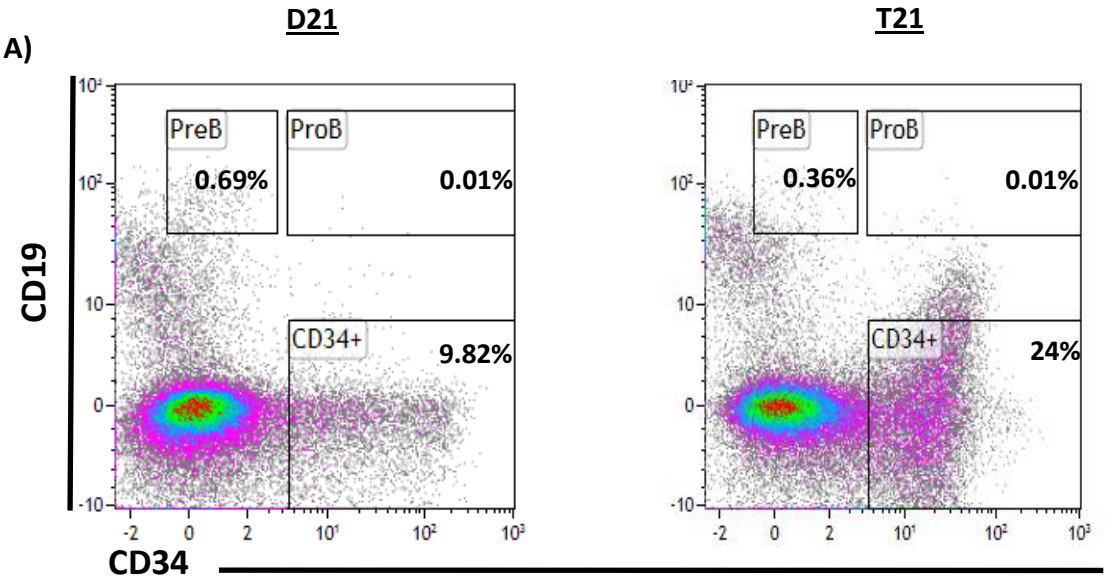


Figure 3. 5: Derivation of haematopoietic stem progenitor cells from day 10 OP9-hiPSC co-culture. Samples are pre-gated on CD45+ CD34+ CD38-. A) HSCs (CD45+ CD34+ CD38- CD90+ CD45Ra-), MPP (CD45+ CD34+ CD38- CD90-) and LMPP (CD45+ CD34+ CD38- CD90- CD45Ra+) and B) B lymphocyte hSPCs CD45+ CD34+/- CD19+ from T21 iPSCs and D21 iPSCs. Numbers demonstrate the percentage of cells gated.

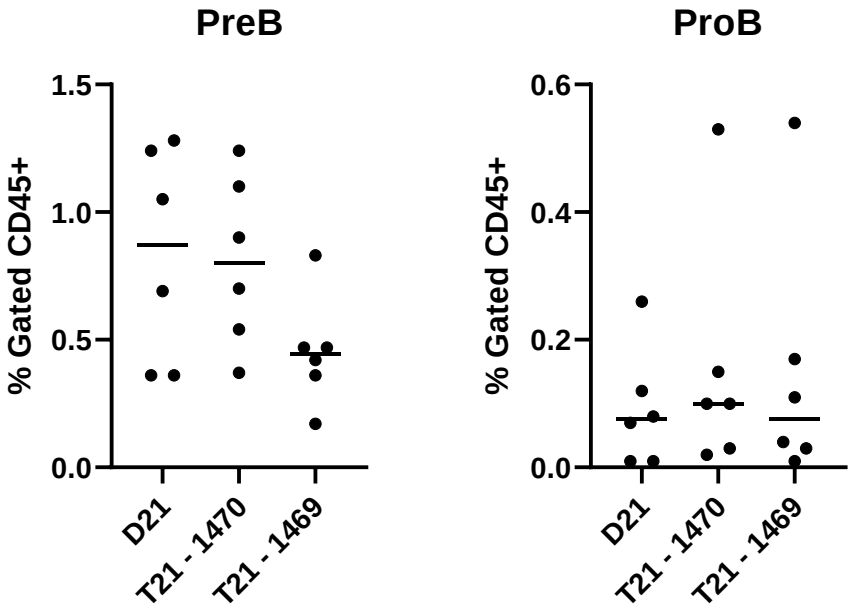
Having confirmed 1474 and 1469 isogenic iPS cell lines are capable of haematopoietic progenitor production, I tested the frequency of B-cell output by deriving $CD34^{+}$ haematoendothelial enriched progenitors from day 10 of OP9-iPSC co-culture and cultured them on MS-5 stroma in the presence of SCF, IL-3, IL-7 and Flt3l for a further 19 days as described in section 2.1.4. Immunophenotypic analysis

of HSPCs derived from day 29 of 1469 and 1474 B-cell differentiation for the presence of $CD34^+$ $CD19^+$ pro and $CD34^-$ $CD19^+$ preB cells is displayed in figure 3.6.

A)


D21
T21
PreB
ProB
CD34+
0.69%
0.01%
9.82%
0.36%
0.01%
24%
CD19
CD34

B)


PreB
ProB
% Gated CD45+
D21
T21 - 1470
T21 - 1469

C)

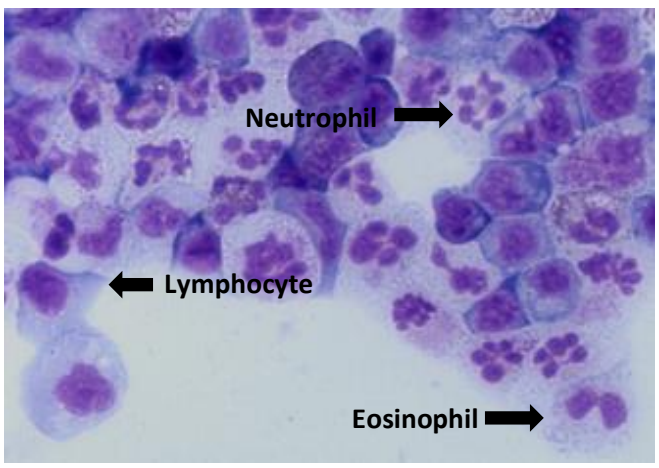

Neutrophil
Lymphocyte
Eosinophil

Figure 3. 6: Differentiated CD34+ haemato-endothelial progenitors derived from human induced pluripotent stem cells analysed at day 29 of the differentiation protocol for the expression of CD34+ CD19+ and CD34- CD19+ pro and preB cells respectively. A) D21 sample (left) and T21 1469 (right). Samples are gated on CD45+ cells. B) Dot plot illustrating the impaired preB cell output (p-value = 0.1688) from T21 iPSCs relative to isogenic D21 counterpart while the proB (p-value = 0.8135) compartment remains at a similar frequency. N = 6. C) Cytospin of harvested suspension cells at day 29 of B-cell differentiation highlighting the presence of myeloid and lymphoid cells. Statistical analysis was performed using the Kruskall-Wallis test for multiple comparisons.

These experiments demonstrate the successful production, although at a very low frequency, of immature $CD19^+$ B cells from isogenic DS iPSCs. The decreased frequency of preB cell production observed between T21 (1469) and D21 (1474) recapitulates the defect in B-cell output observed between DS1, DS2 and Miff3. However only a minor impairment in preB cell output was observed within the 1470 T21 DS iPSC line. This observation, although experimentally noisy, is consistent with the defective B cell immunophenotype observed within T21 foetal liver samples vs normal euploid samples (Roy *et al.*, 2012). D21 and T21 iPSC proB output was constant between samples. I theorise the decrease in preB cell frequency observed is due to an impaired proliferative capacity of the T21 preB compartment due to disrupted pre-BCR signalling.

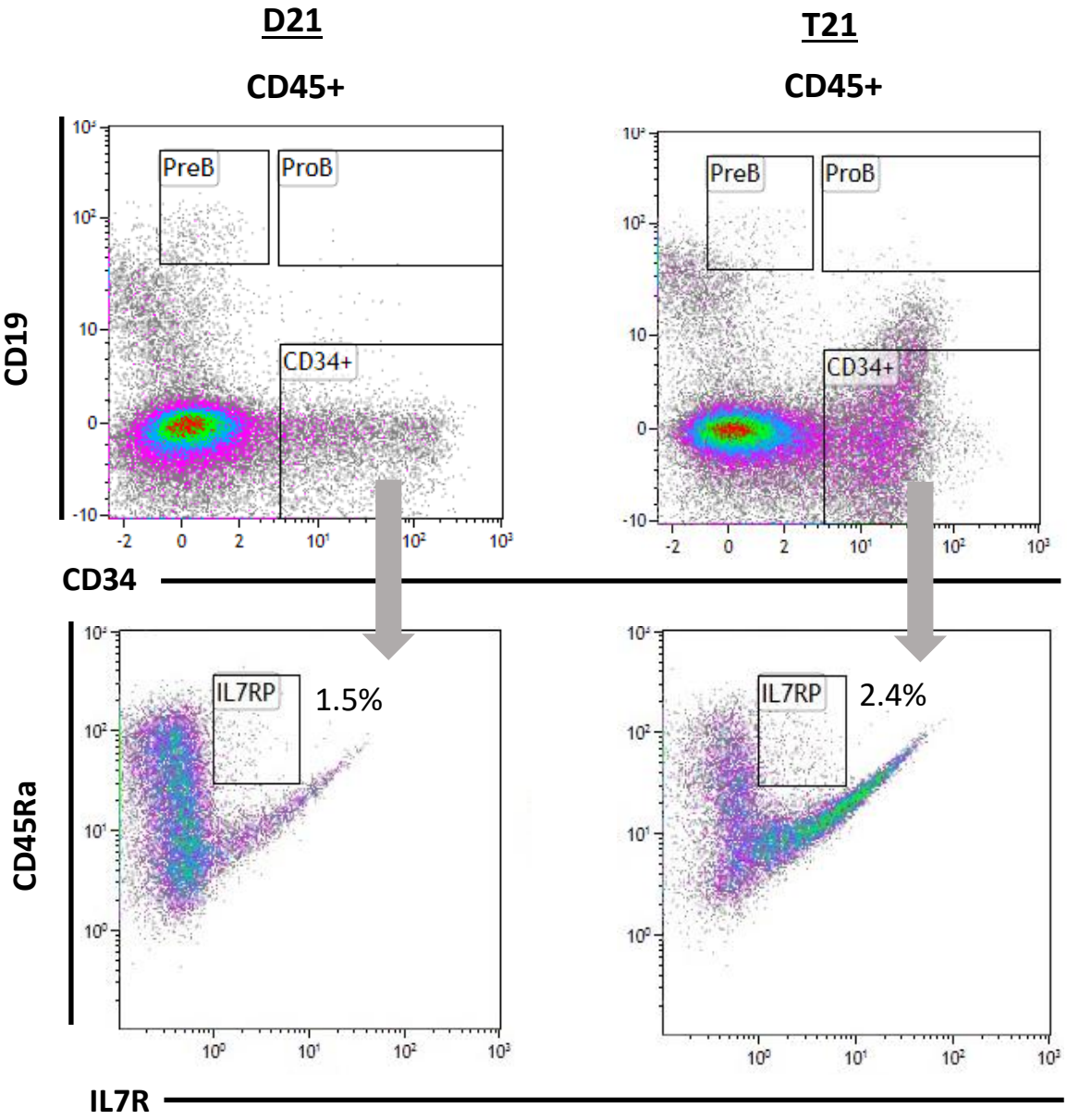
Cytospins from day 19 of MS-5 co-culture (figure 3.6 C above) indicate the presence of immature lymphoid progenitors along with a significant proportion of myeloid precursors. The frequency of myeloid progenitors derived from the B-cell differentiation system is unsurprising as Flt3L, IL-3 and SCF facilitate myeloid as well as B-cell output. Human ES derived $CD34^+$ HSPCs cultured on MS-5 stroma in the presence of Flt3l, IL-3 and IL-7 have similarly demonstrated multilineage output (Vodyanik *et al.*, 2005).

3.1.5 Immunophenotypic characterisation of CD34+ CD38- CD33hi CD45Rahi progenitor

With the above, I speculated that the partial block in B-cell lymphopoiesis occurs within a primitive cellular compartment and therefore I next investigated the frequency of immature HSPCs within the $CD34^{+}$ $CD19^{-}$ compartment. If DS-iPSCs could reproduce the expansion of the $CD34^{+}$ $CD19^{-}$ immature progenitor compartment observed in T21 FL as described by Roy et al,. 2012, I hypothesised that DS-ALL may be oligoclonal and that perturbation of the transcriptional network within a small number of T21 cells in a primitive lineage-negative cellular compartment could potentially induce a partial differentiation arrest at an immature progenitor stage; therefore resulting in lineage skewing and development of a pre-leukemic phenotype.

To test this hypothesis, I analysed the frequency of the IL7RP derived from DS-iPSC isogenic differentiations at day 29 of the B-cell differentiation protocol. T21 1469 iPSC line displayed a minor expansion of the IL7RP relative to D21 1474 control, however this observation was technically variable between T21 samples and the 1470 iPSC line displayed only a minor increase in IL7RP output (figure 3.7).

A)


D21
CD45+
T21
CD45+
PreB
ProB
CD34+
CD19
CD34
IL7RP
1.5%
2.4%
CD45Ra
IL7R

B)

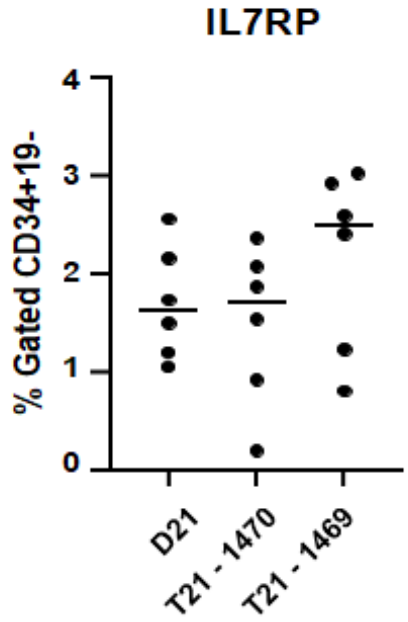
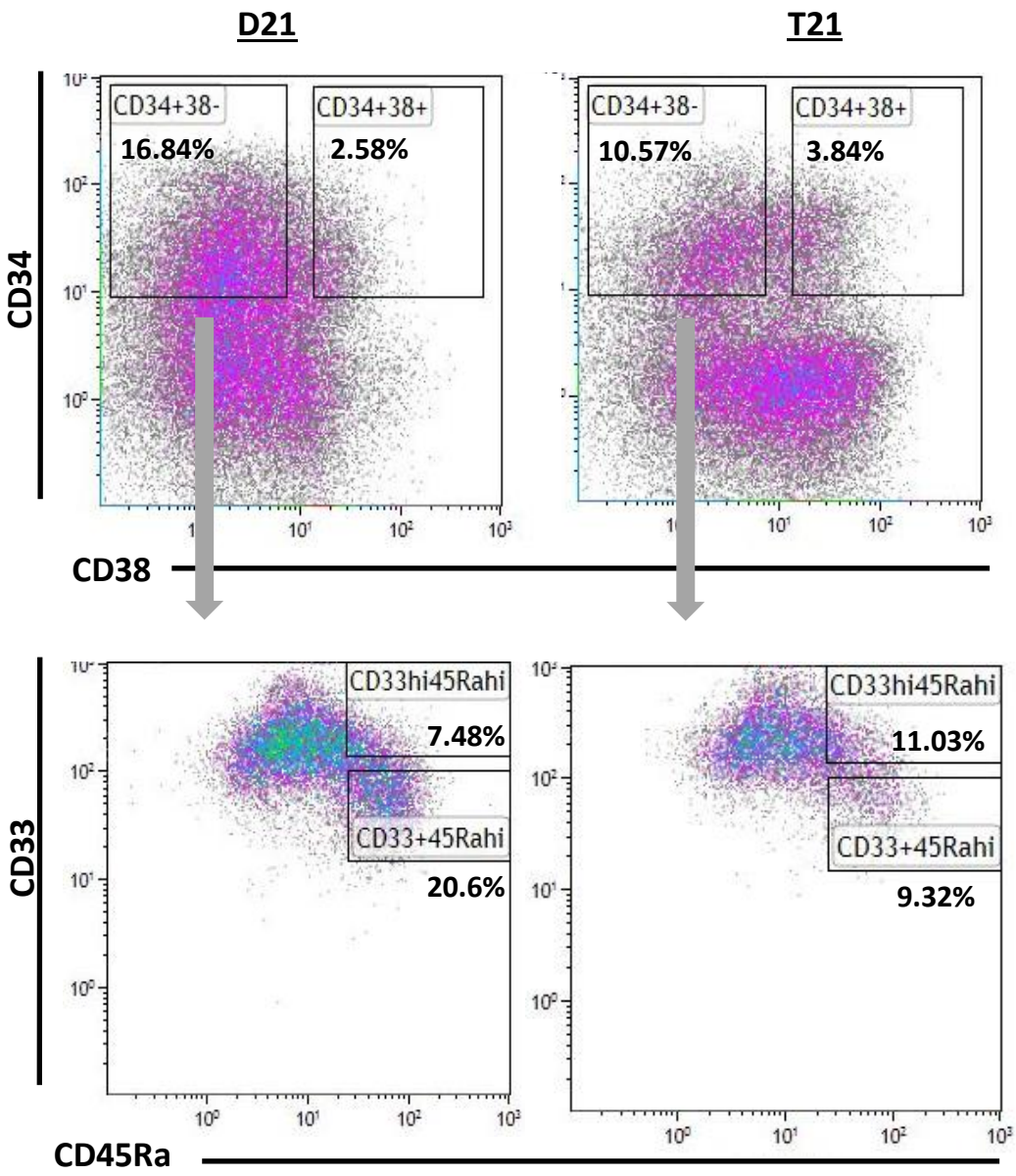


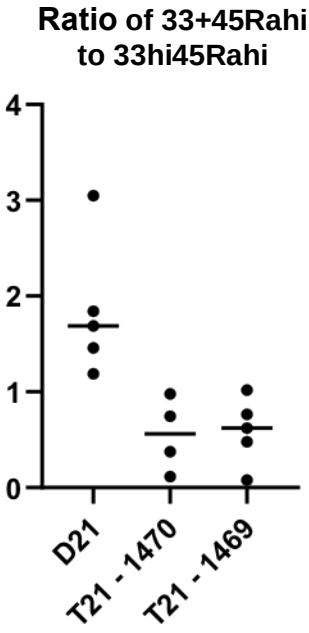
Figure 3. 7: Isogenic DS iPSCs analysed for the presence of the CD45+ CD34+ CD19- CD45Ra+ IL7R+ IL7RP progenitor population. A) Cells were gated as CD45+ CD34+ CD19- prior to analysis of CD45Ra and IL7R expression. Numbers demonstrate the percentage of cells gated. B) T21 1469 iPSC line exhibits an expansion of the IL7RP compartment while 1470 T21 iPSCs display a minor increase in IL7RP relative to D21 control. mean+SD N=6, p-value = 0.3199. Statistical analysis was performed using the Kruskall-Wallis test for multiple comparisons.

During foetal haematopoiesis T21 induces an expansion of the HSC compartment with a megakaryocyte-erythroid bias and a concomitant decrease in B-cell progenitor output (Tunstall-Pedoe *et al.*, 2008; Roy *et al.*, 2012). With the above observed decrease in frequency of the T21 IL7RP relative to D21 controls, I explored the possibility that T21 impacts a lymphomyeloid progenitor-like state by observing the frequency and expression of more primitive myeloid and lymphoid cell surface markers (CD33 and CD45Ra) within the immature HSPC compartments CD45+ CD34+ CD38+/-. The gating strategy used to investigate these populations is outlined in figure 3.8 below.

A)


D21
T21
CD34+38-
16.84%
CD34+38+
2.58%
CD34+38-
10.57%
CD34+38+
3.84%
CD34
CD38
CD33hi45Rahi
7.48%
CD33+45Rahi
20.6%
CD33hi45Rahi
11.03%
CD33+45Rahi
9.32%
CD33
CD45Ra

B)



C)

Figure 3. 8: Perturbation of haematopoietic hierarchy derived from DS-iPSCs. Representative plots showing the percentage of immunophenotypic expression of cell surface markers of A) (i) CD45+ CD34+ CD38+/- hSPCs and CD33hi/+ CD45Rahi/+ showing perturbed expression of CD33hi45Rahi/+ vs D21 control. The gating strategy is highlighted, numbers demonstrate the percentage of cells gated. B) Ratio of CD34+ CD38- CD33+ CD45Rahi LM compartment to CD34+ CD38- CD33hi 45Rahi in T21 and D21 isogenic controls. The T21 LM compartment displays an impaired potential to transition to the CD33+ CD45Rahi population. (N = 5 D21 and T21 and N= 4 T21, p-value=0.028). C) Cytospin of FACS sorted T21 preB cell persisting in suspension myeloid conditions for 18 days n = 1. Statistical analysis was performed using the Kruskall-Wallis test for multiple comparisons.

DS-iPSCs do not display an expansion of the CD34+ CD38- compartment as previously described for in second trimester T21 foetal liver relative to age matched controls (Roy *et al.*, 2012). Investigation of immature lymphoid (CD45Ra) and myeloid (CD33) expression markers within the $CD34^{+}$ $CD38^{-}$ primitive compartment revealed a subpopulation of T21 cells that have an increased co-expression of CD33 and CD45Ra, identified as $CD34^{+}$ $CD38^{-}$ $CD33^{hi}$ $CD45Ra^{hi}$, in comparison to D21 isogenic control [from now termed 'LM progenitor'] (Figure 3.8 A and B). This suggests that T21 LM progenitors display misexpression of the immature myeloid cell surface antigen CD33 relative to D21 counterparts. I hypothesise that the misexpression of CD33 results from an impaired ability to mature to a CD33+ CD45Rahi immunophenotype, indicative of lymphoid commitment, potentially resulting in a subpopulation of cells that have a myeloid bias and become partially blocked in lymphoid commitment. The cells that escape the partial block and undergo B-cell differentiation potentially maintain a bi-lineage lympho-myeloid gene expression signature during B lymphopoiesis that subsequently results in a lineage skewing of more mature B lymphocytes that display a capacity to undergo lineage switching and give rise to myeloid progenitors resulting in perturbed B-cell commitment and differentiation.

To test the hypothesis that an underlying myeloid potential is maintained during T21 B-cell lymphopoiesis, I examined the myeloid clonogenicity of D21 and T21 pro and preB progenitors in myeloid liquid culture conditions. 20-150 pro or preB cells were FACS sorted and cultured in myeloid suspension media containing IL3, SCF, hGM-CSF, GCSF, mCSF and TPO for a further 18 days and samples were analysed for the presence of myeloid progenitor cells (figure 3.8 C). Although this is very preliminary data N=1, T21 and D21 preB

cells survived in myeloid conditions. Initial results indicate trisomic samples produced an increased frequency of HSPCs that persist in myeloid conditions (data not shown). Unfortunately it was not possible to ascertain a robust quantitative output between T21 and D21 samples as the low cell output impaired successful D21 cytospins. FACS sorted pro and preB cells cultured in methocult H4435 (Stem Cell Technologies) for 15 days D21 and T21 cultures did not give rise to viable cells.

To investigate the frequency of the described LM population in T21 foetal tissue I immunophenotypically analysed second trimester T21 and D21 foetal liver. Second trimester T21 FL do mimic the expansion of CD45+ CD34+ CD38- compartment with a concomitant decrease in B-cell populations, as described above in the DS-iPSC model.

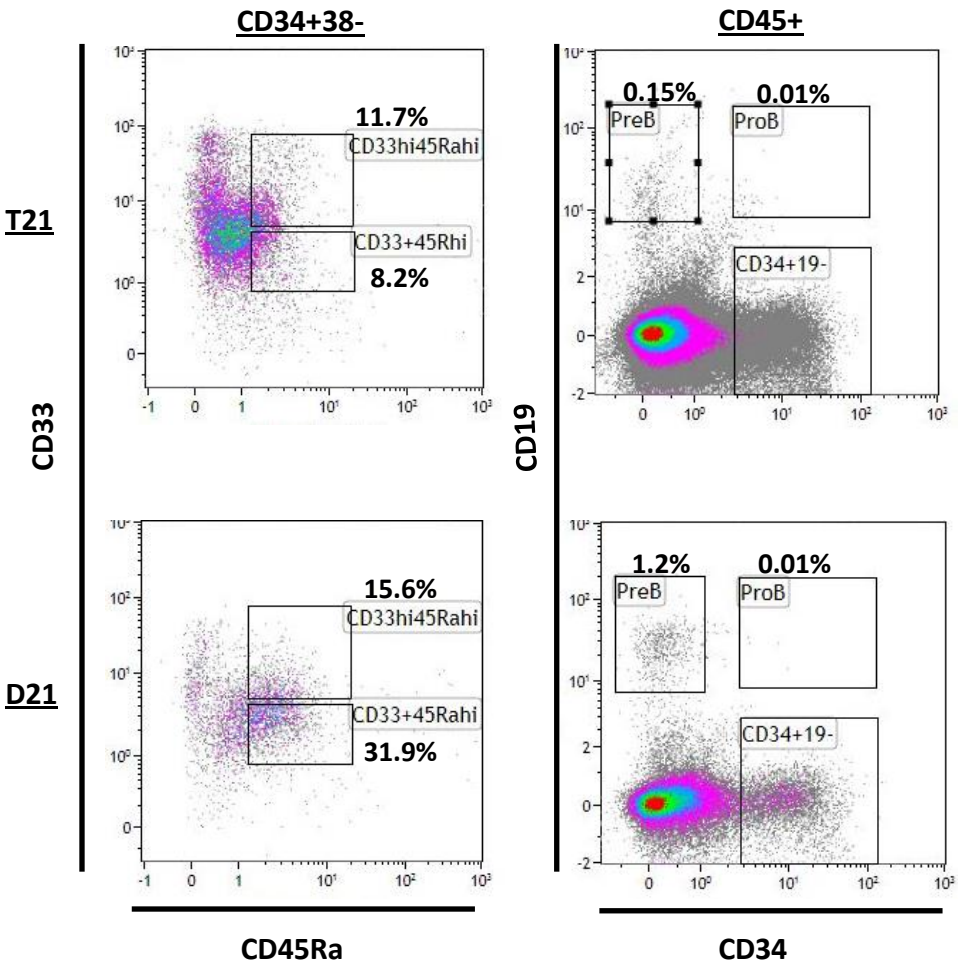


Figure 3. 9: Flow cytometry analysis of T21 and D21 age matched second trimester foetal liver displaying CD45+ CD34+ CD38- CD33hi/+ CD45Rahi, CD34+/- CD19- pro and preB populations. Second trimester T21 foetal liver do mimic the impaired ability to downregulate CD33 within CD34+ CD38- CD33hi 45Rahi compartment with a concomitant decrease in frequency in preB populations as observed in the DS-iPSC model.

The above results suggest that this is a tractable in-vitro system to examine the effects of T21 on early haematopoietic and B-cell development. The identified T21 LM immunophenotype supports the hypothesis that T21 disrupts immature HSPCs prior to lineage specification and suggests a partial block in B-cell differentiation and myeloid lineage bias at the LM progenitor. This suggests the LM progenitor may be the in-utero

target cell for perturbed DS haematopoietic commitment and subsequent pre-leukaemic development.

3.1.1 Modelling CRLF2 and IL7Rα overexpression in DS-iPSCs

To characterise the synergistic effect of T21 and known DS B-ALL mutations affecting CRLF2 and IL7Rα on the DS haematopoeitic hierarchy and leukaemic transformation I aimed to model the overexpression of CRLF2 wild-type and IL7Rα with a gain-of-function 12bp insertion (CCCCCGTGA) in exon 6 of the transmembrane domain, hereafter referred to as CRLF2_IL7Rα12bp ins and IL7Rα12bp ins, in isogenic disomic and trisomic DS-iPSCs (Shochat *et al.*, 2011). I hypothesised that CRLF2 and/or IL7Rα overexpression would synergise with the T21 induced gene expression signatures described in chapter 4 resulting in enhanced proliferation and transformation of the B-cell compartment thus providing a unique system to model DS B-ALL leukaemic onset and transformation.

To achieve overexpression of CRLF2 and IL7R two approaches were taken as outlined below. 1) Isogenic DS-iPSCs were transduced with CEI vector control, or CRLF2_IL7Rα12bp ins lentiviral preparations which express GFP from an alternative promoter. Stable cell lines were derived by sorting GFP+ cells and these were expanded to undergo haematopoietic and B-cell differentiation (figure 3.10). 2) CD34+ haemato-endothelial progenitor cells were derived from parental isogenic DS-iPSCs and transduced with CEI or IL7Rα12bp ins lentiviral preparations at an MOI of 100. Samples were harvested, washed and then cultured on MS-5 stroma for downstream B-cell differentiation as described in figure 3.14.

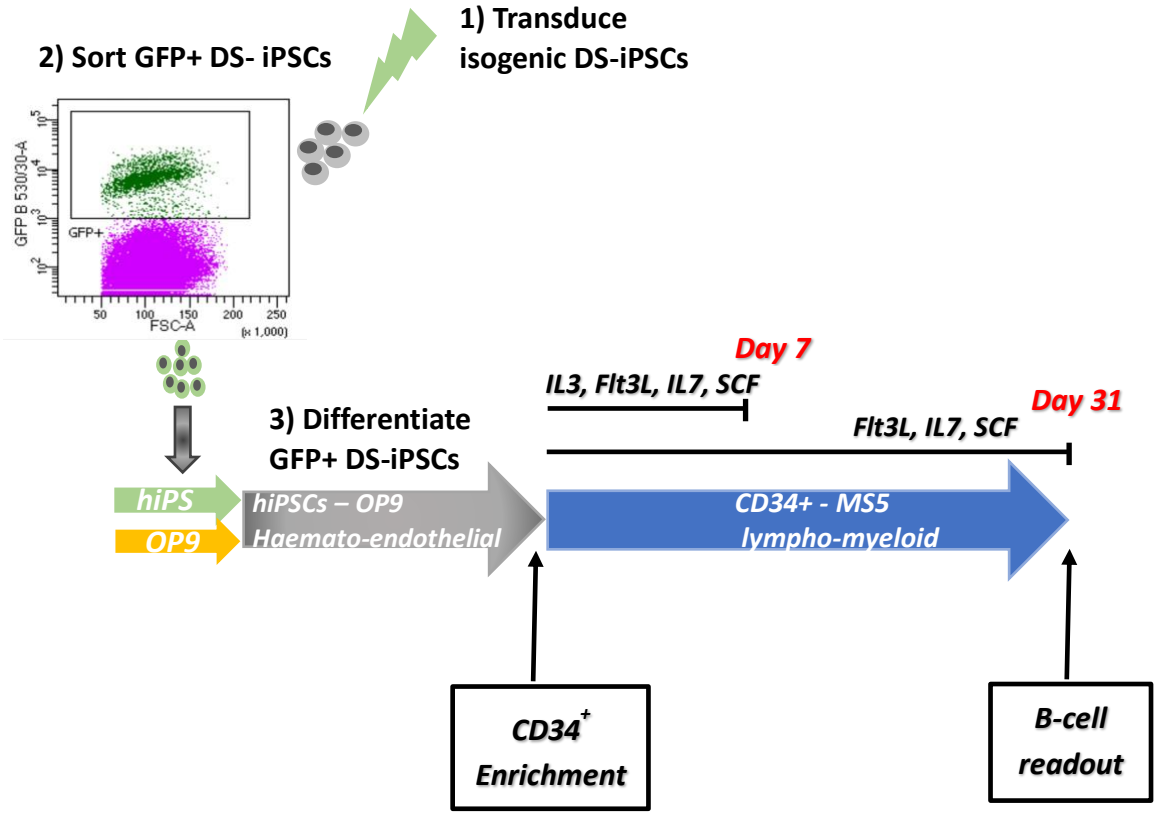


Figure 3.10; A) Schematic representation of the strategy used to model lentiviral overexpression of CRLF2 and IL7Rα12bp ins in isogenic disomic and trisomic DS-iPSCs. Green arrow indicates lentiviral transduction of iPSCs. T21 and D21 isogenic iPSCs were incubated with control CEI or multicistronic CRLF2_IL7Rα12bp ins lentivirus overnight. 48 hours post transduction iPSCs were washed and fresh media added. GFP+ iPSCs were derived by FACS and expanded prior to co-culture on OP9 stroma to derive CD34+ haemato-endothelial progenitor cells and subsequently cultured on MS-5 stroma for B-cell output as previously described.

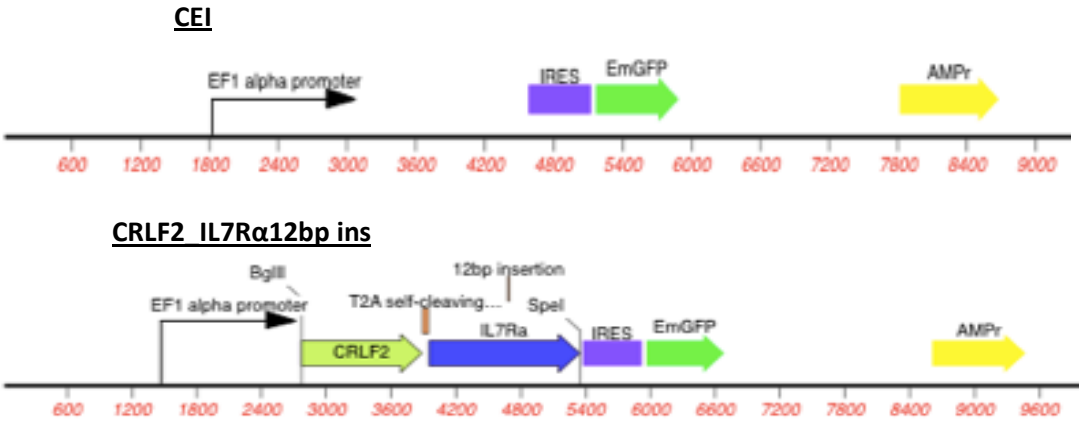
3.1.2 Lentiviral overexpression of CRLF2 and IL7Rα12bp ins in DS-iPSCs

Schematics of the CEI and CRLF2_IL7Rα12bp ins lentiviral constructs generated are shown in figure 3.11 below. Lentiviral transduction of DS-iPSCs enabled the overexpression of CRLF2

and IL7Rα12bp ins under the control of the constitutive EF1α promoter. The CRLF2_IL7Rα12bp ins overexpression construct comprises of CRLF2 and IL7Rα12bp ins separated by a T2A peptide and a GFP reporter downstream of an internal ribosomal entry sequence (IRES) facilitating expression of CRLF2, IL7Rα and GFP as a single transcript. The EF1α promoter was selected for this study as it has previously been demonstrated to maintain the highest level of activity during ESC differentiation relative to other viral promoters (Norrman *et al.*, 2010).

To confirm functionality of the generated CEI, IL7Rα12bp ins and CRLF2_ IL7Rα12bp ins lentivirus HEK 293T cells were transduced and analysed by FACS for CRLF2 and IL7Rα cell surface expression after 5 days in culture. All samples were gated on live and GFP+ cells. IL7Rα12bp ins and CRLF2_ IL7Rα12bp ins transduced HEK293T cells displayed surface expression of CRLF2 and/or IL7Rα while CEI expressing cells were negative for both (figure 3.11 b).

A)



B)

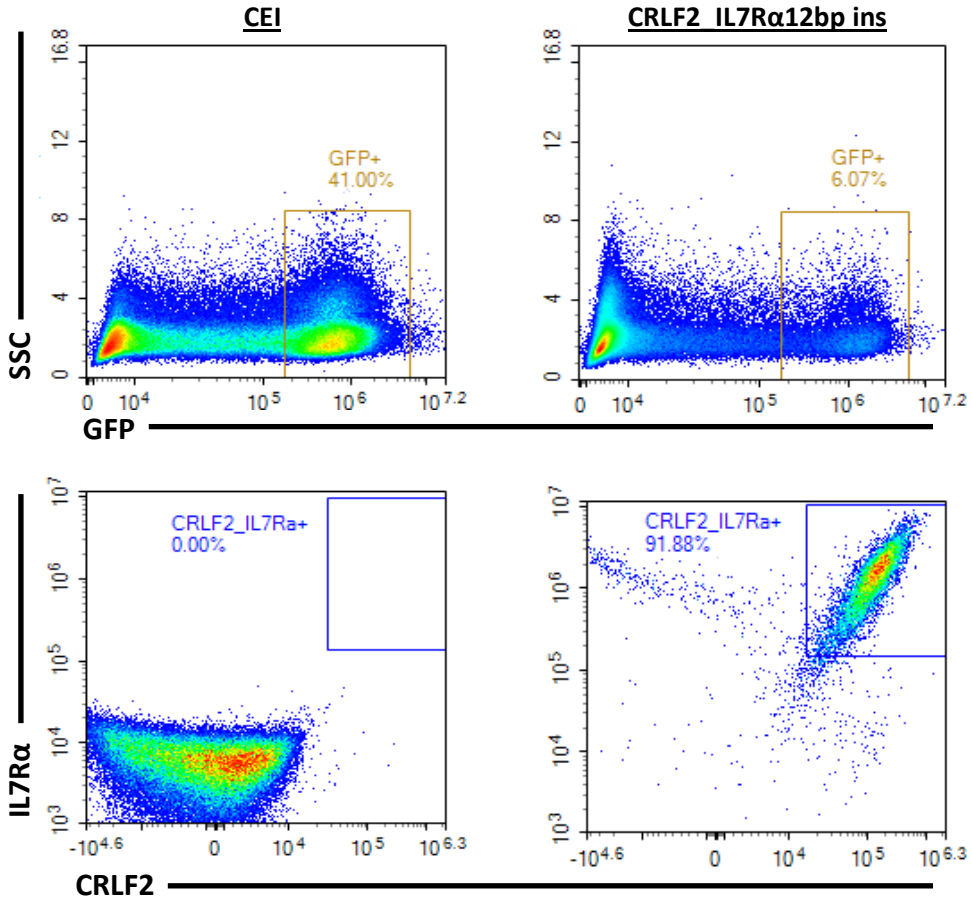


Figure 3.11; A) Schematic of CEI empty and CRLF2_IL7Rα12bp ins lentiviral plasmid maps. Highlighted; EF1α promoter – elongation factor 1α, CRLF2, T2A peptide, IL7Rα12bp ins, IRES – internal ribosome entry sequence, GFP – green fluorescent protein, BglII and SpeI restriction enzyme sites used to clone CRLF2 and or IL7Rα. B) FACS plots of HEK 293T cells transduced with

CEI or CRLF2_IL7Rα12bp ins lentivirus displaying double positive cell surface expression of CRLF2 and IL7Rα.

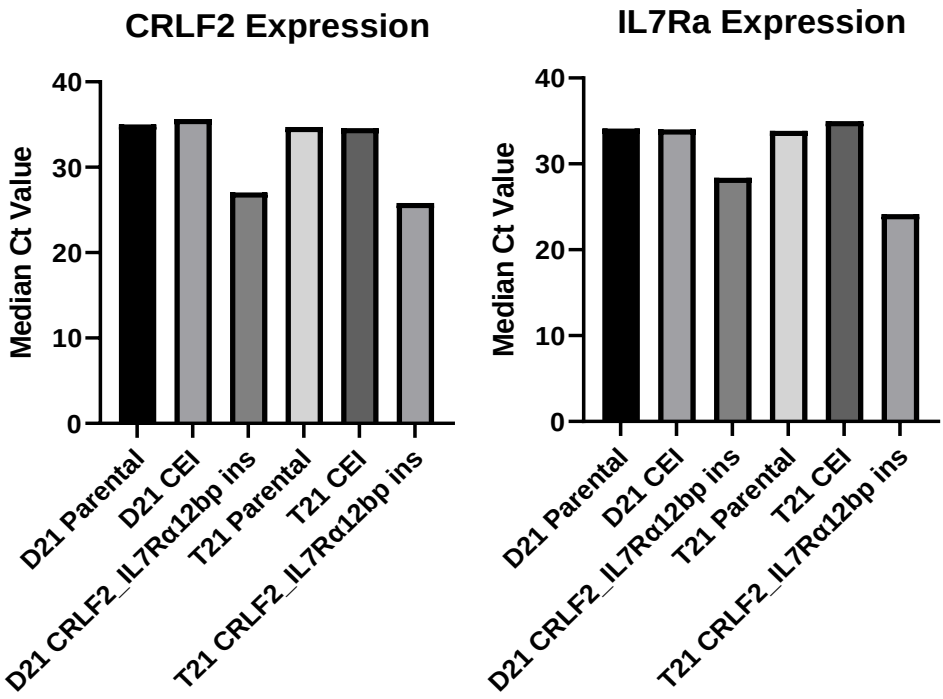
Transduced and stably selected GFP+ isogenic DS-iPSCs display increased expression of CRLF2 and IL7Rα relative to control CEI and isogenic parental cell lines as determined by q-RT-PCR (figure 3.12 a i) however do not display increased cell surface expression of CRLF2 or IL7Rα relative to CEI controls (data not shown). Increased CRLF2 and IL7Rα protein expression was detected upon fixation and permibilisation of CRLF2_IL7Rα12bp ins transduced isogenic DS-iPSCs (figure 3.12 a i). This suggests an iPSC specific down regulation of CRLF2 and IL7Rα surface expression.

To determine the potential of DS-iPSCs overexpressing control CEI, CRLF2 and IL7Rα12bp ins to undergo haematopoeitic differentiation, transduced DS-iPSCs were co-cultured on OP9 stroma as described in methods section 2.1.4.2**.** On day 10 of co-culture samples were dissociated and underwent MACS enrichment for CD34+ haemato-endothelial progenitors and assessed by FACS for CD45+ GFP+ output (figure 3.12 c). Although at a varied frequency CD45+ GFP+ haematopoeitic progenitors were derived from CEI (D21 12.9% + 7.38% and T21 78.6 + 67.15%) and CRLF2_IL7Rα12bp ins (D21 5.8% + 3.78% and T21 70.7% + 43.6%) transduced T21 and D21 iPSCs. The decreased frequency of CD45+ GFP+ haematopoietic progenitors derived from D21 CEI and CRLF2_IL7Rα12bp ins transduced iPSCs suggests that T21 iPSCs have increased potential for haematopoietic differentiation post-transduction with these particular vectors.

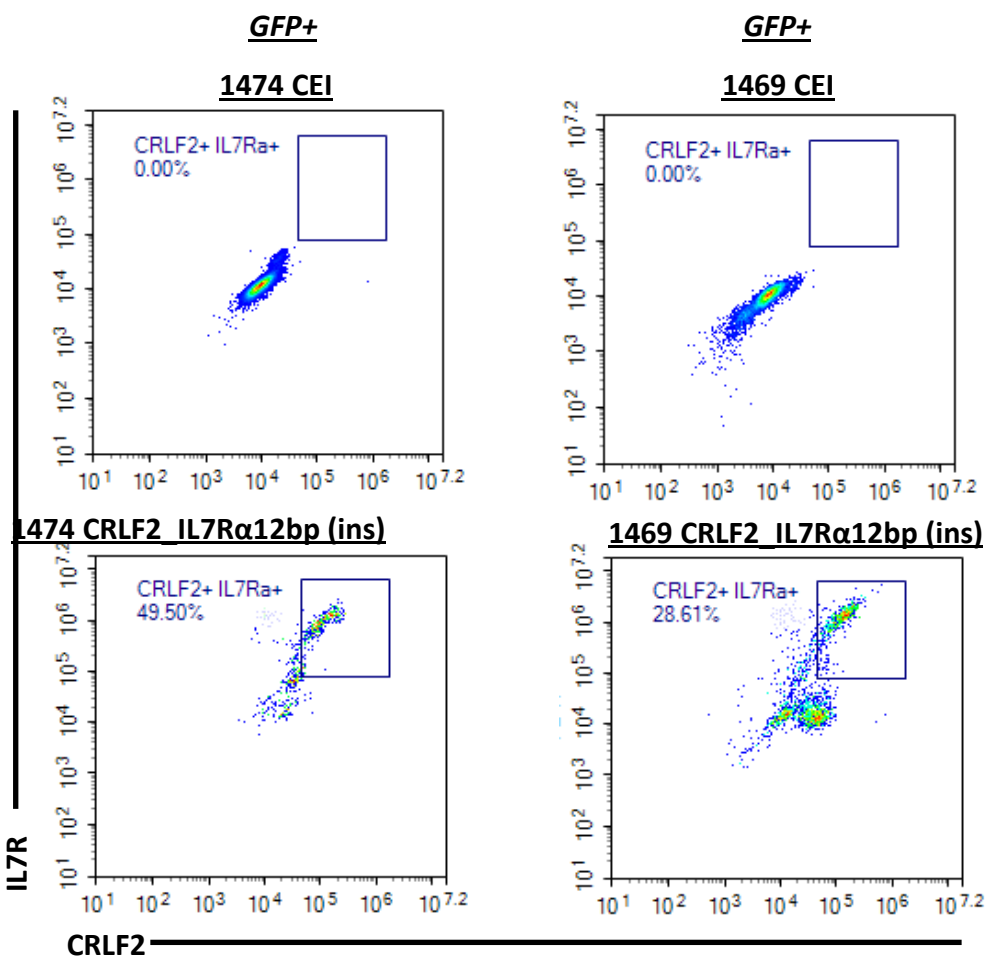
The frequency of GFP expression of CEI and CRLF2_IL7Rα12bp ins stably selected iPSCs decreased during routine cell culture (self-observation), which may explain the experimental variation in GFP expression levels observed at day 10 of OP9 co-culture. I speculate the

observed decrease in GFP expression observed in CEI and CRLF2_IL7R α12bp ins transduced iPSCs during routine culture was due to EF1α promoter silencing as previously described in human ESCs by Norman et al., (Norrman *et al.*, 2010).

A i)

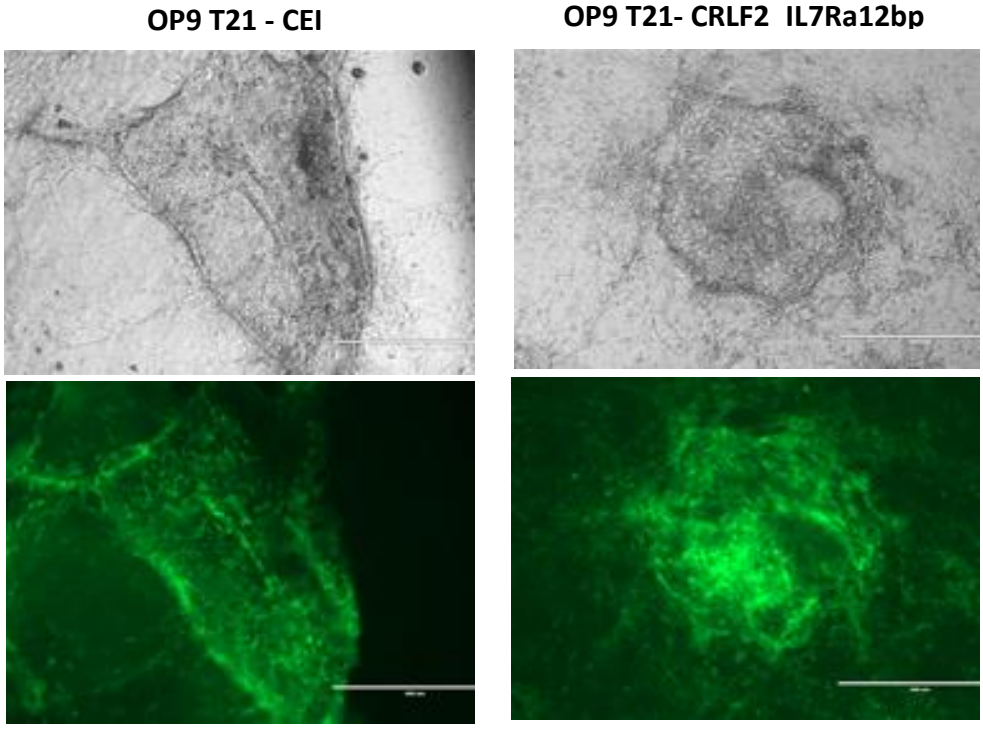


ii)



B)

Day 10 iPSC OP9 co-culture



C)

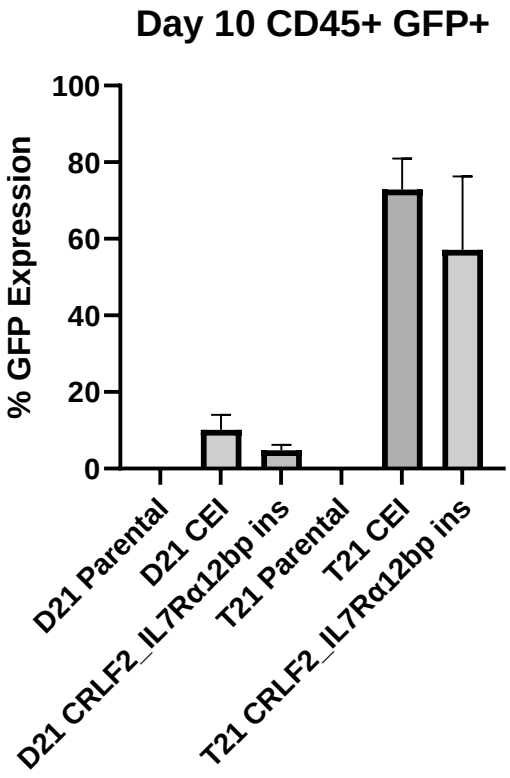
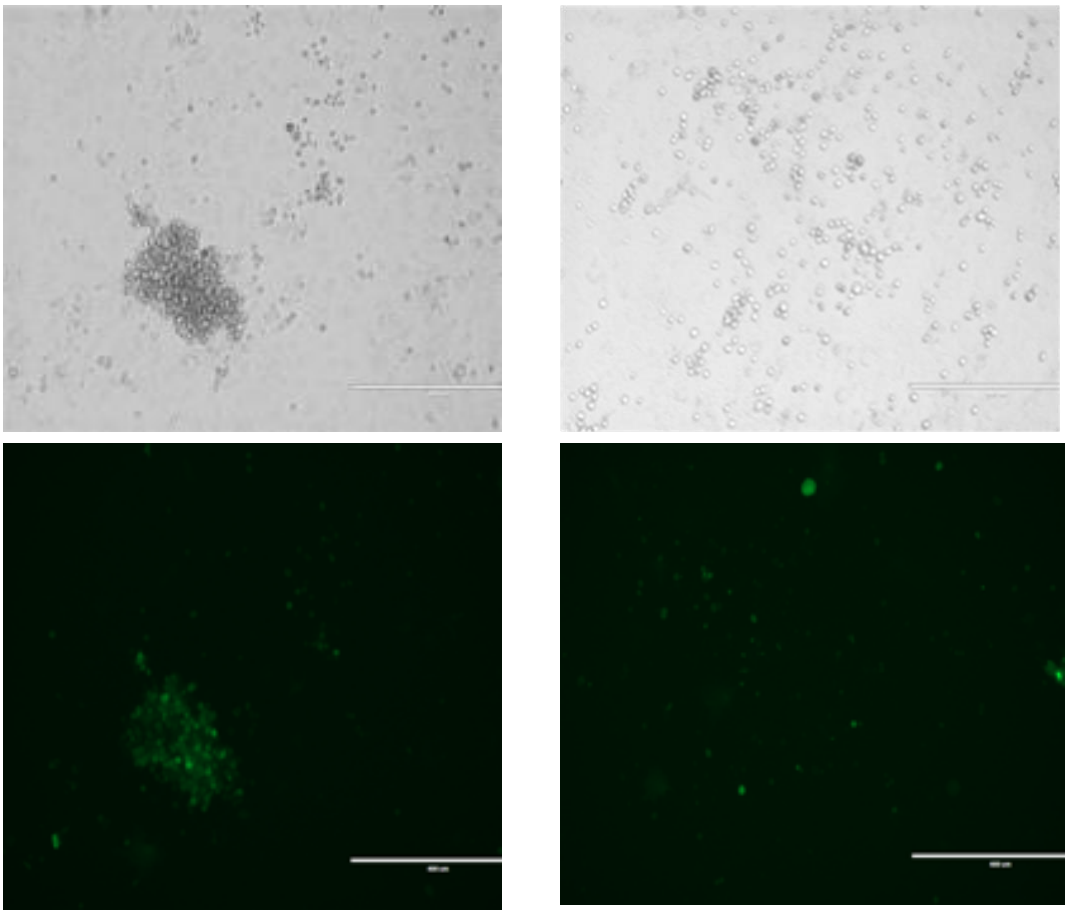


Figure 3.12; Transduced D21 and T21 DS-iPSCs overexpress CRLF2 and IL7Rα and maintain GFP expression during haemato-endothelial transition. A i) D21 and T21 iPSCs transduced with CRLF2_IL7Rα12bp ins lentivirus display increased expression of CRLF2 and IL7Rα relative to parental and CEI control stable cell lines as assessed by q-RT-PCR and display increased intracellular protein expression as detected by FACS post cell fixation and permibilisation (A ii) B) Corresponding brightfield and GFP images of CEI and CRLF2_IL7rα12bp ins transduced T21 DS-IPSCs during OP9 co-culture. C) Frequency of CD45+ GFP+ haematopoietic progenitors derived at day 10 of OP9-iPSC co-culture. CEI and CRLF2_IL7rα12bp ins transduced isogenic DS-iPSCs can undergo haematopoietic differentiation and produce CD45+ GFP+ haematopoietic. Error bars indicate standard deviation.

I next examined the potential of CD34+ haemato-endothelial progenitors derived from transduced DS-iPSCs to undergo B-cell differentiation as described in methods 2.1.4. Control

T21 CEI haematopoietic progenitors maintained GFP expression during the B-cell differentiation protocol in contrast to T21 CRLF2_IL7Rα12bp ins transduced cells whose GFP expression diminished between days 20 - 25 of differentiation (self-observation) and dissipated by day 31 (figure 3.13 b). This suggests a negative selection against CRLF2 and IL7Rα12bp ins overexpressing T21 haematopoietic progenitors during in-vitro B-cell differentiation. The decreased frequency of CD45+ GFP+ haematopoietic progenitors derived from D21 CEI transduced iPSCs relative to T21 CEI may be explained by an initial decreased frequency of CD45+ GFP+ haematopoietic progenitors derived from transduced D21 iPSCs at day 10 of OP9 co-culture (figure 3.12 c above). GFP expression of D21 haematopoeitic progenitors overexpressing CRLF2 and IL7Rα12bp ins similarly disappeared by day 31.

A)



B)

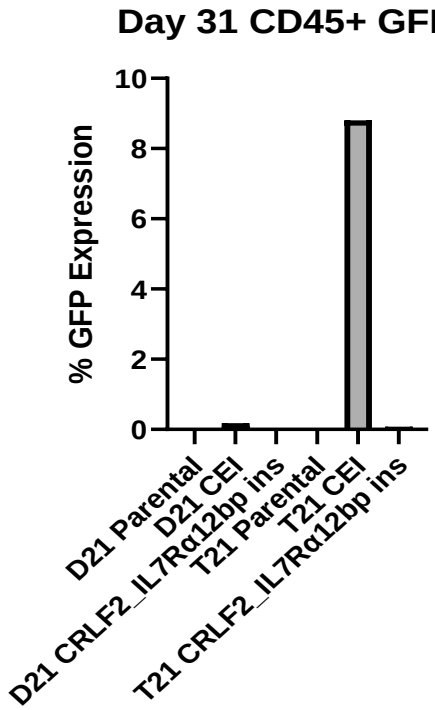


Figure 3.13; GFP+ CRLF2_IL7Rα12bp ins overexpressing haematopoeitic cells undergo a negative selection during B-cell differentiation. A) Brightfield and corresponding GFP images at day 7 of MS-5 co-culture of CD34+ haemato-endothelial progenitors derived from transduced DS-iPSCs. GFP+ haematopoietic progenitors derived from CEI and CRLF2_IL7Rα12bp ins transduced DS-iPSCs maintain a significant level of GFP expression during week 1 of B-cell differentiation. B) Frequency of CD45+ GFP+ expression for T21 CEI and CRLF2_IL7Rα12bp ins haematopoeitic progenitors at day 31 of B-cell differentiation. CEI expressing cells maintained a significantly increased level of GFP throughout the differentiation. GFP expression of CRLF2_IL7Rα12bp ins transduced cells declined after approximately 25 days and dissipated by day 31 of differentiation.

3.1.3 Lentiviral overexpression of DS-iPSC derived CD34+ haemato-endothelial progenitors

I theorised haematopoietic progenitors derived from DS-iPSCs overexpressing both CRFL2 and IL7Rα12bp ins underwent negative selection during B-cell differentiation. I therefore next assessed whether lentiviral overexpression of CEI and IL7Rα12bp ins in DS-iPSC derived CD34+ haemato-endothelial progenitors would persist during B-cell differentiation. In brief, CD34+ progenitors were derived from DS-iPSCs (methods 2.1.4.2) and transduced overnight with CEI or IL7Rα12bp ins lentivirus, samples were washed three times in differentiation media and plated on MS-5 stroma in the presence of IL3, IL7, SCF and Flt3l as described in section 2.1.4.5. 3 weeks post transduction samples were assessed for GFP expression and B-cell output (see figure 3.14 below).

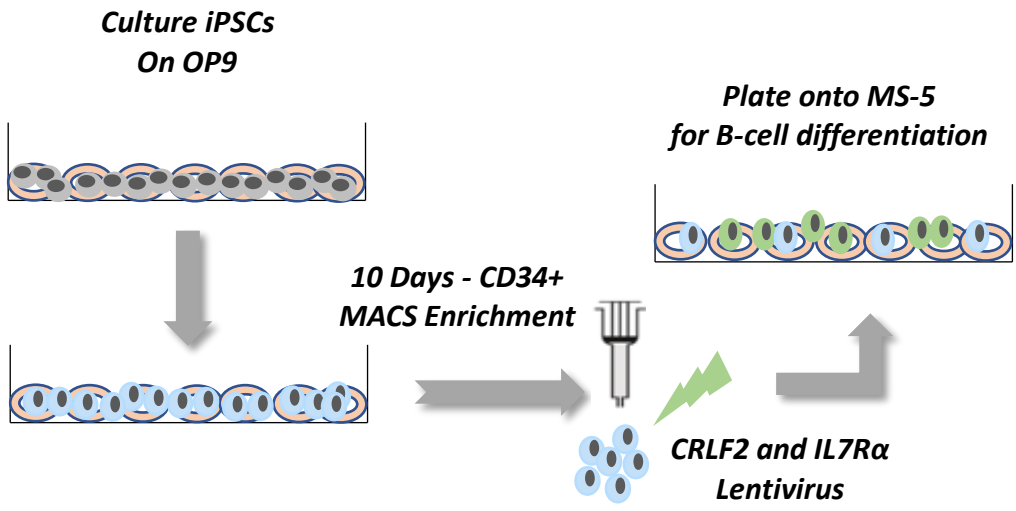


Figure 3.14; Schematic representation of the strategy used to force overexpression of CEI and IL7Rα12bp ins in CD34+ haemato-endothelial progenitors. DS-iPSCs were co-cultured on OP9 stroma for 10 days. CD34+ haemato-endothelial progenitors were enriched by MACS and transduced with CEI or IL7Rα12bp ins lentivirus overnight. Samples were washed three times and cultured on MS-5 stroma for downstream GFP and B-cell analysis.

The IL7Rα12bp ins lentiviral plasmid was generated as described above for the CRLF2_IL7R α12bp ins construct allowing for expression of IL7Rα12bp ins and GFP as a single transcript. To confirm functionality of the generated IL7Rα12bp ins lentivirus HEK 293T cells were transduced with CEI and IL7Rα12bp ins lentivirus and analysed by FACS for IL7Rα cell surface expression after 5 days in culture. All samples were gated on live and GFP+ cells. IL7Rα12bp ins transduced HEK293T cells displayed surface expression of IL7Rα while CEI expressing cells did not (figure 3.15).

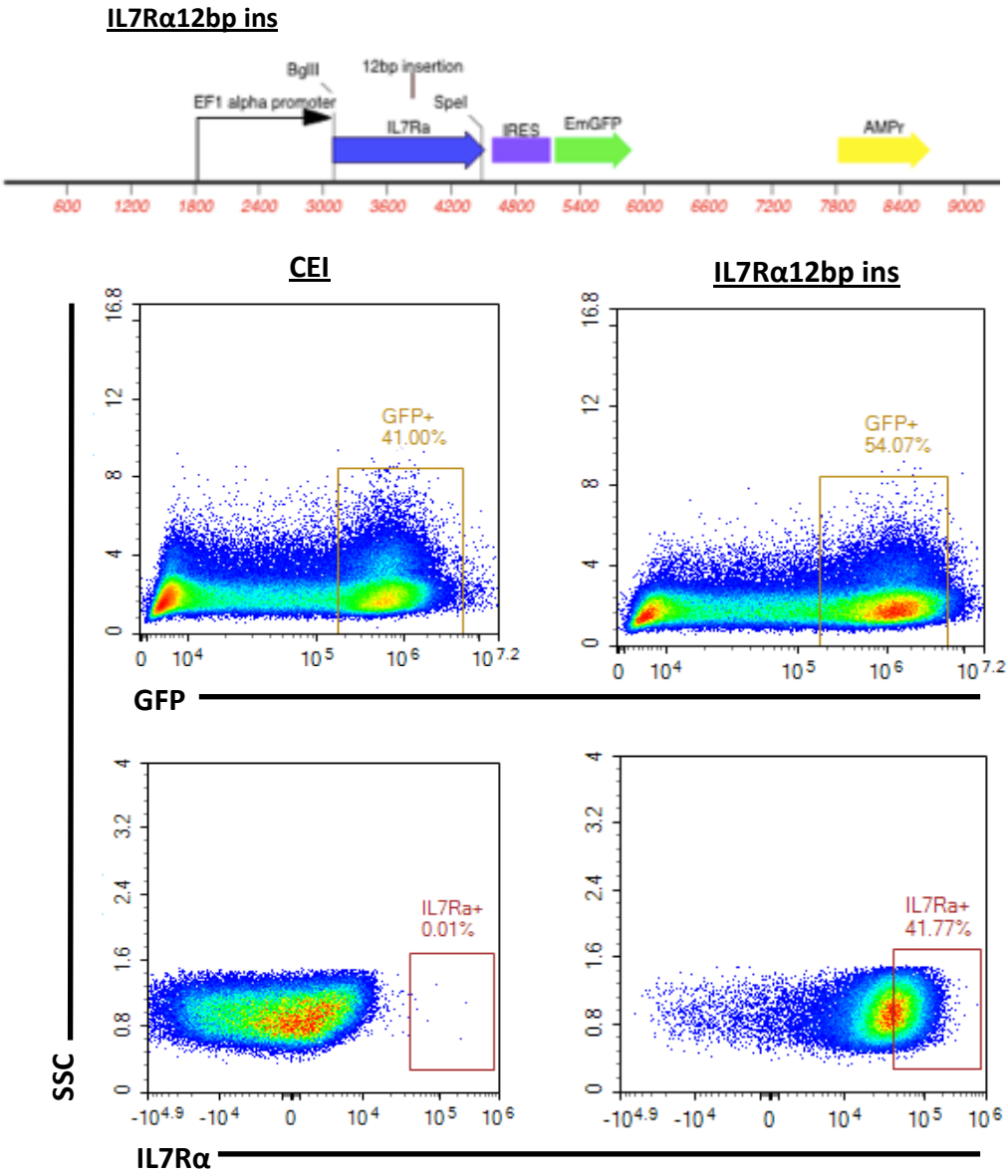
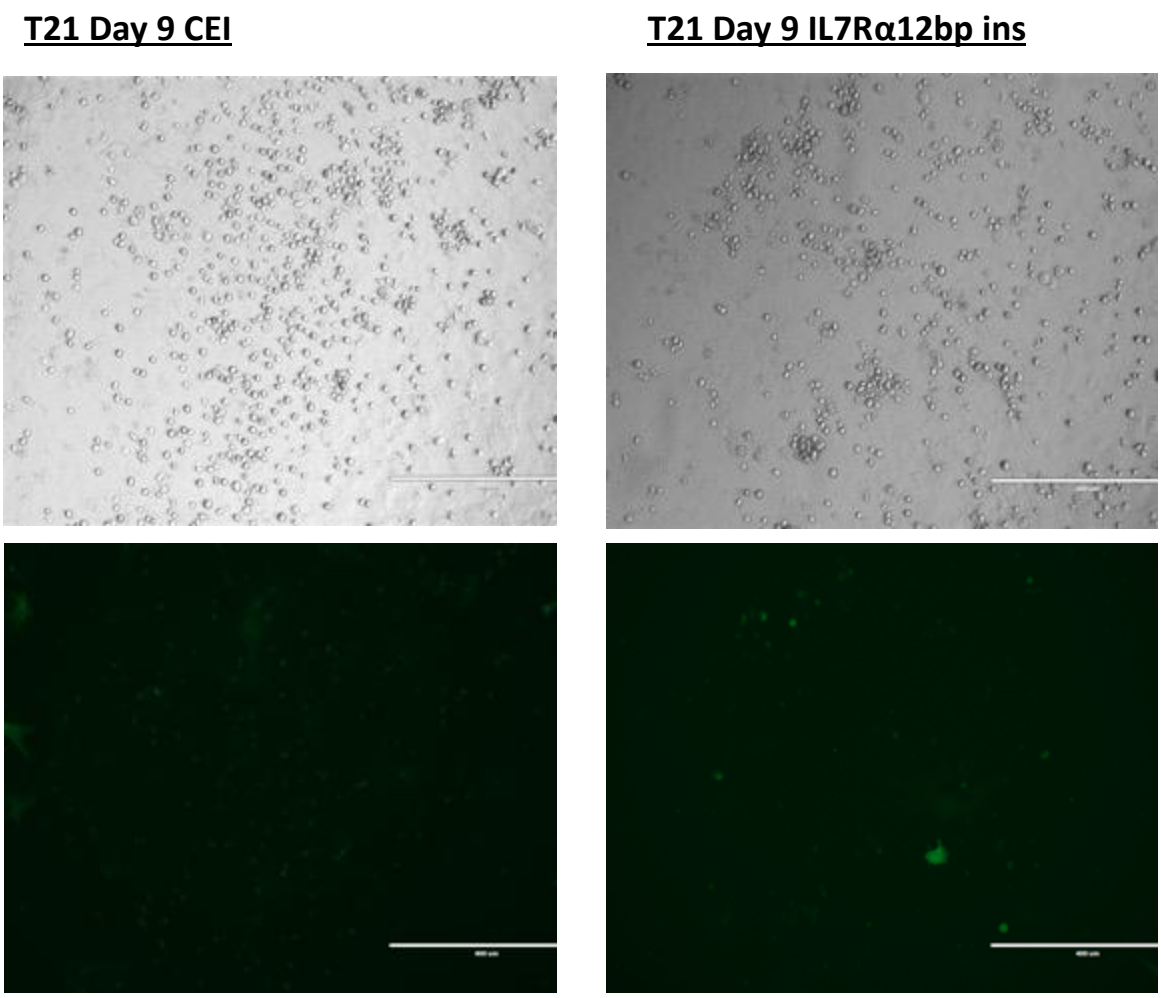


Figure 3.15; FACS plots of HEK 293T cells transduced with CEI or IL7Rα12bp ins lentivirus. IL7Rα12bp ins transduced HEK 293T display cell surface expression of IL7Rα while control CEI transduced cells do not.

T21 and D21 CEI and IL7Rα12bp ins transduced CD34+ haemato-endothelial progenitors express GFP during the first 9 days of MS-5 co-culture (figure 3.15 a). T21 and D21 CEI transduced cells maintained GFP expression (25.1% and 5.5%) during B-cell differentiation. In contrast GFP expression in IL7R12bp ins expressing T21 and D21 HSPCs was significantly decreased (1.1% and 0.1% respectively) by day 31 of differentiation.

A)



B)

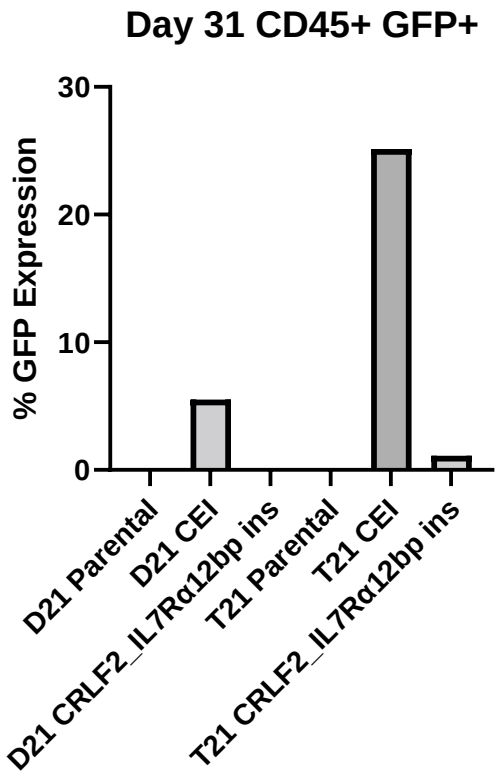


Figure 3.15; GFP expression in CEI and IL7Rα12bp ins transduced CD34+ haemato-endothelial progenitors during B-cell differentiation. A) Brightfield and corresponding GFP images of transduced haematopoeitic progenitors. T21 CEI and IL7Rα12bp ins transduced haematopoeitic progenitors display GFP expression during the first 9 days of MS-5 co-culture. B) FACS analysis displaying T21 CEI transduced haematopoetic progenitors maintain GFP expression during B-cell differentiation in contrast GFP expression of IL7Rα12bp ins overexpressing progenitors diminished during differentiation.

Counter-intuitively these results suggest DS-iPSCs and DS-iPSC derived CD34+ haemato-endothelial progenitors that constitutively overexpress CRLF2 and/or IL7Rα12bp ins undergo negative selection during in-vitro B-cell differentiation. While overexpression of CRLF2 and/or IL7R α12bp ins is initially tolerated during haemato-endothelial transition and the early stages of MS-5 co-culture I speculate that DS-iPSC derived haematopoeitic progenitors

undergo cell autonomous downregulation of the EF1α promoter activity due to potential genotoxic effects of CRLF2 and/or IL7R α12bp ins overexpression.

3.2 Discussion

Children with DS are born with a number of haematological abnormalities as outlined in table 1.1. Due to lack of DS foetal material I have been unable to gain an insight into T21 primitive haematopoiesis. To address this, I aimed to develop a DS iPSC system of early foetal haematopoiesis and examine whether it can be used as a tractable model of DS B lymphopoiesis.

Despite technical variability intrinsic to the B-cell differentiation system, my analysis

1) Demonstrate that isogenic DS-IPSCs can effectively recapitulate the B lymphopoiesis impairment/s observed within second trimester T21 FL; as well as that described in other models of DS and non-DS cALL (Roy *et al.*, 2012; Lane *et al.*, 2014; Böiers *et al.*, 2018; Cheung *et al.*, 2018).
2) Identify a previously uncharacterised LM progenitor compartment as the potential upstream target cell of DS-ALL.

In-vitro B-cell differentiation of iPSCs is technically challenging and the number of $CD19^{+}$ B-cells yielded through this protocol is low. This now withstanding, albeit at a low frequency, my analysis allowed successful derivation and characterisation of immunophenotypically defined pro and preB progenitors from DS-iPSCs. This data therefore provide proof of principle that the differentiation system described here can be successfully utilised to investigate the early stages of B-cell development, where preliminary analysis suggests perturbed DS haematopoietic differentiation and pre-leukaemic initiation likely occurs (Roy *et al.*, 2012). The differences observed in B-cell and IL7RP output between T21 iPS cell lines 1469 and 1470 is potentially due to technical variability of the system. Further subcloning of

disomic and trisomic populations from each cell line and subsequent parallel B-cell differentiations with parental cell lines may control for the observed variability.

T21 is suspected to disrupt foetal haematopoiesis and lineage commitment late in the first trimester as immunophenotypic and functional analysis of second trimester T21 FL revealed an MkE lineage bias within HSCs and subsequent impaired B-cell lymphopoiesisis (Bhatnagar *et al.*, 2016). The speculated impaired ability of the identified LM population to downregulate CD33 as effectively as disomic controls suggests that T21 potentially disrupts the myeloid to lymphoid transition in the CD34+ CD38- LM compartment and may subsequently induce a myeloid lineage bias and a partial block in B-cell commitment. Although the data are preliminary, the ability of sorted T21 B-cells to persist when cultured in myeloid may suggest that T21 cells that are able to escape the differentiation block and undergo B-cell differentiation retain a primitive bi-lineage lympho-myeloid signature. This hypothesis is supported by my RNA-sequencing data described in chapter 4 which display that T21 preB cells exhibit positive enrichment of a myeloid gene expression profile which may underlie the observed impaired proliferative potential of the T21 preB compartment (figure 3.6 b) . Further repetitions of myeloid clonogenic assays are required to confirm enhanced myeloid potential of the T21 B-cell compartment.

If validated, this finding in conjunction with analysis of RNA-seq and assessment of myeloid clonogenic potential of T21 foetal liver LM and B-cell compartments may confirm the validity of the DS-iPSC system and results described herein, provide a unique insight into early DS haematopoiesis, and partly, explain the increased incidence of B-ALL leukaemias in DS children. Furthermore, the observed myeloid clonogenic potential of the B-cell compartment indicates that this differentiation system mimics the lineage programming

and plasticity observed within foetal liver and cALL suggestive of a developmentally relevant system (Notta *et al.*, 2016; Morris *et al.*, 2020).

It is possible that persistence of a bi-lineage signature may induce a lineage confusion, therefore inhibit further B-cell maturation and potentially enhance the sensitivity of B-cell progenitors to acquiring postnatal secondary mutations. While attempts to model constitutive lentiviral overexpression of IL7Rα12bp ins and CRLF2 on DS B lymphopoiesis were unsuccessful a lentiviral system containing an Eμ-B29 B-cell specific enhancer/promoter (kindly provided by Shai Izraeli) will enable overexpression of IL7Rα12bp ins and CRLF2 in the B-cell compartment therefore providing a physiologically relevant model to investigate the synergistic effect of T21 and known oncogenic mutations on leukaemic transformation. I hypothesise that the regulation of IL7Rα12bp ins and/or CRLF2 expression under the Eμ-B29 enhancer/promoter will induce enhanced activation of downstream JAK/STAT/mTORC1 pathways resulting in an increased proliferative state and expansion of the CD19+ B-cell compartment. Similarly, immunophenotypic and transcriptomic characterisation of DS-iPSC B-cell differentiations constitutively overexpressing CRLF2 and IL7Rα12bp ins prior to deterioration of GFP expression may reveal perturbed genetic signatures that disrupt early DS lineage commitment and differentiation and identify candidate drivers of leukaemic transformation. The potential of iPSCs to downregulate cell surface localisation of overexpressed proteins has not previously been reported however has been observed for IL7Rα in other cell types such as CD8+ T-cells by which stimulation with IL7 induces rapid internalisation of IL7Rα from the cell surface. IL7Rα is also regulated at the transcript level in CD8+ T-cells upon hyperactivation of downstream JAK/STAT signalling (Ghazawi et al., 2012). As I detect IL7Rα and CRLF2

transcript and protein and iPSCs are not cultured in the presence of IL7 such reasoning does not explain my observation. I therefore theorise that CRLF2 and IL7Rα undergo protein misfolding when overexpressed in this iPSC system or an iPSC specific disruption in cellular trafficking that perturbs localisation to the cell surface. Single cell qPCR analysis of lymphoid and myeloid lineage associated gene expression in LM, pro and preB compartments may highlight whether the T21 LM compartment potentially initiates a skewed lympho-myeloid signature that is maintained during B-cell commitment and co-expressed in immature B-cells resulting in lineage conflict (see appendices).

Overall these results indicate that this B-cell differentiation protocol can be utilised to decipher the pathophysiological effect of T21 and other primary oncogenic mutations responsible for pre-leukaemic initiation during early foetal haematopoiesis. Specifically, I have immunophenotypically characterised early DS B-cell commitment and differentiation and identified a potential partial block in B-cell commitment at the LM progenitor level, that may similarly act as the target cell for myeloid leukaemias of DS. Moreover, this in-vitro system can be utilised to understand the collaborating effects of T21 and secondary mutations on early B lymphopoiesis and leukaemic transformation at an immunophenotypic, functional and transcriptomic level.

Chapter 4

Molecular Characterisation of DS B Lymphopoiesis from iPSCs

4 Chapter 4

4.1 Molecular Characterisation DS B Lymphopoiesis from iPSCs

4.1.1 Introduction

There is growing evidence that cALL initiates from a pre-leukaemic clone in-utero. Studies of cord blood revealed that the ETV6-RUNX1 cALL subtype is organised in a hierarchy in which a pre-leukaemic stem cell like fraction initiates and propagates the malignancy (Hong *et al.*, 2008). Genomically engineered iPSCs with an inducible knock-in of ETV6-RUNX1 exhibited an expansion of the foetal IL7R^{+} CD19^{-} progenitor due to perturbed myeloid to lymphoid transition resulting in a partial block in B-cell development and the production of proB cells expressing an aberrant lympho-myeloid gene expression profile (Böiers *et al.*, 2018). This evidence suggests that pre-leukaemic initiation for the ETV6-RUNX1 subtype, and also hypothesised for MLL-AF4 and BCR-ABL1 subtypes, occurs within an immature progenitor fraction of the foetal haematopoietic hierarchy and is characterised by lineage confusion (Gale *et al.*, 1997; Cazzaniga *et al.*, 2011).

T21 induces an MkE lineage bias and a concomitant decrease in B-cell commitment in second trimester foetal liver HSCs. Consistent with this, we observed a partial block in lymphoid commitment, associated with altered expression of the myeloid marker, CD33, in the LM compartment (Chapter 3). Hence, I hypothesized that childhood DS-ALL may have a foetal origin and that T21 may induce aberrant expression of lineage programmes in foetal HSPCs, resulting in a pre-leukaemic state and predisposing DS children to develop leukaemia.

The modelling of foetal DS lymphopoiesis has proved technically challenging due to the difficulty in deriving B cells from iPSCs, ES cells or embryoid bodies and therefore very few in-vitro models have been developed (MacLean *et* al., 2012; Banno *et al.*, 2016; Chiang *et al.*, 2018). Derivation of $CD45^{+}CD34^{+}CD19^{+}$ haematopoietic progenitors from DS and isogenic control embryoid bodies revealed a decrease in trisomic $CD19^{+}$ B cells emerging in comparison to isogenic controls. However, immunophenotypic identification and transcriptomic characterisation of more immature progenitor cells, where defective DS haematopoiesis and B-cell development is hypothesised to initiate, has not been described (MacLean *et al.*, 2018).

As I had observed a defect in B cell lymphopoiesis and perturbed LM immunophenotype in T21 cells, I aimed to characterise the impact of T21 on early DS lymphopoiesis. RNA-seq analysis of HSPCs, LM and B-cell populations would provide a transcriptomic trajectory of early DS lymphopoiesis and facilitate the identification of pathways and genes associated with the disrupted LM immunophenotype described in chapter 3.

4.1.2 Transcriptomic analysis of isogenic DS-iPSC B-cell differentiation

To transcriptionally characterise the effect of T21 on DS lymphopoiesis I used global RNA seq to compare isogenic D21 and T21 cells from four immunophenotypically defined cellular compartments representing different stages of haematopoietic differentiation. $CD34^{+}$ MACS was used to enrich for HSPCs after 10 days of OP9-iPSC co-culture and the LM, proB and preB populations were FACS sorted from day 29 of the B cell differentiation as described in chapter 3. Isogenic D21 and T21 samples were harvested in parallel, and LM, proB and preB samples were obtained in triplicate from independent differentiation cultures for each cell

line, facilitating statistical analyses – one disomic preB sample was filtered out during QC analysis. Due to the low frequency of B-cell production 50-150 cells were sorted for each of the pro and preB populations. This necessitated use of an adapted SmartSeq2 protocol optimised for low input.

To assess the relationships between the 4 populations we performed principal component analysis (PCA). This revealed that $CD34^+$ MACS enriched samples cluster separately from LM, pro and preB cell progenitors indicating they are transcriptomically distinct. D21 and T21 $CD34^+$ MACS enriched cells group closely together suggesting that chromosome 21 status is not a major source of transcriptional variability in the CD34+ population (figure 4.1 A). To better visualise variability between LM, proB and preB populations, $CD34^+$ cells were excluded (figure 4.1 B). Pro and preB cells separate along the PC2 axis. Consistent with B-cell maturation the genes VPREB3, CD79A, CD79B and IGHM, which are associated with the formation of the preB cell receptor complex and B-cell differentiation, contributed most to the variance on PC2. Notably, T21 preB cells cluster more closely to proB cells than D21 preB samples suggesting impaired B-cell differentiation. T21 LM populations separate from D21 LM and all B-cell populations on PC2 displayed in figure 4.1 A and PC1 in figure 4.1 B. This suggests that T21 has its greatest impact on the gene expression profile of the LM progenitor which may subsequently impact B cell development.

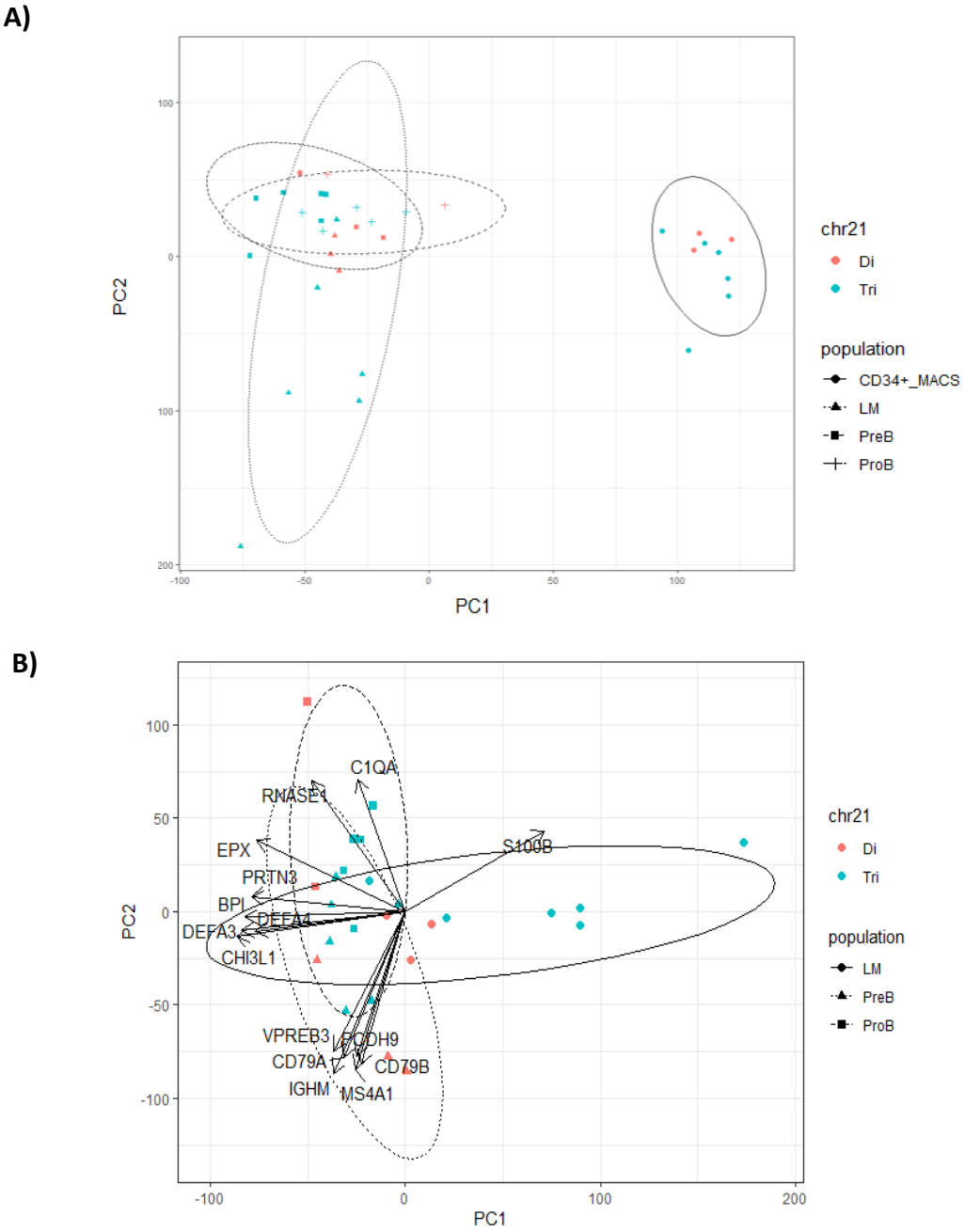
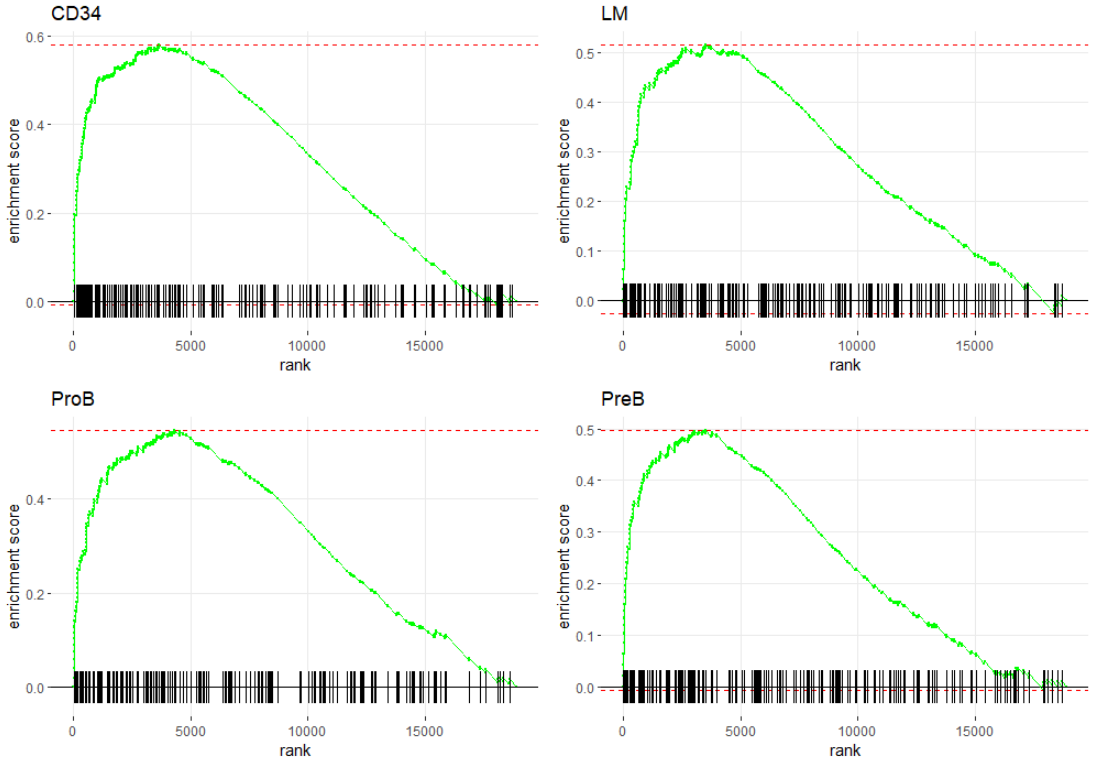


Figure 4. 1: PCA plot from RNA-seq data of DS-iPSC derived CD34+, LM, pro and preB populations. PC1 and PC2 are shown. Each dot represents an individual sample. Ellipses indicate grouping. A) Analysis of day 10 MACS enriched CD34+ and FACS sorted LM, pro and preB populations from day 29 of the differentiation protocol. B) CD34+ samples were excluded from analysis revealing T21 LM cells cluster independently and T21 preB cells cluster nearby proB samples indicating impaired B-cell development. Arrows indicate the

top 15 differentially expressed genes that contribute most to the variance of PC1 And PC2.

It is expected that expression of genes located on chr 21 is increased in DS cells. To assess the relative expression of chr 21 genes between T21 and D21 during lymphopoietic specification we employed gene set enrichment analysis (GSEA), testing for enrichment of genes grouped by chromosome. Using the DESeq2 program to determine differential gene expression, genes were ranked from the most significantly up-regulated to the most significantly down-regulated in T21 vs D21 for each of the four cell populations (Love, Huber and Anders, 2014). In all four populations GSEA revealed that the most significantly enriched gene set was chr 21 showing that throughout early B lymphoid commitment and B-cell differentiation T21 is associated with higher expression of genes located on chr 21 (figure 4.2 A and B).

A)


CD34
LM
ProB
PreB
enrichment score
rank
0
5000
10000
15000
0.0
0.1
0.2
0.3
0.4
0.5
0.6

B)

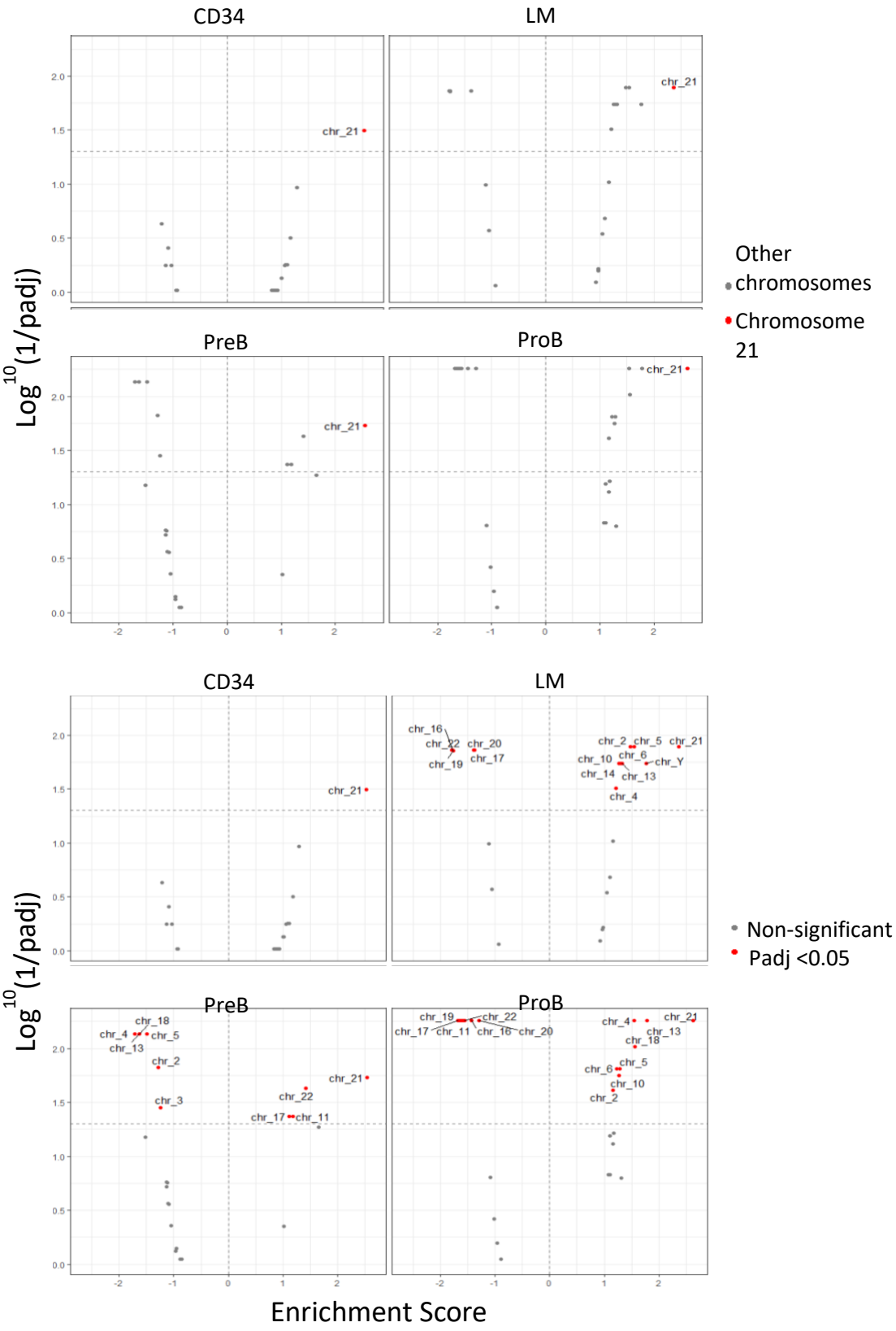


Figure 4. 2: A) GSEA Enrichment plots highlighting an increase in chromosome 21 gene sets in CD34+ MACS enriched, LM, pro and preB samples relative to D21 controls. B) Volcano plots of

GSEA results for chromosome gene sets T21 vs D21 ranked gene lists for each of the CD34+ MACS enriched, LM, pro and preB populations. Enrichment scores were calculated using GSEA for T21 vs D21 progenitors. Positive enrichment score indicates association with genes more highly expressed in T21. The horizontal dashed line indicates padj=0.05. Statistical significance was calculated using the fast set gene set enrichment analysis (fgsea) R package (Subramanian et al., 2005, Korotkevich et al., 2021).

To identify differentially expressed genes (DEGs) between T21 and D21 populations during early lymphopoietic commitment differential gene expression analysis using DESeq2 was completed for each of the four cell compartments. $CD34^{+}$, pro and preB populations had relatively few DEGs suggesting a lesser impact of T21 in these compartments. In contrast, T21 LM progenitors show clear clusters of increased and decreased gene expression profiles in comparison to the D21 LM population (351 genes upregulated and 50 downregulated padj < 0.05). This suggests that it is the LM transcriptome that is most sensitive to T21 during B-cell differentiation.

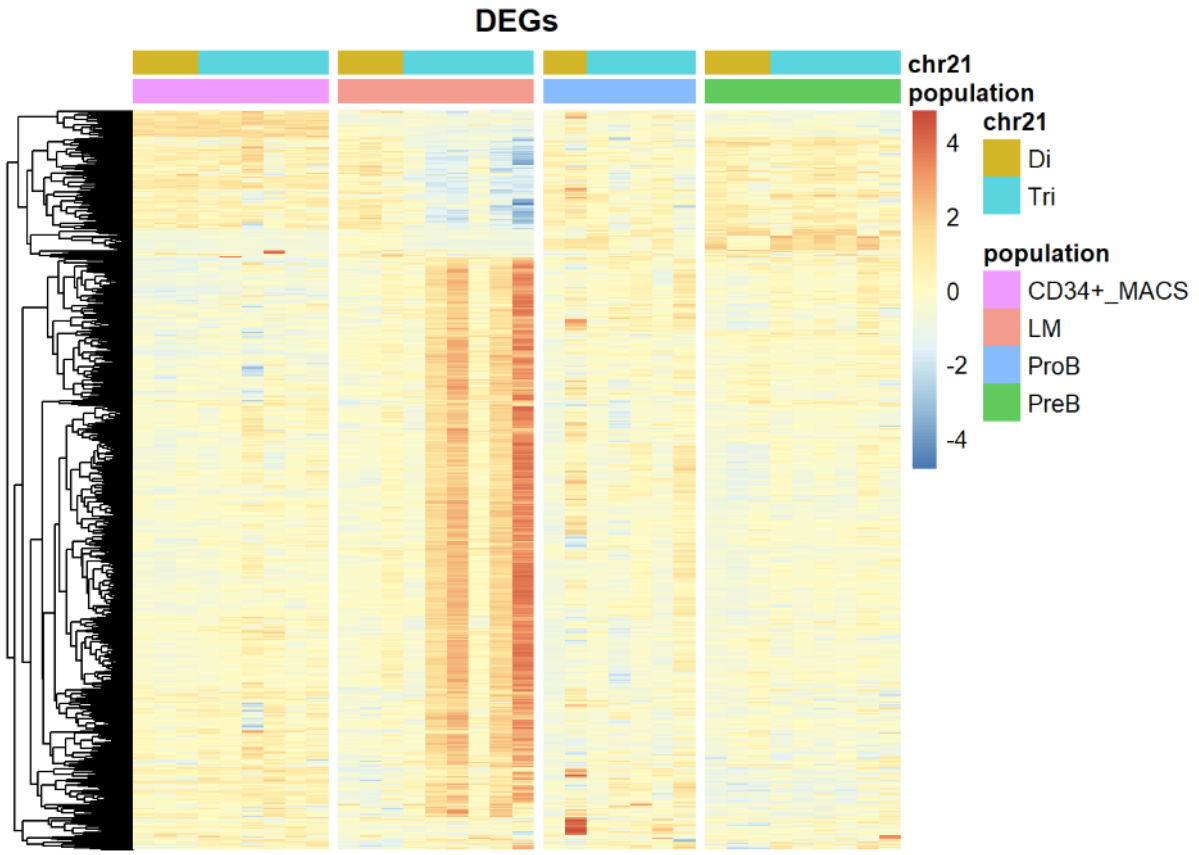


Figure 4.3: Differential gene expression across T21 and D21 CD34+ MACS enriched, LM, proB and preB cells from day 29 B-cell differentiation. T21 and D21 CD34+, pro and preB populations display relatively few DEGs. T21 LM compartment exhibits clusters of increased and decreased gene expression profiles. Each column represents either disomic (brown) or trisomic (blue) samples and each row signifies an individual gene.

I next identified genes on chr 21 that are up or downregulated in each of the four populations that may be responsible for the perturbed B-cell commitment described in chapter 3. Genes that are under or overexpressed relative to D21 samples are illustrated in the volcano plots (figure 4.4, chr 21 genes highlighted in red). The LM population exhibited the highest number of chromosome 21 genes significantly up-regulated with a small number of chr 21 genes differentially expressed in $CD34^{+}$, pro and preB populations. $CD34^{+}$ and preB populations did display increased expression in the chr 21-encoded pre-mRNA

splicing factor U2AF1 and the mitochondrial protein C21orf33, which was also increased in proB cells. A number of previously described chr 21 genes associated with DS leukaemic development in human and/or mouse models had significantly increased expression within the LM population: ATP5J (mitochondrial respiratory chain) (Malinge, et al, 2013), SOD1 (antioxidant enzyme) (Khan, Malinge and Crispino, 2011), PSMG1 (proteasome assembly) (Laurent *et al.*, 2020) and SON (RNA splicing) (Bourquin *et al.*, 2006) (figure 4.4 B). Interestingly, among the genes downregulated in the LM progenitor were a number of non-chromosome 21 genes involved in early B-cell commitment - IGLL5, VPREB3, and CD19 - suggesting impaired lymphoid potential.

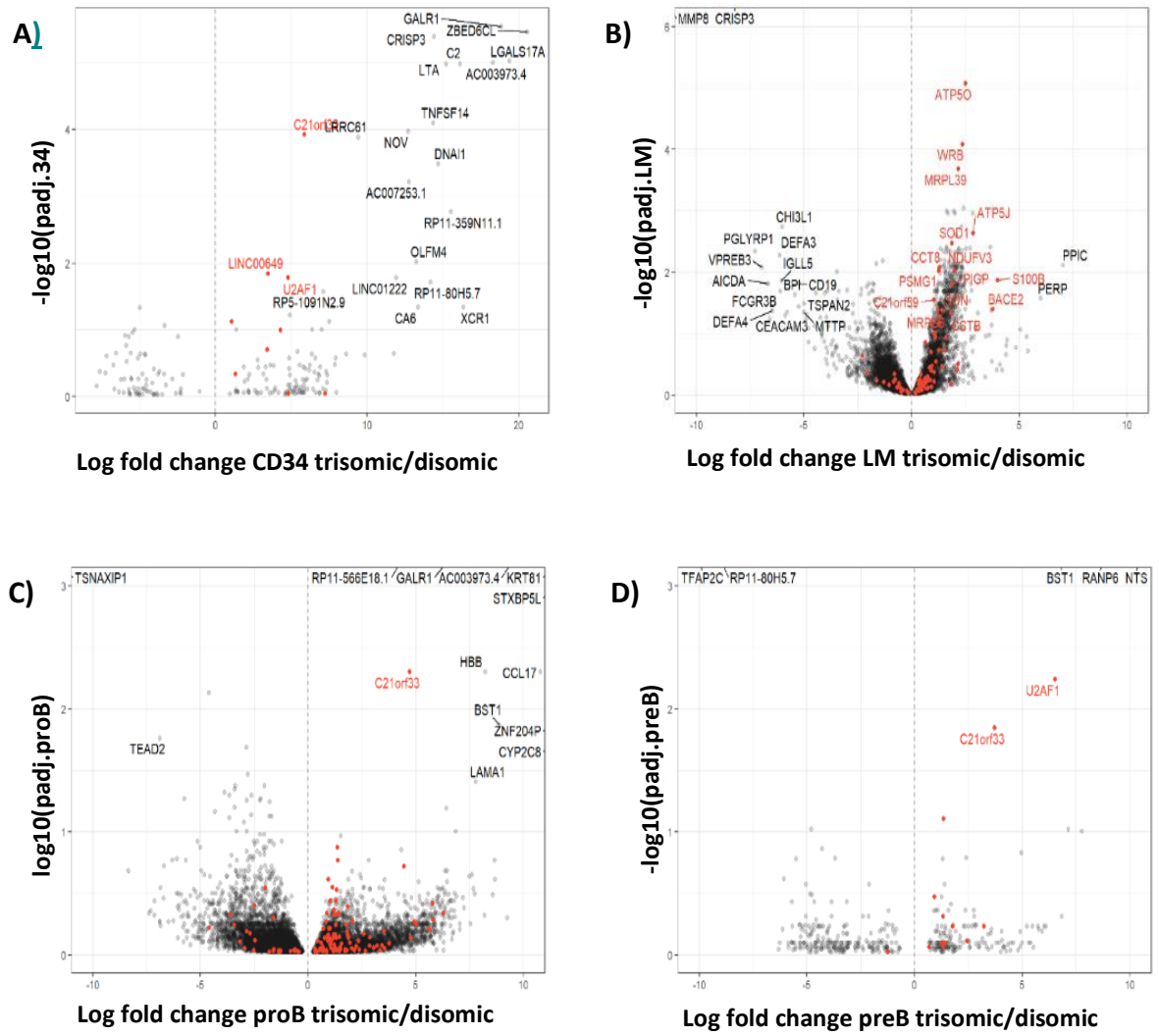


Figure 4.4: Enrichment of chromosome 21 genes across (A) CD34+ MACS enriched and sorted (B) LM, (C) pro and (D) preB samples. Genes upregulated are right of the vertical axis and genes downregulate on the left. Chr 21 genes are highlighted in red.

To examine whether gene expression changes associated with T21 in the LM population were maintained in committed B progenitor cells, log fold change in LM was plotted against log fold change in pro or preB for all DEGs in LM. There was a high degree of correlation across all genes and chr 21 genes upregulated in the LM progenitor were invariably up-regulated in pro and preB populations. This indicates that gene expression differences

established in the LM progenitor are largely maintained as the cells undergo B-cell differentiation. Chr 21 genes significantly up-regulated in the LM progenitor and up in pro and preB populations are displayed in figure 4.5 and listed with their functions in table 4.1. The increased expression of genes previously described in DS leukaemia (e.g. HMGN1 and CSTB) or important regulators of haematopoiesis (SOD1 and SON) may disrupt LM lymphopoietic commitment and potentially induce the partial block in B-cell commitment (described in figure 3.8) and associated decrease in DS-iPSC B-cell output.

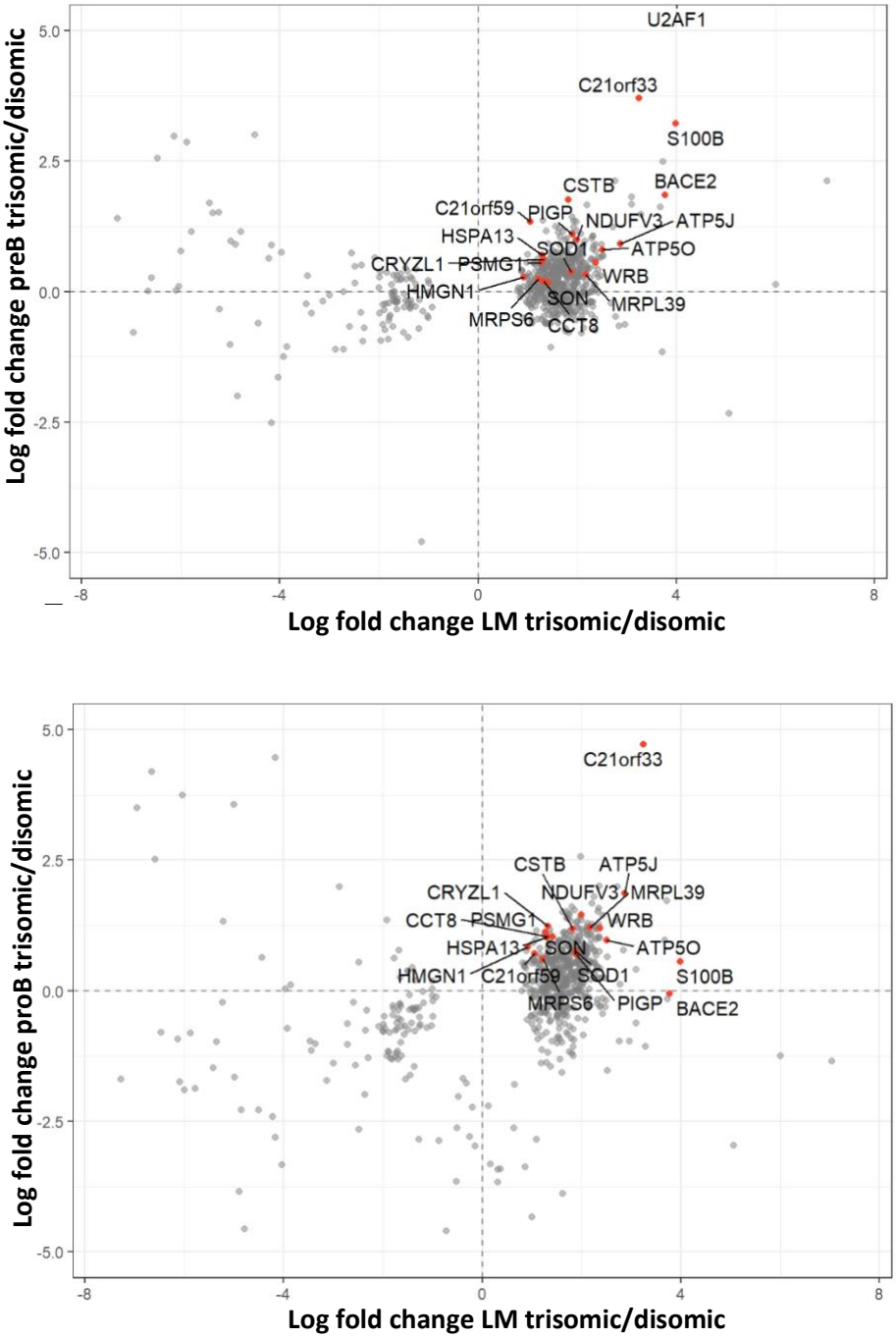


Figure 4.5: Chr 21 genes significantly upregulated in the LM population also displayed increased expression in pro and preB populations. Genes located on chromosome 21 are represented in red.

Gene Name	Function	Cited in Leukaemia
NDUFV3	Mitochondrial respiratory chain	Yes (Flis et al,. 2012)
ATP5J	Mitochondrial ATP synthase	Yes DS-AMKL (Malinge et al,. 2013)
MRPL39	Mitochondrial ribosomal protein	No
WRB	Tryptophan-rich basic protein – unknown function	Yes (Laurent et al,. 2020)
ATP50	Mitochondrial ATP synthase O subunit	Yes iAMP21 (Strefford et al,. 2006)
MRPS6	Mitochondrial ribosomal protein	No
C21orf59	Unknown Function	No
HMGN1	Nucleosome Remodelling Complex	Yes DS-ALL(Mowery et al,. 2018)
CCT8	Involved in the folding of newly synthesized multidomain proteins	No
PSMG1	Proteasome assembly chaperone	Yes (Laurent et al,. 2020)
CSTB	Protease Inhibitor	Yes (Roberts and Izraeli 2014)
CRYZL1	Quinone oxidoreductase	Yes iAMP21 (Ofverholm et al,. 2019)
SON	mRNA splicing co-factor	Yes AML (Kim et al,. 2016)
C21orf33	Glutamine amidotransferase	No
U2AF1	*Pre-mRNA splicing factor*	*Yes MDS and ML (Shirai et al,. 2015)*
BACE2	*Beta-site amyloid beta A4 precursor protein-cleaving enzyme*	*No*

Table 4. 1: List of chr 21 genes and their respective functions that are upregulated in the LM population and maintain an increased level of expression during B-cell differentiation. Genes that display increased expression in LM and preB populations alone are indicated by italics.

While altered dosage of chr 21 genes is likely responsible for the phenotypes I have described, downstream pathways affected will not be confined to chr 21 alone. To identify pathways impacted by T21 in the LM population we performed GSEA using the hallmark genesets from molecular signature database (MsigDB) (Subramanian *et al.*, 2005). The LM population exhibited positive enrichment for; Myc targets (proliferation), mTORC1 signalling

(protein synthesis), E2F targets (cell cycle) and the reactive oxygen species pathway (metabolism). This suggests that T21 increases proliferation of the LM progenitor and may result from increased MYC activity and/or and protein synthesis downstream of mTORC1. The T21 LM population also displayed enrichment of the ROS hallmark. NDUFV3, ATP5J and ATP5O are components of the mitochondrial respiratory chain encoded on chr 21 that were significantly up-regulated in LM T21 cells. The activity of these proteins has previously been described to increase ROS, suggesting a direct link between T21 and the ROS pathway (Flis *et al.*, 2012). Furthermore, PSMG1, similarly located in the DSCR and a target of the mTORC1 pathway, is up-regulated in T21 LM population and interestingly displays increased expression in cALL that gain chr21q22.2 (Schiffmana *et al.*, 2009).

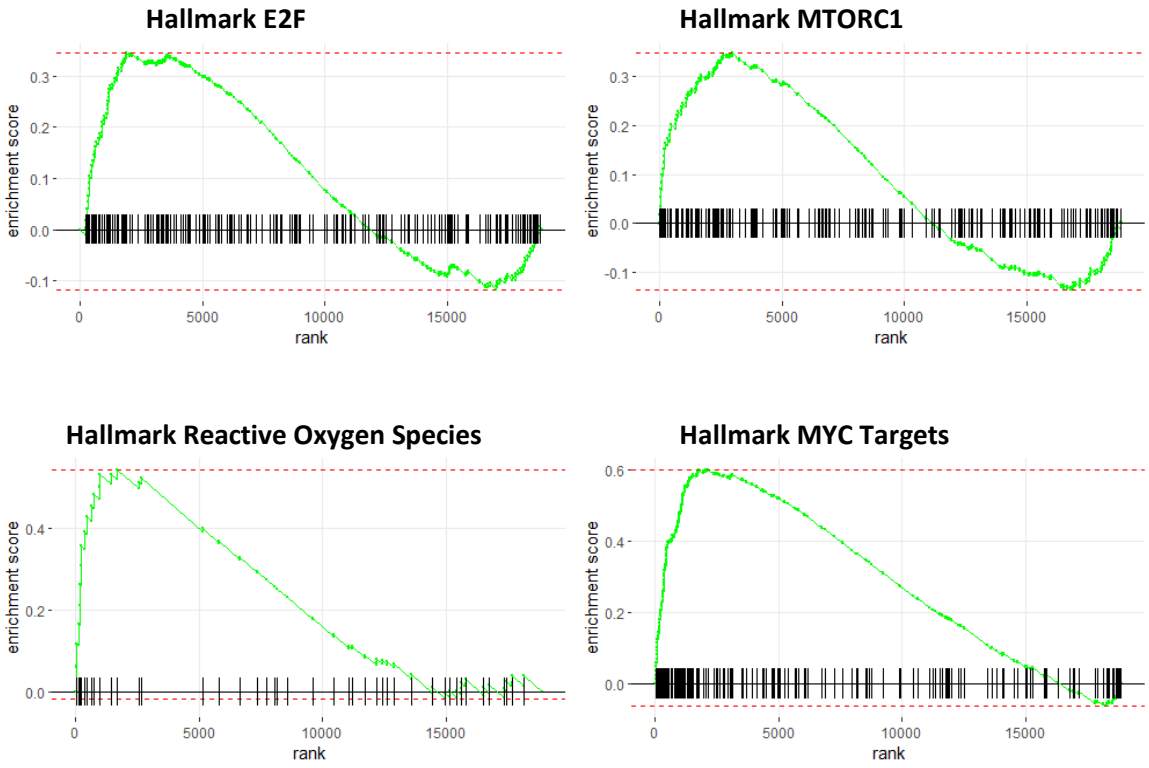


Figure 4.6: GSEA pathway signatures positively enriched within the LM population relative to D21 corresponding population; E2F targets, MTORC1 signalling, reactive oxygen species pathway and MYC targets. P-value <0.05.

I next analysed if the chr 21 induced gene expression profile in the T21 LM population is similar to that observed in leukaemic samples with intrachromosomal amplification of chr 21. To do this we used publicly available cALL gene expression data from the St. Jude cloud PeCan dataset and identified genes that are up or downregulated in iAMP21 ALL relative to other B-ALL subtypes. The T21 LM progenitor displayed substantial overlap in chr 21 genes that are upregulated in iAMP21 leukaemia, exhibiting increased expression in a number of genes involved in generation of ROS and haematopoietic development: NDUFV3, ATP5O, WRB, MRPL39, ATP5J, SOD1, CCT8, PSMG1, PIGP, C21orf59, SON and BACE2 (Flis *et al.*, 2012; Roberts and Izraeli, 2014).

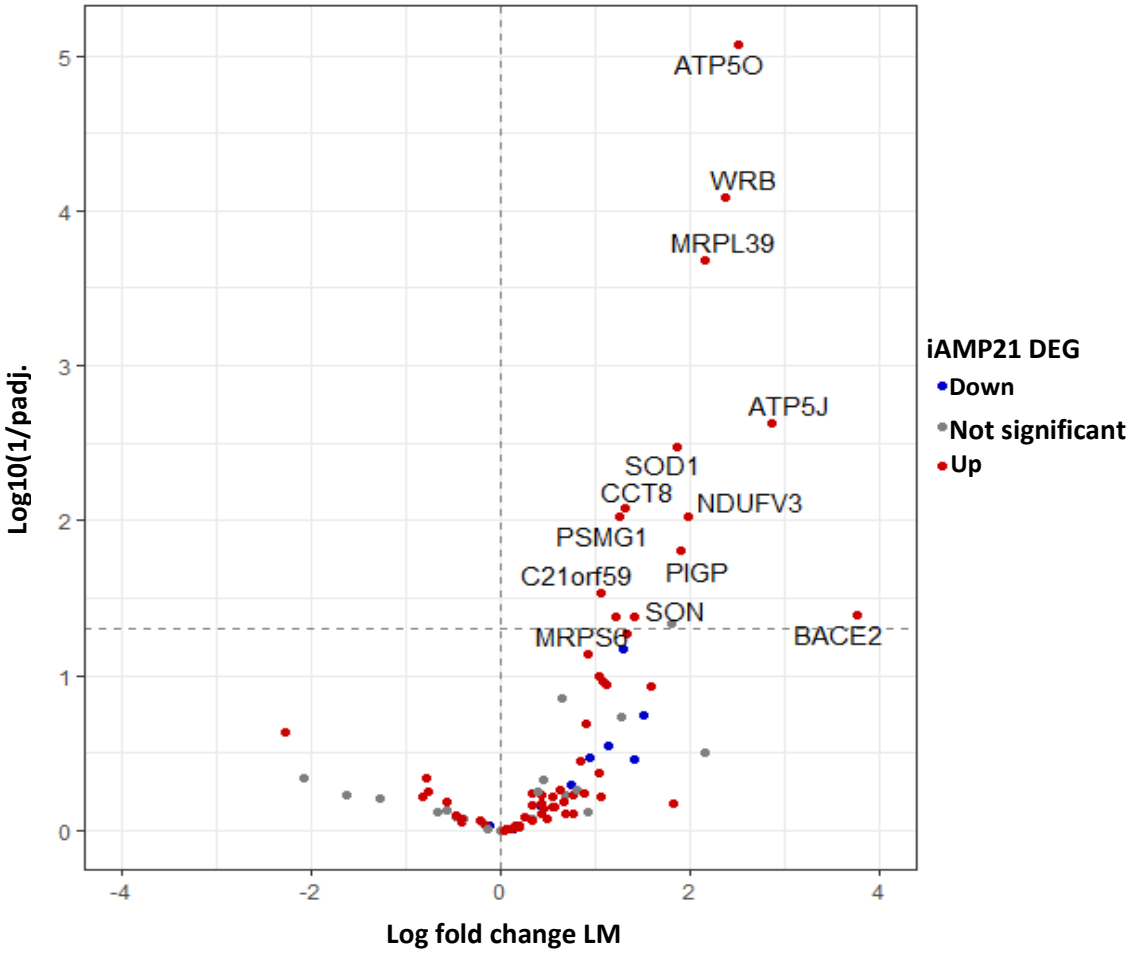


Figure 4.7: Correlation of chr21 genes that are up or downregulated in T21 LM and iAMP21 ALL. **Volcano plot displaying log fold change T21/D21 (x-axis) vs log10(1/padj) in the LM populations for**

chr 21 genes. Colours correspond to DEGs in iAMP21 relative to other B-ALL subtypes: red = upregulated, blue = downregulated and grey = not significant.

The altered expression of CD33 in the T21 LM compartment suggests a failure of lineage resolution in the LM bipotent progenitor that may be responsible for impaired lymphoid commitment and decreased B-cell output (figure 3.8A). I therefore examined the impact of T21 on lineage affiliated gene expression programmes within each of the four populations. To do this I used previously described gene expression signatures for HSC, MEP, GMP, CMP, ETP and proB lineages – derived by comparing gene expression profiles in immunophenotypically defined progenitor compartments - and examined their enrichment in each of the four populations (Laurenti *et al.*, 2013). Table 4.2 below shows the normalised enrichment score (NES) for significantly enriched signatures (p-value <0.05, padj-value <0.1) within the four populations.

The T21 LM progenitor exhibited a positive enrichment for HSC and CMP haematopoietic lineage expression signatures and a negative enrichment in the proB signature. This suggests that the transcriptional programme of the T21 LM progenitor retains a more primitive 'stemness-like' gene expression signature with a myeloid bias and a concomitant decrease in lymphoid specification. Surprisingly T21 pro and preB cells displayed opposing enrichment of lineage associated signatures. T21 ProB cells were positively enriched for ETP and proB signatures and showed negative enrichment for HSC and myeloid signatures, while preB cells displayed positive enrichment of HSC and myeloid signatures. Further analysis will be required to understand this gene expression pattern. However, the enrichment of stem and myeloid gene sets within the preB population suggests a bi-lineage gene expression

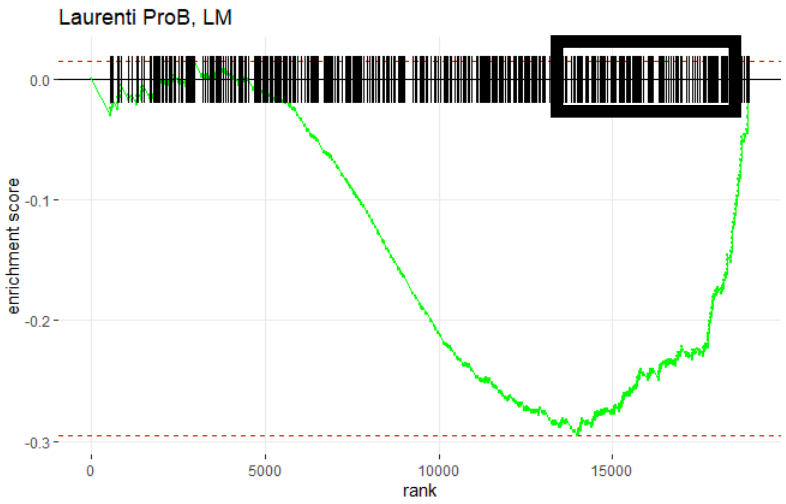
signature, consistent with the finding that T21 preB cells have the ability to undergo lineage switching and proliferate when cultured in myeloid conditions (figure 3.8 C).

Population	Pathway	NES	pval	padj
CD34	ETP	1.356143	0.009	0.03
CD34	ProB	1.391803	0.002	0.01
LM	HSC	1.414997	0.001	0.009
LM	CMP	1.21456	0.03	0.09
LM	ProB	-1.6006	0.003	0.01
ProB	HSC	-1.38033	0.001	0.002
ProB	MEP	-1.8207	0.001	0.002
ProB	GMP	-1.83971	0.001	0.002
ProB	CMP	-1.86299	0.002	0.002
ProB	ETP	1.662828	0.001	0.002
ProB	ProB	1.51129	0.001	0.002
PreB	HSC	1.1872972	0.01	0.01
PreB	GMP	3.205082	0.006	0.01
PreB	MEP	2.504647	0.005	0.01
PreB	CMP	2.44626	0.006	0.01

Table 4. 2: NES of gene set enrichment analysis of the Laurenti et al,. 2013 haematopoietic lineage gene expression signatures within the CD34+, LM pro and preB populations. The LM population is positively enriched for HSC and myeloid gene expression programmes while pro and preB compartments display opposing enrichment for lineage signatures, proB: ETP and proB and preB: stem and myeloid signatures respectively. LM, pro and preB samples were FACS sorted from day 29 of the B cell differentiation protocol. Green indicates positively enriched and red negatively enriched. P-value = <0.05, padj-value <0.1. Statistical significance was calculated using the fast set gene set enrichment analysis (fgsea) R package (Subramanian et al., 2005, Korotkevich et al., 2021).

As T21 had the strongest impact on the LM progenitor I further examined the significant GSEA results from this population. Leading edge genes are those genes that contribute most to the enrichment signal (Figure 4.8A). T21 LM leading edge genes displayed increased expression in HSC and CMP signatures and a decrease the proB signature suggestive of increased stem and myeloid potential. Differences in haematopoietic signature gene expression across the individual samples is illustrated in the heatmap in figure 4.8B. The leading edge from the proB gene set includes a number of key genes involved in early B-lymphopoietic commitment and differentiation; VPREB1, VPREB3, EBF1, ERG, CXCR4, CD79A, IKZF3, PAX5 and CD19. Decreased expression of these genes is consistent with the reduced B-cell potential of T21 LM cells. Furthermore, HOXB5, a negative regulator of PAX5 and EBF1 in proB cells, and ID1, described to impair B-cell differentiation and maintain stem cell renewal, were upregulated in HSC leading gene set suggesting enhanced self-renewal and decreased B-cell potential (Jankovic *et al.*, 2007; Cochrane *et al.*, 2009; Chen *et al.*, 2016; Zhang *et al.*, 2018). The megakaryocytic markers GULP1 and PBX1 displayed increased expression in the CMP leading edge gene set indicating a potential megakaryocyte bias (Voisin *et al.*, 2010; Paul *et al.*, 2015).

A)



B)

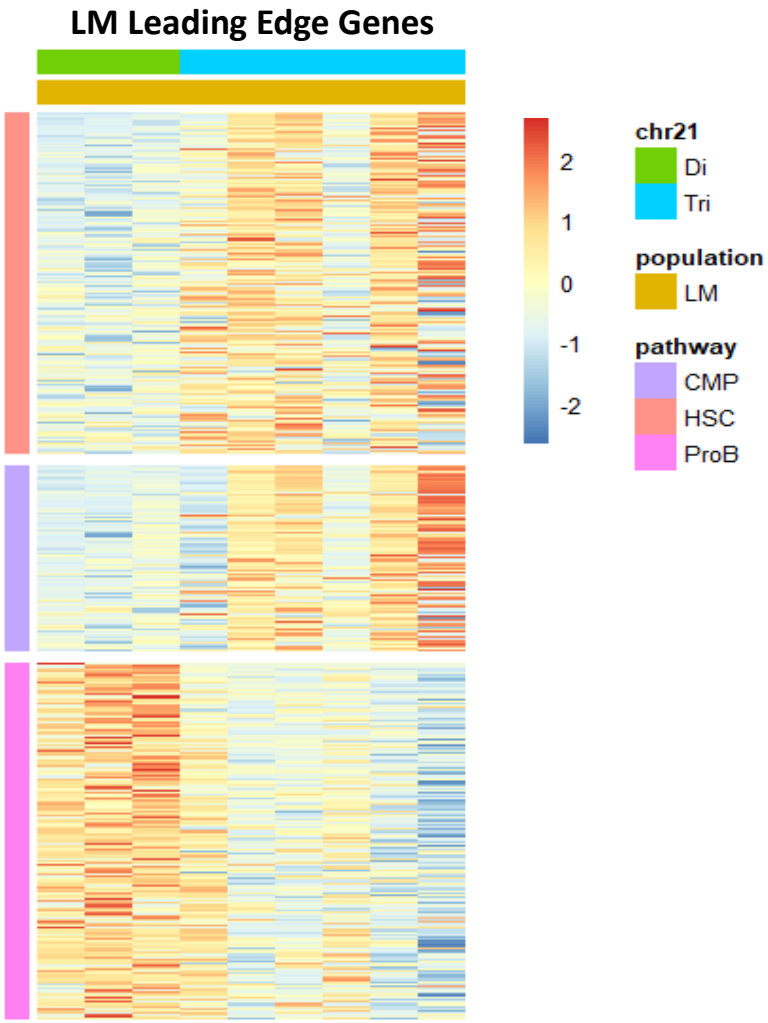


Figure 4. 8: Enrichment plot of LM leading edge genes. A) T21 LM leading edge genes display a negative enrichment of the proB signature, LM leading edge genes are outlined by the black box. B) T21 LM display positive enrichment for the Laurenti HSC and CMP gene set and display a negative enrichment for proB gene set, while D21 LM is positively enriched for proB and negatively enriched for HSC and myeloid gene sets. P-value=<0.05. Statistical significance was calculated using the fast set gene set enrichment analysis (fgsea) R package (Subramanian et al., 2005, Korotkevich et al., 2021).

These results suggest that T21 dysregulates lineage commitment of the LM progenitor and that this is associated with aberrant expression of stem and myeloid lineage associated

genes. This potentially occurs through downregulation of key transcription factors involved in early B-cell commitment that may subsequently result in the observed partial block in B-cell development.

Discussion

Largely, modelling and understanding the multi-step process of Down's syndrome leukemic initiation and development has focused on the increased predisposition to developing myeloid leukaemia. This involved investigating the transformation of the postnatally diagnosed pre-leukaemic disorder TMD into full-blown AMKL. However, few tractable models of DS-ALL exist. To address this, and to characterise early DS B-cell lymphopoiesis and the partial block identified in the LM population, that potentially resulted in impaired B-cell commitment (described in chapter 3), I used low cell number bulk RNA-seq to analyse time points of DS lymphopoietic differentiation, including $CD34^{+}$, LM, pro and preB populations.

Consistent with previous hypothesis that T21 disrupts lineage commitment in a primitive multipotent compartment prior to lineage commitment, PCA analysis demonstrated T21 LM progenitors as clear outliers indicative of a unique global transcriptome (Roy *et al.*, 2012). Notably the LM population had a significant number of DEGs and a number of positively enriched hallmark pathways that were previously described to be dysregulated in DS and leukaemia; ROS pathway (Perluigi and Butterfield, 2012), MYC targets (Marion K. Mateos *et al.*, 2015), mTORC1 pathway (Klusmann *et al.*, 2010) and E2F targets (Maroz *et al.*, 2014). The LM population exhibited upregulation in a number of DSCR genes known to be involved in haematopoiesis and/or previously described as candidate drivers of leukaemic development, that may also act as candidate targets for the observed hallmark enrichment. Of particular interest are a number of genes involved in the formation of the mitochondrial respiratory complex (MRC) - NDUFV3, ATP50 and ATP5J - that have previously been

described to induce genomic instability in CML-LSCs due to enhanced ROS production (Flis *et al.*, 2012). Increased expression of genes involved in the MRC may increase ROS levels, as is observed DS patients, and promote DNA damage and leukaemic progression (Nižetić and Groet, 2012). Interestingly the proteasome assembly chaperone PSMG1, a target of the mTORC1 pathway, is found to be frequently amplified in cALL that gain chr21q22.2 and may enhance LM proliferative potential (Schiffmana *et al.*, 2009). A number of chromosome 21 genes not previously implicated in leukaemic development, such as the mitochondrial ribosomal protein MRPL39 (involved in mitochondrial protein synthesis) and the negative regulator of p53 S100B (regulator of cell-cycle progression and differentiation), were increased in T21 LM, pro and preB compartments and may contribute to the changes in cell cycle and mitochondrial function in the LM population (Wilder *et al.*, 2006). Moreover, the LM progenitor similarly displayed upregulation of the nucleosome remodelling complex HMGN1, described to promote B-ALL leukaemogenesis through global transcriptional amplification in the Ts1Rhr DS mouse model (Mowery *et al.*, 2018). These findings indicate that T21 disrupts the gene expression profile of the LM population resulting in increased expression of a number of genes associated with leukaemic development. Interestingly a number of these candidate genes displayed similar increased expression in iAMP21 B-ALL suggesting that chr21 induces a dosage sensitive gene expression programme that is common to both DS and iAMP21 leukaemic initiation and development. Further characterisation of these candidate genes and pathways may identify novel therapeutic targets for chr 21 associated leukaemias.

The increased expression of HSC and myeloid gene expression signatures with a concomitant decrease in lymphoid lineage expression patterns within the T21 LM

compartment indicates a myeloid lineage bias and the maintenance of a stem cell like gene expression signature suggesting a partial block in B-cell commitment. Decreased expression of the negative regulators of the myeloid lineage EBF1 and PAX5 in the LM leading edge gene set may attenuate B-lymphoid commitment with a coinciding positive enrichment of myeloid and HSC transcriptional programs (Holmes and Corcoran, 2006; Pongubala *et al.*, 2008). Interestingly, the chr 21 encoded pre-mRNA splicing factor U2AF1, upregulated in both the LM and preB populations (figure 4.4), has been described in MDS and also shown to enhance HSC expansion of myeloid precursors in mouse bone marrow and may therefore be responsible for the observed enhanced myeloid programme observed in the LM and preB compartments (Yoshida *et al.*, 2011; Graubert *et al.*, 2012; Shirai *et al.*, 2015). The co-expression of conflicting lineage and transcriptional signatures has been proposed as a defining feature of leukaemic predisposition that results in cells becoming 'trapped' in a differentiation block that may render them more susceptible to acquiring secondary mutations (Enver and Greaves, 1998). The potential that the childhood pre-leukaemic cell of origin initiates in a stem cell fraction and that the stem cell gene expression programme persists during lymphopoietic development has previously been described. Deep sequencing of paired diagnosis-relapse samples of infant t(4;11)/MLL-AF4 ALL B-cell receptor repertoire identified a large number of non-expanded polyclonal V-D-J B-cell clones whose transcriptomic signature is similar to FL HSC and LMPP compartments, therefore suggesting that an immature HSC/LMPP pre-VDJ population could be the cell of origin for the t(4;11) B-ALL subtype and its stemness signature is maintained upon leukaemic transformation (Doblas *et al.*, 2019). Moreover, overexpression of ETV6-RUNX1 in UCB HSCs but not early B-cell progenitors induced pre-leukaemia and the maintenance of stem-cell programme in lymphoblasts of NOD-SCID mice suggesting that HSCs function as a 'first-hit'

target population for the ETV6-RUNX1 subtype (Fan *et al.*, 2014). Consistent with such models of pre-leukaemic initiation, the observed positive enrichment of stem and myeloid gene expression programmes in T21 LM and preB cells provide evidence that DS ALL may originate in a multipotent cellular compartment whose stem and myeloid transcriptional programmes persist in B-cell development and reflects the plasticity and ontogeny of infant and childhood BCP-ALL. Moreover, increased expression of cell cycle and protein synthesis-associated pathways in the T21 LM compartment may be indicative of the first step in leukaemic transformation thus supporting my hypothesis.

LM leading edge genes from the negatively enriched proB gene set included CD79A, VPREB1 and VPREB3, which are involved in the formation of surrogate light and Igα chains during V-D-J rearrangement, consistent with impaired B-cell commitment. The persistence of cells with impaired V-D-J rearrangement during B-cell differentiation may result in defective preB cell receptor formation and subsequent emergence of preB-like subclones expressing underlying HSC and myeloid transcriptional programs. Consistent with pre-BCR induced attenuation of JAK/STAT signalling, overexpression of DS-ALL associated secondary mutations IL7Rα and/or CRLF2 may induce a preBCR independent signalling network via activation of the JAK/STAT signalling axis resulting in the survival, transformation and expansion of preB-like cells that retain stem-myeloid capacity (Nakayama *et al.*, 2009; Müschen, 2015). Co-residence of conflicting lineage gene expression signatures may only be tested at a single cell level. To determine the co-expression of lineage gene expression programmes during differentiation of the LM progenitor to committed B-cells I designed a single cell qPCR approach addressing signatures of stem, myeloid and lymphoid potential, preBCR gene expression and the identified chr 21 candidate targets described above (see

Appendices). Single RNA-seq can similarly be employed and provide an unbiased approach however may not be sufficiently sensitive to identify low expressing genes.

My results identify the LM progenitor as a candidate for transformation in DS leukaemia leading me to look for this population in T21 foetal liver. As described in the DS-iPSC differentiation system, second trimester T21 foetal liver similarly display dysregulation of CD33 within the LM population relative to karyotypically normal age matched controls. While transcriptomic analysis of LM, pro and preB compartments of T21 FL is necessary to test whether this DS-iPSC system recapitulates DS foetal haematopoiesis the presence of a foetal specific progenitor compartment with perturbed lineage affiliated signatures, such as the LM progenitor, may underlie why DS leukaemia is overwhelmingly a childhood disease. The enhanced myeloid potential of the LM population may provide a substrate for the acquisition of GATA1 mutations that synergistically act with T21 for the development of AMKL. The impaired immunophenotype, enhanced stem and myeloid signatures and decreased early lymphoid signature observed within the T21 LM population along with the enrichment for a number of genes described as key players in haematopoietic and leukaemic development such as ATP5O, upregulated in iAMP21 ALL, and HMGN1 (figure 4.4 above) suggest the LM cell as a potential in-utero target cell for DS pre-leukaemic initiation. This model provides an avenue to explore whether the LM progenitor is the cell of origin for other cALL subtypes.

The above results indicate that the differentiation of isogenic DS iPSCs is a tractable model by which DS pre-leukaemic initiation and development may be further investigated. The enhanced myeloid transcriptional signature in the LM compartment along with the

increased expression of chr 21 genes previously described in myeloid leukaemias and expression of megakaryocytic markers GULP1 and PBX1 in the CMP leading edge gene set warrants an investigation into whether the LM population may be the in-utero target cell for both DS AMKL and ALL. Whether the identified lympho-myeloid programme persists in B-cells postnatally may provide an insight into the precise population that is susceptible to acquiring secondary mutations and identify a window for therapeutic intervention prior to leukaemic progression. More specifically, this system provides an exciting platform to facilitate functional and transcriptomic characterisation of the synergistic effect of chr 21 and IL7Rα/CRLF2 DS-ALL secondary mutations on the LM and B-cell compartments and to investigate the role of the identified candidate genes and pathways. Furthermore, this model will enable the development of a screening platform to test targeted therapies.

Chapter 5

Final Remarks and Future Experiments

5 Chapter 5: Final remarks and future experiments

The results described in this thesis show that trisomy 21 disrupts foetal haematopoiesis, as modelled in an iPSC system, in a lympho-myeloid progenitor immunophenotypically characterised as $CD34^{+}$ $CD38^{-}$ $CD33^{hi}$ $CD45Ra^{hi}$ that is positively enriched for stem and myeloid gene expression signatures and exhibits perturbed B-cell commitment and differentiation. This system provides evidence for an in-vitro model from which the molecular characterisation of the effect of T21 on early foetal haematopoiesis and the collaborative effects of T21 and secondary mutations on cellular transformation can be understood.

The analysis performed in this study focused on immunophenotypically characterising the DS lymphopoietic hierarchy derived from isogenic DS-iPSCs and subsequently using RNAseq to dissect the transcriptomic profile of this hierarchy. GSEA analysis suggests that T21 enhances the stem-myeloid lineage potential of the LM and preB compartments with concomitant impaired B-cell commitment, indicative of lineage conflict. Although preliminary, the capacity of T21 B-cells to persist when cultured in myeloid conditions further indicates bi-lineage B-myeloid potential, suggestive of pre-leukemic initiation (Böiers *et al.*, 2018). To fully characterise the co-residence of lineage affiliated gene expression programmes single cell approaches will be required. To address this, I devised a single cell qPCR panel that will facilitate sensitive quantitative analysis of co-expression of lineage affiliated signatures in LM and B-cell compartments (see appendices). Furthermore, CITE-seq will afford the evaluation of complex immunophenotyping and RNA-seq analysis of LM

and B-cell progenitors overcoming the limitations of the current FACS panel used in this study and may further identify sub-compartments primed for pre-leukaemic and leukaemic development.

PCA analysis revealed that the identified T21 LM population is transcriptomically unique from disomic counterparts. The precise mechanism/s by which T21 alters LM transcriptional networks that govern lymphoid and myeloid commitment requires further interrogation. ChIP-seq of LM, pro and preB compartments will facilitate characterisation of binding target sites of key transcription factors involved in lineage commitment that may underlie the observed stem-myeloid bi-lineage enrichment. Specifically, binding targets of HOXB5 a negative regulator of PAX5 and EBF1 and upregulated in T21 LM HSC leading edge gene set may provide insight into disrupted transcription factor networks induced by T21 that regulate lineage choice and impair DS B-cell commitment and differentiation.

Enhanced stem and myeloid gene expression signatures with a concomitant impaired B lymphoid output in-vitro predicts that the in-vivo counterpart of the LM compartment would exhibit a myeloid bias and decreased B-cell output in DS, as is observed in DS FL (Roy *et al.*, 2012). Positive enrichment of stem and myeloid gene expression signatures in the T21 LM compartment potentially induce lineage confusion that results in a 'trapping' of cells that may be susceptible to DNA damage through enhanced ROS signalling and display enrichment of protein synthesis and cell cycle pathways. The LM progenitor therefore represents a candidate target cell for both ALL and AMKL pre-leukaemic initiation. This system provides an exciting platform to further investigate the effect of the identified enriched gene expression signatures on pre-leukaemic development and evaluate their

synergistic effect with 'secondary' ALL and AMKL mutations on leukaemic transformation. Whether the enhanced stem and myeloid transcriptional programmes confer a self-renewal and myeloid bias on the T21 LM progenitor that may increase susceptibility to acquiring oncogenic mutations may be investigated by long-term culture initiating-cell assay (LTC-IC) or through serial transplantation in NOD/SCID mice. Moreover, enhanced ROS signalling in HSCs has been shown to induce a myeloid differentiation bias (Jang and Sharkis, 2007). With this, gene expression analysis of the LM population displaying low, medium and high levels of ROS may unveil mechanisms by which ROS perturbs haematopoietic commitment in the T21 LM progenitor compartment and induces enrichment of myeloid transcriptional programme. The role of novel therapeutic candidates ATP50, ATP5J and NDUFV3, described above, in mitochondrial function and ROS generation within the LM population can be interrogated using the Seahorse assay which will allow for screening of a small molecule library against these targets and assess their role in ROS generation and mitochondrial respiration. Furthermore, I predict that expression of GATA1s would further transform this progenitor and induce a megakaryocyte bias typical of TMD development that could be assessed by clonogenic and FACS analysis of in-vitro myeloid cultures.

This DS hiPSC model similarly provides a tractable system in which the effect of the identified chr 21 candidates on perturbed DS B-lymphopoiesis can be assessed. A CRISPR/Cas9 knockout system with guide RNAs targeting one or a number of the identified chr 21 candidates genes could be employed. Selection of stable T21 iPSC knockout diploid clones for one or a number of target genes and subsequent B-cell differentiation would provide a physiologically relevant model to test the individual and/or synergistic effect of each gene on perturbed haematopoietic differentiation and B-cell commitment. This system

would afford further characterisation of the chr 21 induced vulnerabilities that may render the LM and B-cell compartments susceptible to leukaemic initiation and lead to development of screening strategies and targeted therapies for DS leukaemias.

My results suggest that the observed partial differentiation block in B-lymphopoiesis appears to be associated with the misexpression of stem and myeloid lineage signatures observed in the LM compartment but this is not sufficient to drive leukaemic transformation. With this, and the evidence that the majority of DS patients with TMD enter remission and the few differentially expressed genes identified within D21 and T21 preB cells in this analysis suggests that T21 has weak oncogenic potential. Therefore, the effect of T21 on preB cell leukaemic transformation may depend on the synergistic efforts of T21 with known DS B-ALL oncogenes CRLF2 and/or IL7Rα, analogous to the transformation of TMD to myeloid leukaemia of Down syndrome upon acquisition of GATA1s truncation. I hypothesise, the identified LM gene expression signatures co-operate with DS-leukaemic mutations directly and/or impair B-cell development inducing susceptibility to activating JAK/STAT mutations that provide a positive selection advantage and drive transformation of B-cells enriched for stem-myeloid gene expression programmes. The transformation of preB cells displaying enhanced stem cell gene expression signatures may underlie the poor prognosis of DS B-ALL. While attempts to model the constitutive overexpression of CRLF2 and IL7Rα12bp ins were unsuccessful regulated expression under a B-cell specific promoter, comprised of the immunoglobulin enhancer Emu and Igbeta promoter B29, (kindly provided by Shai Izraeli) will allow the assessment of synergistic effect of T21 and the expression of DS B-ALL mutations specifically in B-cells providing a physiologically relevant model. Transformation would be evidenced by expansion of the B-cell compartment in vitro and the ability to engraft NSG mice.

The described GSEA results suggest that T21 disrupts preB cell maturation via the upregulation of myeloid gene expression signatures however this remains to be formally tested. Advancements in the B-cell differentiation protocol have enabled the derivation of mature IgM+ B-cells from enriched preB progenitors (French *et al.*, 2015). Enrichment of D21 and T21 CD19+ B-cells and extended differentiation into mature IgM+ B-cells will facilitate investigation into the frequency of T21 preB cells that incur a block in B-cell differentiation in the preB compartment and the molecular mechanisms occurring; 1) that allow T21 preB cells to escape and mature to IgM+ B cells and 2) aberrant gene expression signatures that result in impaired preB output and enable T21 preB cells to survive myeloid conditions. The frequency at which potential bi-lineage T21 preB cells exist may similarly be assessed by single cell fluorescent activated cell sorting of preB cells into myeloid conditions, as described in methods section 2.1.5.2, where preB cells that persist in culture are considered to have an underlying myeloid signature. This will provide a unique system into the identification and targeting of candidate genes and pathways involved in B-myeloid lineage transformation in the relevant preB target cell that may be amenable to targeted therapy prior to leukaemic development.

The gene expression programmes identified in this study may underlie perturbed haematopoiesis observed in second trimester T21 FL, which occurs prior to acquiring the GATA1s mutation, and the foetal specific onset of TMD (Tunstall-Pedoe *et al.*, 2008). Given the in-utero origins of childhood leukaemia, investigation into whether foetal gene expression programmes persist postnatally and in childhood DS-leukaemias, in conjunction with modelling DS-iPSCs, will allow us to investigate the effect of T21 on the foetal gene

expression programme and the increased predisposition to leukaemia in DS children. The hiPSC system is uniquely placed to provide insight as to why DS leukaemia is more prevalent in children and similarly shed light on the impaired B-cell immunity found in DS patients.

To conclude, these findings demonstrate T21 induced haematopoietic lineage skewing and B-cell defects observed during DS foetal lymphopoiesis. Further analysis of this hierarchy and induction of secondary ALL and AMKL mutations will allow characterisation of the above-described candidate mechanisms and target genes that result in the identified lineage conflict and potentially increased predisposition to DS children developing leukaemia. Moreover, this system provides an opportunity to evaluate whether the LM progenitor is the target cell for non-DS ALL pre-leukaemic initiation and identification of candidate leukaemogenic targets that arise during foetal development.

Appendix

6 Appendix

Single Cell qPCR:

Based on findings of the above study and recent publications investigating the role of T21 and key lymphoid and myeloid genes on haematopoiesis I devised a panel of genes to complete single cell qPCR addressing signatures of *stemness*, myeloid and lymphoid potential along with previously described DSCR genes that are involved in haematopoietic specification (Roy *et al.*, 2012; Di and Berto, 2013; Böiers *et al.*, 2018; Ivanov Öfverholm *et al.*, 2020). I have also included a number of chr 21 and preBCR genes identified from the above analysis previously described as potential candidates in DS leukaemic initiation and progression or identified to be significantly upregulated within the LM, pro and preB populations – NDUFV3, ATP5J, WRB, ATP5O, SOD1, HMGN1, PSMG1, CSTB, CRYZL1, SON, C21orf33, U2AF1, vPREB1, CD79A, vPREB3, PAX5, EBF1, ERG (table 6.1 below).

With the above findings I now aim to use this platform to investigate the impact of the T21 LM lympho-myeloid signature on the increased myeloid signature observed within the T21 preB cell population.

Gene Name		Identifier
ERG	ThermoFisher	Hs01554629_m1
Notch1	ThermoFisher	Hs01062014_m1
ETS1	ThermoFisher	Hs00428293_m1
MEF2C	ThermoFisher	Hs00231149_m1
HES1	ThermoFisher	Hs00172878_m1
DYRK1A	ThermoFisher	Hs00176369_m1
EBF1	ThermoFisher	Hs03045361_m1
IGH2	ThermoFisher	Hs05008883_gH
GABPA	ThermoFisher	Hs01022016_m1
SCL	ThermoFisher	Hs01097987_m1
GATA2	ThermoFisher	Hs00231119_m1
KLF1	ThermoFisher	Hs00610592_m1
CEBPα	ThermoFisher	Hs00269972_s1
CD19	ThermoFisher	Hs00174333_m1
CRLF2	ThermoFisher	Hs00845692_m1
CSF1R (M-CSFR)	ThermoFisher	Hs00911250_m1
CSF2RA (GM-CSFR)	ThermoFisher	Hs00538900_m1
CSF3R (G-CSFR)	ThermoFisher	Hs00167918_m1
E2A (TCF-3)	ThermoFisher	Hs00413032_m1
EPOR	ThermoFisher	Hs00959427_m1
AML1	ThermoFisher	Hs00959427_m1
GATA1	ThermoFisher	Hs01085823_m1
GATA3	ThermoFisher	Hs00231122_m1
IKAROS	ThermoFisher	Hs00232635_m1
IL3RA	ThermoFisher	Hs00608141_m1
IL7R	ThermoFisher	Hs00902334_m1
MPL	ThermoFisher	Hs00180489_m1
PAX5	ThermoFisher	Hs00277134_m1
PU.1	ThermoFisher	Hs02786711_m1
vPreB3	ThermoFisher	Hs00353682_m1
CD79A	ThermoFisher	Hs00233566_m1
SON	ThermoFisher	Hs00371372_m1
BCL6	ThermoFisher	Hs00153368_m1
CSTB	ThermoFisher	Hs00164368_m1
vPreB1	ThermoFisher	Hs00356766_g1
MEIS1	ThermoFisher	Hs00180020_m1
CRYZL1	ThermoFisher	Hs00270793_m1
NDUFV3	ThermoFisher	Hs00221479_m1
WRB	ThermoFisher	Hs00190294_m1
ATP5O	ThermoFisher	Hs00426889_m1
C21orf33	ThermoFisher	Hs01105802_g1
U2AF1	ThermoFisher	Hs00739599_m1
SOD1	ThermoFisher	Hs00533490_m1
HMGN1	ThermoFisher	Hs05024802_m1
PSMG1	ThermoFisher	Hs00186605_m1
ATP5J	ThermoFisher	Hs01081389_g1
GAPDH	ThermoFisher	Hs99999905_m1
β-ACTIN	ThermoFisher	Hs99999903_m1

Table 6. 1: List of Taqman probes and corresponding identifier for scQPCR analysis of LM and pro and preB cell populations as described above.

Karyotype Reports for 1469, 1470 and 1474 DS-iPSCs kindly provided by Dr Thorsten Schlaeger Boston Children's Hospital:

1469:

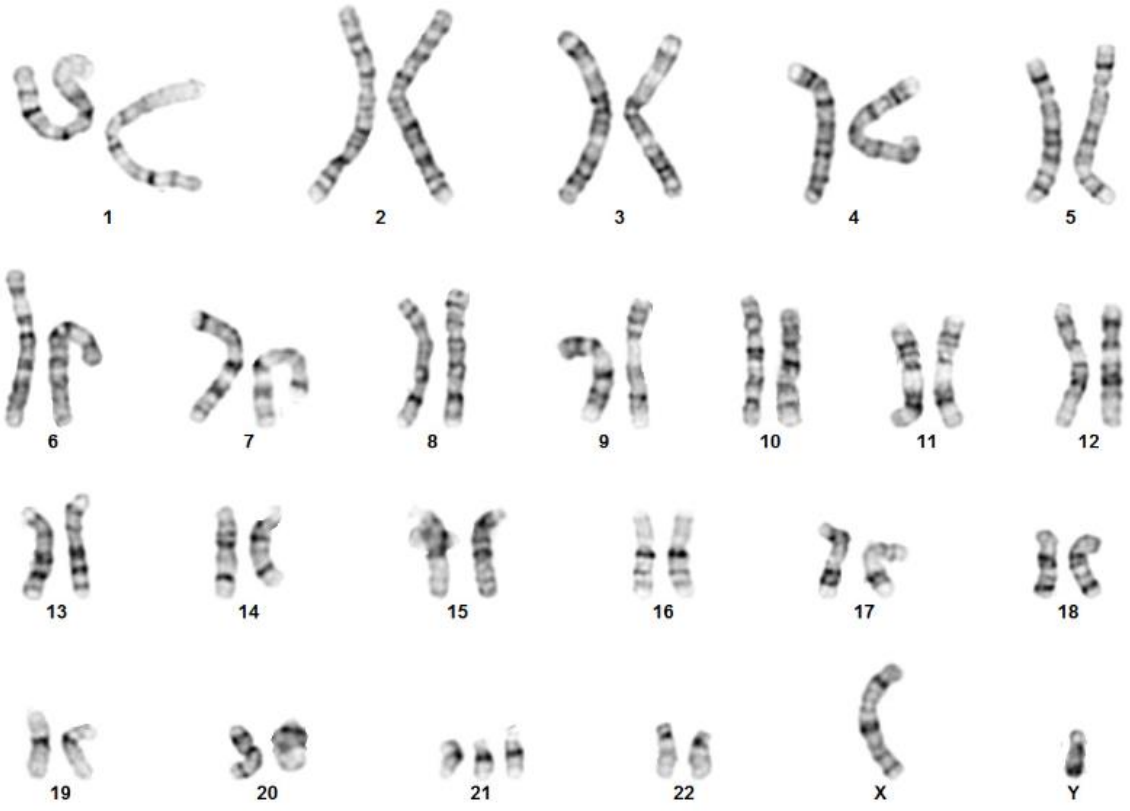
Banding Technique: GTL **Band Resolution:** Good
Metaphases Counted: 20 Analyzed: 7 Karyotyped: 2

RESULTS: 47,XY,+21[20] ABNORMAL Human Male Karyotype

Non-clonal Aberrations: none

INTERPRETATION:

Cytogenetic analysis was performed on twenty G-banded metaphase cells from human cell line 1469 p53 and all twenty cells demonstrated an abnormal male karyotype with trisomy 21.



1470:

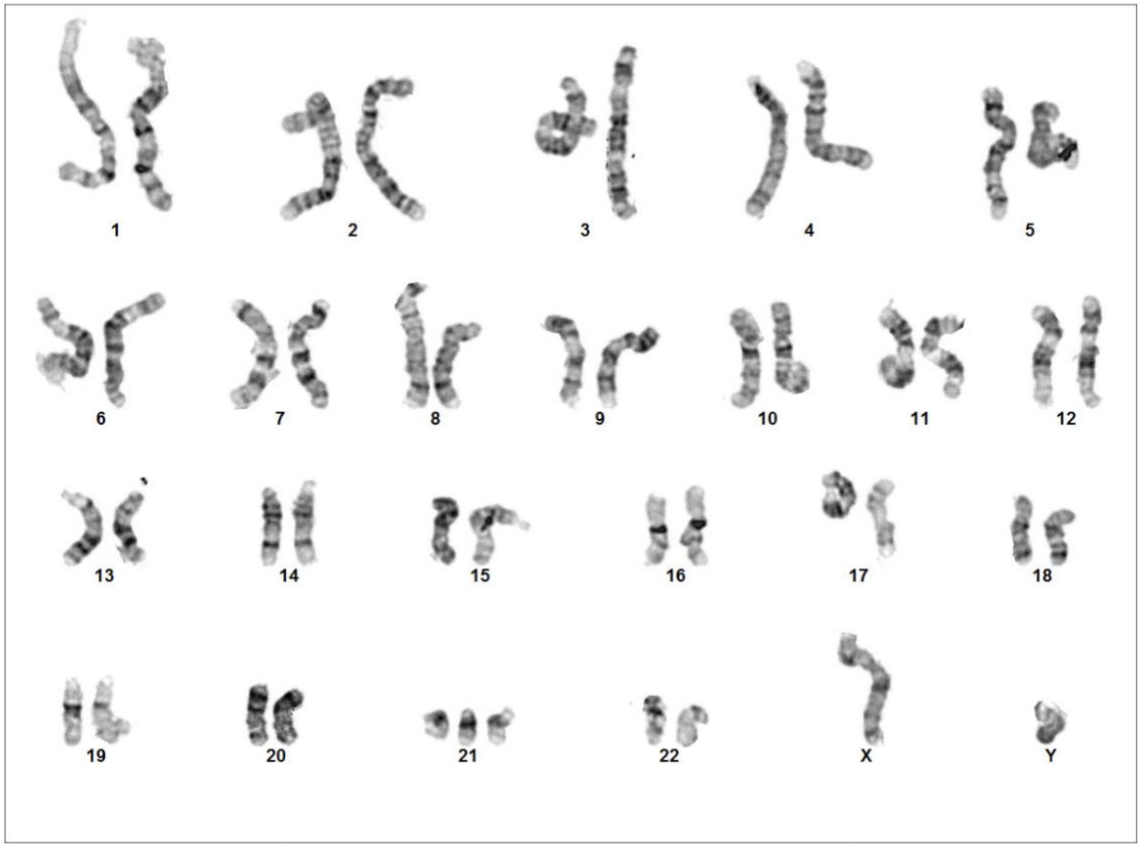
RESULTS: 47,XY,+21[18] ABNORMAL Human Male Karyotype

Non-clonal Aberrations: 48,XY,+15,+21[1]
46,XY,-12,+21[1]

INTERPRETATION:

Cytogenetic analysis was performed on twenty G-banded metaphase cells from human cell line 1470 p51 and all twenty cells demonstrated an abnormal male karyotype with trisomy 21.
In addition to trisomy 21, two cells demonstrated non-clonal chromosome aberrations (listed above) which are most likely artifacts of culture.

Result: 47,XY,+21



1474:

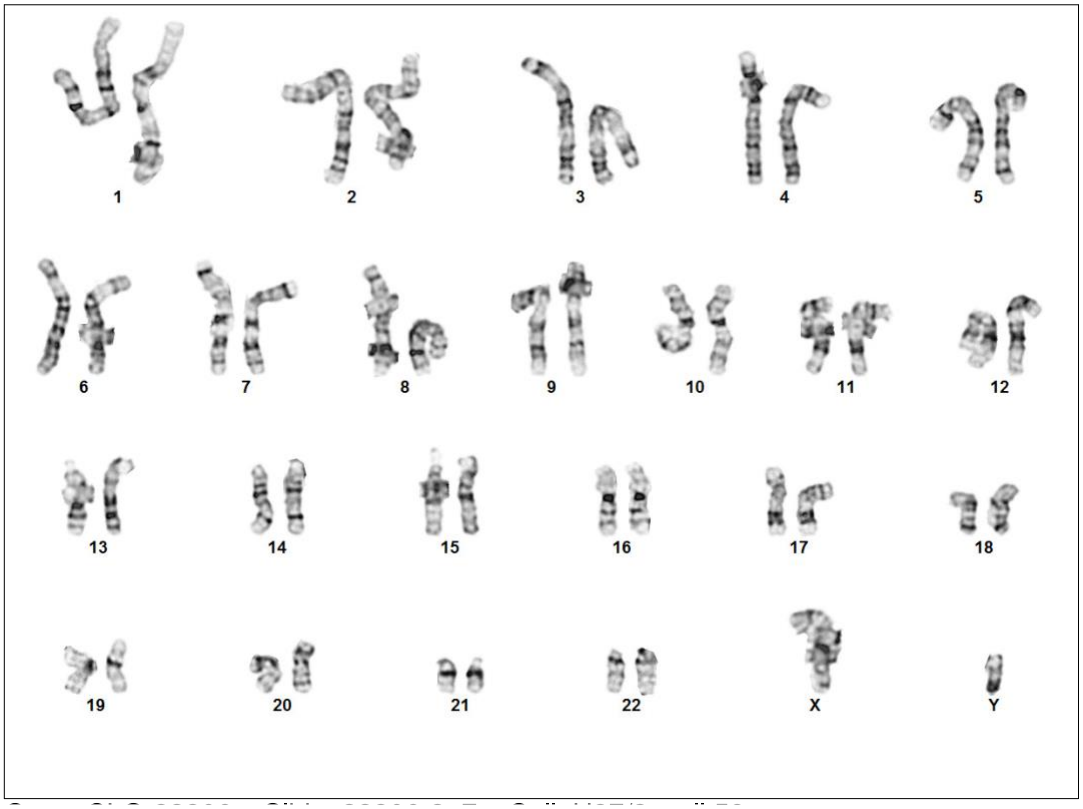
RESULTS: 46,XY[19] Apparently NORMAL Human Male Karyotype

Non-clonal Aberrations: 45,XY,-15(one cell)

INTERPRETATION:

Cytogenetic analysis was performed on twenty G-banded metaphase cells from human cell line 1474 p47. Nineteen cells demonstrated an apparently normal male karyotype, while one cell demonstrated a non-clonal chromosome aberration (listed above) which is most likely an artifact of culture.

Result: 46,XY



7 References;

Adams, G. B. *et al.* (2006) 'Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor', *Nature*, 439(7076), pp. 599–603. doi: 10.1038/nature04247.

Adolfsson, J. *et al.* (2001) 'Upregulation of Flt3 expression within the bone marrow Lin-Sca1+c-kit+ stem cell compartment is accompanied by loss of self-renewal capacity', *Immunity*, 15(4), pp. 659–669. doi: 10.1016/S1074-7613(01)00220-5.

Ahmed, M. *et al.* (2004) 'Natural history of GATA1 mutations in Down syndrome', *Blood*, 103(7), pp. 2480–2489. doi: 10.1182/blood-2003-10-3383.

Alberti-Servera, L. *et al.* (2017) 'Single-cell RNA sequencing reveals developmental heterogeneity among early lymphoid progenitors', *The EMBO Journal*, 36(24), pp. 3619–3633. doi: 10.15252/embj.201797105.

Antonarakis, S. E. *et al.* (2020) 'Down syndrome', *Nature Reviews Disease Primers*. Springer US, 6(1), pp. 1–20. doi: 10.1038/s41572-019-0143-7.

Arai, F. *et al.* (2004) 'Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche', *Cell*, 118(2), pp. 149–161. doi: 10.1016/j.cell.2004.07.004.

Aranda, P. *et al.* (2009) 'Epigenetic signatures associated with different levels of differentiation potential in human stem cells', *PLoS ONE*. doi: 10.1371/journal.pone.0007809.

Asim, A. *et al.* (2015) '"down syndrome: An insight of the disease"', *Journal of Biomedical Science*. Journal of Biomedical Science, 22(1), pp. 1–9. doi: 10.1186/s12929-015-0138-y.

B. I.Lord, Nydia G. Testa, and J. H. H. (1975) 'The Relative Distributions of CFUc and CFUc in the Normal Mouse Femur', *Blood*, 46(1), pp. 65–72. doi: 10.1017/CBO9781107415324.004.

Baburamani, A. A. *et al.* (2019) 'New approaches to studying early brain development

in Down syndrome', *Developmental Medicine and Child Neurology*, 61(8), pp. 867–879. doi: 10.1111/dmcn.14260.

Banno, K. *et al.* (2016) 'Systematic Cellular Disease Models Reveal Synergistic Interaction of Trisomy 21 and GATA1 Mutations in Hematopoietic Abnormalities', *Cell Reports*. The Authors, 15(6), pp. 1228–1241. doi: 10.1016/j.celrep.2016.04.031.

Baum, C. M. *et al.* (1992) 'Isolation of a candidate human hematopoietic stem-cell population', *Proceedings of the National Academy of Sciences of the United States of America*, 89(7), pp. 2804–2808. doi: 10.1073/pnas.89.7.2804.

Baumgarth, N. (2017) 'A Hard(y) Look at B-1 Cell Development and Function', *The Journal of Immunology*, 199(10), pp. 3387–3394. doi: 10.4049/jimmunol.1700943.

Bercovich, D. *et al.* (2008) 'Mutations of JAK2 in acute lymphoblastic leukaemias associated with Down's syndrome', *The Lancet*. doi: 10.1016/S0140-6736(08)61341-0.

Bhatnagar, N. *et al.* (2016) 'Transient Abnormal Myelopoiesis and AML in Down Syndrome: an Update', *Current Hematologic Malignancy Reports*. Current Hematologic Malignancy Reports, 11(5), pp. 333–341. doi: 10.1007/s11899-016-0338-x.

Birchler, J. A. (2010) 'Reflections on studies of gene expression in aneuploids', *Biochemical Journal*, 426(2), pp. 119–123. doi: 10.1042/BJ20091617.

Birger, Y. *et al.* (2013) 'Perturbation of fetal hematopoiesis in a mouse model of Down syndrome's transient myeloproliferative disorder', *Blood*, 122(6), pp. 988–998. doi: 10.1182/blood-2012-10-460998.

Boekel, T., Melchers, E. and Rolink, F. A. G. (1998) 'Precursor B cells showing H chain allelic inclusion display allelic exclusion at the level of pre-B cell receptor surface expression', *Immunity*, 8(2), pp. 199–207. doi: 10.1016/S1074-7613(00)80472-0.

Böiers, C. *et al.* (2013) 'Lymphomyeloid contribution of an immune-restricted progenitor

emerging prior to definitive hematopoietic stem cells.', *Cell stem cell*, 13(5), pp. 535–548. doi: 10.1016/j.stem.2013.08.012.

Böiers, C. *et al.* (2018) 'A Human IPS Model Implicates Embryonic B-Myeloid Fate Restriction as Developmental Susceptibility to B Acute Lymphoblastic Leukemia-Associated ETV6-RUNX1', *Developmental Cell*. doi: 10.1016/j.devcel.2017.12.005.

Bombery, M. and Vergilio, J. A. (2014) 'Transient abnormal myelopoiesis in neonates: GATA get the diagnosis', *Archives of Pathology and Laboratory Medicine*, 138(10), pp. 1302–1306. doi: 10.5858/arpa.2014-0304-CC.

Bonnet, D. and Dick, J. E. (1997) 'Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell', *Nature medicine*. doi: 10.1038/nm0797-730.

Borzillo, G. V, Ashmun, R. A. and Sherr, C. J. (1990) 'Macrophage lineage switching of murine early pre-B lymphoid cells expressing transduced fms genes.', *Molecular and Cellular Biology*, 10(6), pp. 2703–2714. doi: 10.1128/mcb.10.6.2703.

Bourquin, J. P. *et al.* (2006) 'Identification of distinct molecular phenotypes in acute megakaryoblastic leukemia by gene expression profiling', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0511150103.

Busciglio, J. and Yankner, B. A. (1995) 'Apoptosis and increased generation of reactive oxygen species in down's syndrome neurons in vitro', *Nature*, 378(6559), pp. 776–779. doi: 10.1038/378776a0.

Cabelof, D. C. *et al.* (2009) 'Mutational spectrum at GATA1 provides insights into mutagenesis and leukemogenesis in Down syndrome', *Blood*, 114(13), pp. 2753–2763. doi: 10.1182/blood-2008-11-190330.

Cariappa, A. *et al.* (2007) 'The Recirculating B Cell Pool Contains Two Functionally Distinct,

Long-Lived, Posttransitional, Follicular B Cell Populations', *The Journal of Immunology*. doi: 10.4049/jimmunol.179.4.2270.

Carmeliet, P. *et al.* (1996) 'Abnormal blood vessel development and lethality in embryos lacking a single vascular endothelial growth factor allele', *Nature*, 380(1), pp. 435–439. Available at: https://www.nature.com/articles/380435a0.pdf%0Ahttp://dx.doi.org/10.1038/380435a0.

Carpenter, L. *et al.* (2011) 'Human induced pluripotent stem cells are capable of B-cell lymphopoiesis.', *Blood*. doi: 10.1182/blood-2010-08-299941.

Cazzaniga, G. *et al.* (2011) 'Developmental origins and impact of BCR-ABL1 fusion and IKZF1 deletions in monozygotic twins with Ph + acute lymphoblastic leukemia', *Blood*, 118(20), pp. 5559–5564. doi: 10.1182/blood-2011-07-366542.

Chen, J. Y. *et al.* (2016) 'Hoxb5 marks long-term haematopoietic stem cells and reveals a homogenous perivascular niche', *Nature*, 530(7589), pp. 223–227. doi: 10.1038/nature16943.Hoxb5.

Cheung, L. C. *et al.* (2018) 'New therapeutic opportunities from dissecting the pre-B leukemia bone marrow microenvironment', *Leukemia*, 32(11), pp. 2326–2338. doi: 10.1038/s41375-018-0144-7.

Chiang, J. C. *et al.* (2018) 'Trisomy silencing by XIST normalizes Down syndrome cell pathogenesis demonstrated for hematopoietic defects in vitro', *Nature Communications*. Springer US, 9(1), pp. 1–12. doi: 10.1038/s41467-018-07630-y.

Cho, S. K. *et al.* (1999) 'Functional characterization of B lymphocytes generated in vitro from embryonic stem cells.', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.96.17.9797.

Choi, K.-D. *et al.* (2008) 'Hematopoietic Differentiation of Human Induced Pluripotent Stem

Cells', *Blood*, 112(11), pp. 731–731. doi: 10.1182/blood.v112.11.731.731.

Choi, K. D. *et al.* (2012) 'Identification of the Hemogenic Endothelial Progenitor and Its Direct Precursor in Human Pluripotent Stem Cell Differentiation Cultures', *Cell Reports*. doi: 10.1016/j.celrep.2012.08.002.

Chou, S. T. *et al.* (2012) 'Trisomy 21-associated defects in human primitive hematopoiesis revealed through induced pluripotent stem cells', *Proceedings of the National Academy of Sciences of the United States of America*, 109(43), pp. 17573–17578. doi: 10.1073/pnas.1211175109.

Ciau-Uitz, A., Patient, R. and Medvinsky, A. (2016) 'Ontogeny of the Hematopoietic System', in *Encyclopedia of Immunobiology*. doi: 10.1016/B978-0-12-374279-7.01002-X.

Civin, C. I. *et al.* (1984) 'Antigenic analysis of hematopoiesis . III . A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells . Information about subscribing to The Journal of Immunology is online at : . A Hematopoietic Progenito', *The Journal of Immunology*, 133(1), pp. 157–165.

Clark, M. R. *et al.* (2014) 'Orchestrating B cell lymphopoiesis through interplay of IL-7 receptor and pre-B cell receptor signalling', *Nature Reviews Immunology*. doi: 10.1038/nri3570.

Cochrane, S. W. *et al.* (2009) 'Balance between Id and E proteins regulates myeloid-versus-lymphoid lineage decisions', *Blood*, 113(5), pp. 1016–1026. doi: 10.1182/blood-2008-06-164996.

Delabar, J. M. *et al.* (1993) 'Molecular mapping of twenty-four features of Down syndrome on chromosome 21.', *European journal of human genetics : EJHG*. doi: 10.1159/000472398.

Di, F. and Berto, G. (2013) *Molecular Pathways of Down Syndrome Critical Region Genes*, *Down Syndrome*. doi: 10.5772/53000.

Dias, S. *et al.* (2005) 'Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors', *Journal of Experimental Medicine*, 201(6), pp. 971–979. doi: 10.1084/jem.20042393.

Dieterlen Lievre, F. (1975) 'On the origin of haemopoietic stem cells in the avian embryo: an experimental approach', *Journal of Embryology and Experimental Morphology*, 33(3), pp. 607–619.

Doblas, A. A. *et al.* (2019) 'Unraveling the cellular origin and clinical prognostic markers of infant B-cell acute lymphoblastic leukemia using genome-wide analysis', *Haematologica*, 104(6), pp. 1176–1188. doi: 10.3324/haematol.2018.206375.

Doulatov, S. *et al.* (2010) 'Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development', *Nature Immunology*. Nature Publishing Group, 11(7), pp. 585–593. doi: 10.1038/ni.1889.

Dyer, M. A. *et al.* (2001) 'Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo', *Development*, 128(10), pp. 1717–1730.

Encarnacion Montecino-Rodriguez and Kenneth Dorshkind (2012) 'B-1 B Cell Development in the Fetus and Adult', *Immunity*, 36(1), pp. 13–21. doi: 10.1038/jid.2014.371.

Enver, T. and Greaves, M. (1998) 'Loops, Lineage, and Leukemia', *Cell*, 94(1), pp. 9–12. doi: 10.1016/S0092-8674(00)81215-5.

Enver, T. and Jacobsen, S. E. W. (2009) 'Instructions writ in blood', *Nature*, 461(7261), pp. 183–184. doi: 10.1038/461183a.

Ewels, P. *et al.* (2019) 'nf-core : Community curated bioinformatics pipelines', *bioRxiv*, p. 610741. doi: 10.1101/610741.

Fan, R. Bonde, S. Gao, P. Sotomayor, B. Chen, C. Mouw, T. Zavazava, N. Tan, K. (2012)

'Dynamic HoxB4-regulatory network during embryonic stem cell differentiation to hematopoietic cells', *Hematopoiesis and stem cells*, 119(19), pp. 139–147. doi: 10.1182/blood-2011-12-396754.This.

Fan, D. *et al.* (2014) 'Stem cell programs are retained in human leukemic lymphoblasts', *Oncogene*, 34(16), pp. 2083–2093. doi: 10.1038/onc.2014.148.

Ferkowicz, M. J. and Yoder, M. C. (2005) 'Blood island formation: Longstanding observations and modern interpretations', *Experimental Hematology*, 33(9 SPEC. ISS.), pp. 1041–1047. doi: 10.1016/j.exphem.2005.06.006.

Finkbeiner, S. R. *et al.* (2015) 'Transcriptome-wide Analysis Reveals Hallmarks of Human Intestine Development and Maturation In Vitro and In Vivo', *Stem Cell Reports*. The Authors, 4(6), pp. 1140–1155. doi: 10.1016/j.stemcr.2015.04.010.

Flis, K. *et al.* (2012) 'Chronic myeloid leukemia stem cells display alterations in expression of genes involved in oxidative phosphorylation', *Leukemia and Lymphoma*, 53(12), pp. 2474–2478. doi: 10.3109/10428194.2012.696313.

Ford, A. M. *et al.* (1993) 'In utero rearrangements in the trithorax-related oncogene in infant leukaemias', 363(May), pp. 358–360.

Forestier, E. *et al.* (2008) 'Cytogenetic features of acute lymphoblastic and myeloid leukemias in pediatric patients with Down syndrome: An iBFM-SG study', *Blood*. doi: 10.1182/blood-2007-09-114231.

French, A. *et al.* (2015) 'Human Induced Pluripotent Stem Cell-Derived B Lymphocytes Express sIgM and Can Be Generated via a Hemogenic Endothelium Intermediate', *Stem Cells and Development*, 24(9), pp. 1082–1095. doi: 10.1089/scd.2014.0318.

Gale, K. B. *et al.* (1997) 'Backtracking leukemia to birth: Identification of clonotypic gene fusion sequences in neonatal blood spots', *Proceedings of the National Academy of Sciences*

of the United States of America, 94(25), pp. 13950–13954. doi: 10.1073/pnas.94.25.13950.

Ghazawi, F.M. *et al.* (2012) 'IL-7 downregulates IL-7Ra expression in human CD8 T cells by two independent mechanisms', *Immunology and cell biology,* 91, pp. 149-158.

Gao, X. *et al.* (2013) 'Gata2 cis-element is required for hematopoietic stem cell generation in the mammalian embryo', *Journal of Experimental Medicine*, 210(13), pp. 2833–2842. doi: 10.1084/jem.20130733.

Gialesaki, S. *et al.* (2018) 'GATA1s exerts developmental stage-specific effects in human hematopoiesis', *Haematologica*, 103(8), pp. e336–e340. doi: 10.3324/haematol.2018.191338.

Godin, I. *et al.* (1999) 'Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites', *Journal of Experimental Medicine*, 190(1), pp. 43–52. doi: 10.1084/jem.190.1.43.

Gothert, J.R. Gustin, S.E. Hall, M.A. Green, A.R. Gottgens, B. Izon, D. J. and C. G. B. (2005) 'In vivo fate-tracing studies using the Scl stem cell enhancer: embryonic hematopoietic stem cells significantly contribute to adult hematopoiesis', *Stem Cells*, 105(7), pp. 2724–2732. doi: 10.1182/blood-2004-08-3037.Supported.

Graf, T. and Enver, T. (2009) 'Forcing cells to change lineages', *Nature*. Nature Publishing Group, 462(7273), pp. 587–594. doi: 10.1038/nature08533.

Graubert, T. A. *et al.* (2012) 'Recurrent mutations in the U2AF1 splicing factor in myelodysplastic syndromes', *Nature Genetics*. Nature Publishing Group, 44(1), pp. 53–57. doi: 10.1038/ng.1031.

Grawunder, U. *et al.* (1995) 'Down-regulation of RAG1 and RAG2 gene expression in PreB cells after functional immunoglobulin heavy chain rearrangement', *Immunity*, 3(5), pp. 601–608. doi: 10.1016/1074-7613(95)90131-0.

Greenbaum, A. *et al.* (2013) 'CXCL12 Production by Early Mesenchymal Progenitors is Required for Hematopoietic Stem Cell Maintenance', *Physiology & behavior*, 176(10), pp. 139–148. doi: 10.1016/j.physbeh.2017.03.040.

Hamdi, S., Teller, G. and Louis, J.-P. (1987) ' Master Regulatory Genes, Auxin Levels, and Sexual Organogeneses in the Dioecious Plant Mercurialis annua ', *Plant Physiology*, 85(2), pp. 393–399. doi: 10.1104/pp.85.2.393.

Hanna, J. *et al.* (2007) 'Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin', *Science*, 318(5858), pp. 1920–1923. doi: 10.1126/science.1152092.

Hasle, H., Haunstrup Clemmensen, I. and Mikkelsen, M. (2000) 'Risks of leukaemia and solid tumours in individuals with Down's syndrome', *Lancet*, 355(9199), pp. 165–169. doi: 10.1016/S0140-6736(99)05264-2.

Hassold, T. and Hunt, P. (2001) 'To err (meiotically) is human: the genesis of human aneuploidy', *Nature Reviews Genetics*. Nature Publishing Group, 2(4), pp. 280–291. doi: 10.1038/35066065.

Herskowitz, I. (1989) 'A regulatory hierarchy for cell specialization in yeast', *Nature*, 342(6251), pp. 749–757. doi: 10.1038/342749a0.

Hertzberg, L. *et al.* (2010) 'Down syndrome acute lymphoblastic leukemia, a highly heterogeneous disease in which aberrant expression of CRLF2 is associated with mutated JAK2: A report from the International BFM Study Group', *Blood*. doi: 10.1182/blood-2009-08-235408.

Herzenberg, Leonore A. and Herzenberg, Leonard A. (1989) 'Toward a layered immune system', *Cell*, 59(6), pp. 953–954. doi: 10.1016/0092-8674(89)90748-4.

Heyworth, C. *et al.* (2002) 'Transcription factor-mediated lineage switching reveals plasticity in primary committed progenitor cells', *EMBO Journal*, 21(14), pp. 3770–3781. doi:

10.1093/emboj/cdf368.

Hibaoui, Y. *et al.* (2014) 'Modelling and rescuing neurodevelopmental defect of Down syndrome using induced pluripotent stem cells from monozygotic twins discordant for trisomy 21', *EMBO Molecular Medicine*. doi: 10.1002/emmm.201302848.

Hideyuki Oguro, Lei Ding, and S. J. M. (2008) 'SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors', *Cell Stem Cell. 2013 July 3; 13(1): 102–116. doi:10.1016/j.stem.2013.05.014.*, 23(1), pp. 1–7. doi: 10.1038/jid.2014.371.

Hong, D. *et al.* (2008) 'Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia', *Science*. doi: 10.1126/science.1150648.

Hou, P. *et al.* (2013) 'Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds.', *Science (New York, N.Y.)*. doi: 10.1126/science.1239278.

Hrušák, O. and Porwit-MacDonald, A. (2002) 'Antigen expression patterns reflecting genotype of acute leukemia', *Leukemia*, 16(7), pp. 1233–1258. doi: 10.1038/sj.leu.2402504.

Hu, Y. *et al.* (2004) 'Quantitative real-time PCR technique for rapid prenatal diagnosis of down syndrome', *Prenatal Diagnosis*, 24(9), pp. 704–707. doi: 10.1002/pd.968.

Hu, Y., Yoshida, T. and Georgopoulos, K. (2017) 'Transcriptional circuits in B cell transformation', *Current Opinion in Hematology*, 24(4), pp. 345–352. doi: 10.1097/MOH.0000000000000352.

Huang, S. *et al.* (2007) 'Bifurcation dynamics in lineage-commitment in bipotent progenitor cells', *Developmental Biology*, 305(2), pp. 695–713. doi: 10.1016/j.ydbio.2007.02.036.

Hurtz, C. *et al.* (2019) 'DYRK1A Is Regulated By Oncogenic KMT2A and Required for Survival of KMT2A-Rearranged Acute Lymphoblastic Leukemia', *Blood*, 134(Supplement_1), p. 2742. doi: 10.1182/blood-2019-123475.

Ikawa, T. *et al.* (2004) 'Long-term cultured E2A-deficient hematopoietic progenitor cells are pluripotent', *Immunity*, 20(3), pp. 349–360. doi: 10.1016/S1074-7613(04)00049-4.

Ivanov Öfverholm, I. *et al.* (2020) 'Overexpression of chromatin remodeling and tyrosine kinase genes in iAMP21-positive acute lymphoblastic leukemia', *Leukemia and Lymphoma*. Taylor & Francis, 61(3), pp. 604–613. doi: 10.1080/10428194.2019.1678153.

Ivanovs, A. *et al.* (2017) 'Human haematopoietic stem cell development: From the embryo to the dish', *Development (Cambridge)*, 144(13), pp. 2323–2337. doi: 10.1242/dev.134866.

Izraeli, S. *et al.* (2014) 'How I treat all in down's syndrome: Pathobiology and management', *Blood*, 123(1), pp. 35–40. doi: 10.1182/blood-2013-07-453480.

Jang, Y. Y. and Sharkis, S. J. (2007) 'A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche', *Blood*, 110(8), pp. 3056–3063. doi: 10.1182/blood-2007-05-087759.

Jankovic, M. *et al.* (2004) 'RAGs and regulation of autoantibodies.', *Annual review of immunology*. doi: 10.1146/annurev.immunol.22.012703.104707.

Jankovic, V. *et al.* (2007) 'Id1 restrains myeloid commitment, maintaining the self-renewal capacity of hematopoietic stem cells', *Proceedings of the National Academy of Sciences of the United States of America*, 104(4), pp. 1260–1265. doi: 10.1073/pnas.0607894104.

Jiang, J. *et al.* (2013) 'Translating dosage compensation to trisomy 21', *Nature*. doi: 10.1038/nature12394.

Johnson, K. *et al.* (2008) 'Regulation of Immunoglobulin Light-Chain Recombination by the Transcription Factor IRF-4 and the Attenuation of Interleukin-7 Signaling', *Immunity*, 28(3), pp. 335–345. doi: 10.1016/j.immuni.2007.12.019.

Johnson, K. D. *et al.* (2007) 'Friend of GATA-1-independent transcriptional repression: A novel mode of GATA-1 function', *Blood*, 109(12), pp. 5230–5233. doi: 10.1182/blood-2007-

02-072983.

Junying, Y. *et al.* (2009) 'Human induced pluripotent stem cells free of vector and transgene sequences', *Science*. doi: 10.1126/science.1172482.

Kaufman, D. S. *et al.* (2001) 'Hematopoietic colony-forming cells derived from human embryonic stem cells', *Proceedings of the National Academy of Sciences of the United States of America*, 98(19), pp. 10716–10721. doi: 10.1073/pnas.191362598.

Kaushansky, K. (2006) 'Lineage-Specific Hematopoietic Growth Factors', pp. 2034–2045.

Kawamoto, H. *et al.* (2010) 'A map for lineage restriction of progenitors during hematopoiesis: The essence of the myeloid-based model', *Immunological Reviews*, 238(1), pp. 23–36. doi: 10.1111/j.1600-065X.2010.00959.x.

Kelminson, K. L., Elias, E. R. and Goldson, E. (2011) 'Down syndrome', in *Berman's Pediatric Decision Making*. doi: 10.1016/B978-0-323-05405-8.00160-1.

Kennedy, M. *et al.* (1997) 'A common precursor for primitive erythropoiesis and definitive haematopoiesis', *Nature*, 386(6624), pp. 488–493. doi: 10.1038/386488a0.

Kennedy, M. *et al.* (2012) 'T Lymphocyte Potential Marks the Emergence of Definitive Hematopoietic Progenitors in Human Pluripotent Stem Cell Differentiation Cultures', *Cell Reports*, 2(6), pp. 1722–1735. doi: 10.1016/j.celrep.2012.11.003.

Khan, I., Malinge, S. and Crispino, J. D. (2011) 'Myeloid leukemia in down syndrome', *Critical Reviews in Oncogenesis*, 16(1–2), pp. 25–36. doi: 10.1615/CritRevOncog.v16.i1-2.40.

Kiel, M. J. *et al.* (2005) 'SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells', *Cell*, 121(7), pp. 1109–1121. doi: 10.1016/j.cell.2005.05.026.

Kim, K. *et al.* (2010) 'Epigenetic memory in induced pluripotent stem cells', *Nature*, 467(7313), pp. 285–290. doi: 10.1038/nature09342.

Kin Chan, S. S. (2013) 'What is a Master Regulator?', *Journal of Stem Cell Research & Therapy*, 03(02), pp. 10–13. doi: 10.4172/2157-7633.1000e114.

Kitajima, K. *et al.* (2006) 'Redirecting differentiation of hematopoietic progenitors by a transcription factor, GATA-2', *Blood*, 107(5), pp. 1857–1863. doi: 10.1182/blood-2005-06-2527.

Klimchenko, O. *et al.* (2009) 'A common bipotent progenitor generates the erythroid and megakaryocyte lineages in embryonic stem cell-derived primitive hematopoiesis', *Blood*, 114(8), pp. 1506–1517. doi: 10.1182/blood-2008-09-178863.

Klusmann, J. H. *et al.* (2010) 'Developmental stage-specific interplay of GATA1 and IGF signaling in fetal megakaryopoiesis and leukemogenesis', *Genes and Development*, 24(15), pp. 1659–1672. doi: 10.1101/gad.1903410.

Kollman, C. *et al.* (2001) 'Donor characteristics as risk factors in recipients after transplantation of bone marrow from unrelated donors: The effect of donor age', *Blood*, 98(7), pp. 2043–2051. doi: 10.1182/blood.V98.7.2043.

Kondo, M. *et al.* (2000) 'Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines', *Nature*, 407(6802), pp. 383–386. doi: 10.1038/35030112.

Korbel, J. O. *et al.* (2009) 'The genetic architecture of Down syndrome phenotypes revealed by high-resolution analysis of human segmental trisomies', *Proceedings of the National Academy of Sciences of the United States of America*, 106(29), pp. 12031–12036. doi: 10.1073/pnas.0813248106.

Korotkevich, G., Sukhov, V. and Sergushichev, A. (2016) 'Fast gene set enrichment analysis', pp. 1–29. doi: 10.1101/060012.

Kouskoff, V. *et al.* (1998) 'Antigens varying in affinity for the B cell receptor induce differential B lymphocyte responses', *Journal of Experimental Medicine*, 188(8), pp. 1453–

1464. doi: 10.1084/jem.188.8.1453.

Kristiansen, T. A. *et al.* (2016) 'Cellular Barcoding Links B-1a B Cell Potential to a Fetal Hematopoietic Stem Cell State at the Single-Cell Level', *Immunity*, 45(2), pp. 346–357. doi: 10.1016/j.immuni.2016.07.014.

Krivtsov, A. V. *et al.* (2006) 'Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9', *Nature*, 442(7104), pp. 818–822. doi: 10.1038/nature04980.

Kyttälä, A. *et al.* (2016) 'Genetic Variability Overrides the Impact of Parental Cell Type and Determines iPSC Differentiation Potential', *Stem Cell Reports*, 6(2), pp. 200–212. doi: 10.1016/j.stemcr.2015.12.009.

Laiosa, C. V. *et al.* (2006) 'Reprogramming of Committed T Cell Progenitors to Macrophages and Dendritic Cells by C/EBPα and PU.1 Transcription Factors', *Immunity*, 25(5), pp. 731–744. doi: 10.1016/j.immuni.2006.09.011.

Lane, A. A. *et al.* (2014) 'Triplication of a 21q22 region contributes to B cell transformation through HMGN1 overexpression and loss of histone H3 Lys27 trimethylation', *Nature Genetics*. doi: 10.1038/ng.2949.

Lansdorp, P. M., Dragowska, W. and Mayani, H. (1993) 'Ontogeny-related changes in proliferative potential of human hematopoietic cells', *Journal of Experimental Medicine*, 178(3), pp. 787–791. doi: 10.1084/jem.178.3.787.

Laurent, A. P. *et al.* (2020) 'Constitutive Activation of RAS/MAPK Pathway Cooperates with Trisomy 21 and Is Therapeutically Exploitable in Down Syndrome B-cell Leukemia', *Clinical Cancer Research*. doi: 10.1158/1078-0432.ccr-19-3519.

Laurenti, E. and Göttgens, B. (2018) 'From haematopoietic stem cells to complex differentiation landscapes', *Nature*, 553(7689), pp. 418–426. doi: 10.1016/j.physbeh.2017.03.040.

Laurenti, E. *et al.* (2013) 'The transcriptional architecture of early human hematopoiesis identifies multilevel control of lymphoid commitment', *Nature Immunology*, 14(7), pp. 756–763. doi: 10.1038/ni.2615.

Lee, J. , Dykstra, B., Sackstein, R. Rossi, D. J. (2015) 'Progress and obstacles towards generating hematopoietic stem cells from pluripotent stem cells', *Curr Opin Hematology*, 22(4), pp. 317–323. doi: 10.1038/s41395-018-0061-4.

Leitão, A., Boudry, P. and Thiriot-Quiévreux, C. (2001) 'Evidence of differential chromosome loss in aneuploid karyotypes of the Pacific oyster, Crassostrea gigas'. doi: 10.1139/gen-44-4-735.

Li, J. (2011) 'Quiescence regulators for hematopoietic stem cell', *Experimental Hematology*. ISEH - Society for Hematology and Stem Cells, 39(5), pp. 511–520. doi: 10.1016/j.exphem.2011.01.008.

Li, Y. *et al.* (2014) 'Constitutional and somatic rearrangement of chromosome 21 in acute lymphoblastic leukaemia', *Nature*. Nature Publishing Group, 508(1), pp. 98–102. doi: 10.1038/nature13115.

Lieber, M. R. (2016) 'Mechanisms of Human Lymphoid Chromosomal Translocations', *Nat Rev Cancer*, 16(6), pp. 387–398. doi: 10.1016/j.physbeh.2017.03.040.

Lim, W. F. *et al.* (2013) 'Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells.', *Stem cell research & therapy*. doi: 10.1186/scrt222.

Liu, B. *et al.* (2015) 'Stem and progenitor cell dysfunction in human trisomies', *EMBO reports*. doi: 10.15252/embr.201439583.

Liu, C. *et al.* (2011) 'Genetic analysis of Down syndrome-associated heart defects in mice', *Human Genetics*, 130(5), pp. 623–632. doi: 10.1007/s00439-011-0980-2.

Lockstone, H. E. *et al.* (2007) 'Gene expression profiling in the adult Down syndrome brain',

Genomics, 90, pp. 647–660. doi: 10.1016/j.ygeno.2007.08.005.

Loges, S. *et al.* (2004) 'Identification of the adult human hemangioblast', *Stem Cells and Development*, 13(3), pp. 229–242. doi: 10.1089/154732804323099163.

Lorenz, E., Congdon, C. and Uphoff, D. . (1952) 'Modification of acute irradiation injury in mice and guinea-pigs by bone marrow injections.', *Radiology*, 58(6), pp. 863–877. doi: 10.1148/58.6.863.

Love, M. I., Huber, W. and Anders, S. (2014) 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2', *Genome Biology*, 15(12), pp. 1–21. doi: 10.1186/s13059-014-0550-8.

Lyle, R. *et al.* (2004) 'Gene expression from the aneuploid chromosome in a trisomy mouse model of Down syndrome', *Genome Research*. doi: 10.1101/gr.2090904.

MacLean, G.A. Menne, T.F. Guo, G. . Sanchez, D.J. Park, I.H. Daley, G.Q. and Orkin, S. H. (2012) 'Altered hematopoiesis in trisomy 21 as revealed through in vitro differentiation of isogenic human pluripotent cells', *Proceedings of the National Academy of Sciences of the United States of America*, 109(43), pp. 17567–17572. doi: 10.1097/GME.0000000000000638.

MacLean, G. A. *et al.* (2018) 'Downregulation of Endothelin Receptor B Contributes to Defective B Cell Lymphopoiesis in Trisomy 21 Pluripotent Stem Cells', *Scientific Reports*. Springer US, 8(1), pp. 1–10. doi: 10.1038/s41598-018-26123-y.

Maia, A. T. *et al.* (2001) 'Molecular tracking of leukemogenesis in a triplet pregnancy', *Blood*, 98(2), pp. 478–482. doi: 10.1182/blood.V98.2.478.

Malcom A.S. Moore (2004) *Ontogeny of the Hematopoietic System*, *Handbook of Stem CellsVolume 2Copyright © 2004 by Elsevier Inc.All rights of reproduction in any form reserved.15*. Elsevier Inc. doi: 10.1016/B978-0-12-374279-7.01002-X.

Malin, S., McManus, S. and Busslinger, M. (2010) 'STAT5 in B cell development and leukemia', *Current Opinion in Immunology*. Elsevier Ltd, 22(2), pp. 168–176. doi: 10.1016/j.coi.2010.02.004.

Malinge, S. Chlon, T. Dor ´e, L.C. Ketterling, R.P. Tallman, M.S. Paietta, E. Gamis, A. Taub, J.W. Chou, S.T. Weiss, M.J. Crispino, J.D., Figueroa, M. (2013) 'Development of acute megakaryoblastic leukemia in Down syndrome is associated with sequential epigenetic changes', *Blood*, 122(14), pp. 33–43. doi: 10.1182/blood-2013-05-503011.This.

Malinge, S. *et al.* (2008) 'Activating mutations in human acute megakaryoblastic leukemia', *Blood*, 112(10), pp. 4220–4226. doi: 10.1182/blood-2008-01-136366.

Malinge, S. *et al.* (2012) 'Increased dosage of the chromosome 21 ortholog Dyrk1a promotes megakaryoblastic leukemia in a murine model of down syndrome', *Journal of Clinical Investigation*, 122(3), pp. 948–962. doi: 10.1172/JCI60455.

Malinge, S., Izraeli, S. and Crispino, J. D. (2009) 'Insights into the manifestations, outcomes, and mechanisms of leukemogenesis in down syndrome', *Blood*. doi: 10.1182/blood-2008-11-163501.

Maloney, K. W. *et al.* (2010) 'Down syndrome childhood acute lymphoblastic leukemia has a unique spectrum of sentinel cytogenetic lesions that influences treatment outcome: A report from the Children's Oncology Group', *Blood*. doi: 10.1182/blood-2009-07-235291.

Mao, R. *et al.* (2005) 'Primary and secondary transcriptional effects in the developing human Down syndrome brain and heart', *Genome Biology*, 6(13). doi: 10.1186/gb-2005-6-13-r107.

Marion K. Mateos, D. B. *et al.* (2015) 'Down syndrome and leukemia: insights into leukemogenesis and translational targets', *Translational Pediatrics*, 4(2), pp. 76–92.

Maroz, A. *et al.* (2014) 'GATA1s induces hyperproliferation of eosinophil precursors in Down syndrome transient leukemia', *Leukemia*, 28(6), pp. 1259–1270. doi: 10.1038/leu.2013.373.

Mårtensson, I. L. and Ceredig, R. (2000) 'Role of the surrogate light chain and the pre-B-cell receptor in mouse B-cell development', *Immunology*, 101(4), pp. 435–441. doi: 10.1046/j.1365-2567.2000.00151.x.

Mayshar, Y. *et al.* (2010) 'Identification and classification of chromosomal aberrations in human induced pluripotent stem cells', *Cell Stem Cell*. Elsevier Inc., 7(4), pp. 521–531. doi: 10.1016/j.stem.2010.07.017.

McCulloch Till, J E, E. A. (1961) 'A Direct Measurement of the Radiation Sensitivity of Normal Mouse Bone Marrow Cells', *Radiat Research*, 14, pp. 213–222.

McElyea, S. D. *et al.* (2016) 'Influence of prenatal EGCG treatment and Dyrk1a dosage reduction on craniofacial features associated with Down syndrome', *Human Molecular Genetics*, 25(22), pp. 4856–4869. doi: 10.1093/hmg/ddw309.

McGrath, K. E. and Palis, J. (2005) 'Hematopoiesis in the yolk sac: More than meets the eye', *Experimental Hematology*, 33(9 SPEC. ISS.), pp. 1021–1028. doi: 10.1016/j.exphem.2005.06.012.

Medvedovic, J. *et al.* (2011) *Pax5: A Master Regulator of B Cell Development and Leukemogenesis*. 1st edn, *Advances in Immunology*. 1st edn. Elsevier Inc. doi: 10.1016/B978-0-12-385991-4.00005-2.

Medvinsky, A., Rybtsov, S. and Taoudi, S. (2011) 'Embryonic origin of the adult hematopoietic system: Advances and questions', *Development*, 138(6), pp. 1017–1031. doi: 10.1242/dev.040998.

Melissa L. Holmes, S. C. and Lynn M. Corcoran, and S. L. N. (2006) 'Repression of Flt3 by Pax5 is crucial for B-cell lineage commitment', *GENES & DEVELOPMENT*, 20(8), pp. 933–938. doi: 10.1101/gad.1396206.GENES.

Mikkola, H. K. A. and Orkin, S. H. (2006) 'The journey of developing hematopoietic stem

cells', *Development*, 133(19), pp. 3733–3744. doi: 10.1242/dev.02568.

Miyauchi, J. and Kawaguchi, H. (2014) 'Fetal liver stromal cells support blast growth in transient abnormal myelopoiesis in Down syndrome through GM-CSF', *Journal of Cellular Biochemistry*, 115(6), pp. 1176–1186. doi: 10.1002/jcb.24764.

Montecino-Rodriguez, E., Leathers, H. and Dorshkind, K. (2006) 'Identification of a B-1 B cell-specified progenitor', *Nature Immunology*, 7(3), pp. 293–301. doi: 10.1038/ni1301.

Moore, M.A.S and Owen, J. J. . (1965) 'Chromosome marker studies on the development of the haematopoietic system in the chick embryo', *Nature*, 208, pp. 989–990.

Morris, V. *et al.* (2020) 'Single-cell analysis reveals mechanisms of plasticity of leukemia initiating cells', *bioRxiv Cancer Biology*. doi: 10.1101/2020.04.29.066332.

Morrison, S.J. and Scadden, D. T. (2014) 'The bone marrow niche for haematopoietic stem cells', 505(7483), pp. 327–334. doi: 10.1038/nature12984.The.

Mowery, C. T. *et al.* (2018) 'Trisomy of a Down Syndrome Critical Region Globally Amplifies Transcription via HMGN1 Overexpression', 25(7), pp. 1898–1911. doi: 10.1016/j.celrep.2018.10.061.Trisomy.

Müller, A. M. *et al.* (1994) 'Development of hematopoietic stem cell activity in the mouse embryo', *Immunity*, 1(4), pp. 291–301. doi: 10.1016/1074-7613(94)90081-7.

Mullighan, Charles G *et al.* (2009) 'Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia.', *Nature genetics*. doi: 10.1038/ng.469.

Mullighan, Charles G. *et al.* (2009) 'Rearrangement of CRLF2 in B-progenitor-and Down syndrome-associated acute lymphoblastic leukemia', *Nature Genetics*, 41(11), pp. 1243–1246. doi: 10.1038/ng.469.

Müschen, M. (2015) 'Rationale for targeting the pre-B-cell receptor signaling pathway in acute lymphoblastic leukemia', *Blood*, 125(24), pp. 3688–3693. doi: 10.1182/blood-2015-01-

567842.

Nakayama, J. *et al.* (2009) 'BLNK suppresses pre B-cell leukemogenesis through inhibition of JAK3', *Blood*, 113(7), pp. 1483–1492. doi: 10.1182/blood-2008-07-166355.

Ng, A. P. *et al.* (2010) 'Trisomy of Erg is required for myeloproliferation in a mouse model of Down syndrome', *Blood*, 115(19), pp. 3966–3969. doi: 10.1182/blood-2009-09-242107.

Ng, A. P. *et al.* (2015) 'Early Lineage Priming by Trisomy of Erg Leads to Myeloproliferation in a Down Syndrome Model', *PLoS Genetics*, 11(5), pp. 1–19. doi: 10.1371/journal.pgen.1005211.

Nilsson, S. K. *et al.* (2005) 'Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells', *Blood*, 106(4), pp. 1232–1239. doi: 10.1182/blood-2004-11-4422.

Nishihara, M. *et al.* (1998) 'A combination of stem cell factor and granulocyte colony-stimulating factor enhances the growth of human progenitor B cells supported by murine stromal cell line MS-5.', *European journal of immunology*. doi: 10.1002/(SICI)1521-4141(199803)28:03<855::AID-IMMU855>3.0.CO;2-X.

Nižetić, D. and Groet, J. (2012) 'Tumorigenesis in Down's syndrome: Big lessons from a small chromosome', *Nature Reviews Cancer*, 12(10), pp. 721–732. doi: 10.1038/nrc3355.

Norrman, K. *et al.* (2010) 'Quantitative comparison of constitutive promoters in human ES cells', *PLoS ONE*, 5(8). doi: 10.1371/journal.pone.0012413.

North, T.E. Stacy, T. Matheny, C.J. Speck, N., De Bruinja, M. (2004) 'Runx1 Is Expressed in Adult Mouse Hematopoietic Stem Cells and Differentiating Myeloid and Lymphoid Cells, But Not in Maturing Erythroid Cells', *Stem Cells*, 22, pp. 158–168.

Notta, F. *et al.* (2016) 'Distinct routes of lineage development reshape teh human blood hierarchy acorss ontogeny', 351(6269), pp. 1–22. doi: 10.1126/science.aab2116.DISTINCT.

Nowell, P. C. (1976) 'The clonal evolution of tumor cell populations.', *Science (New York, N.Y.)*. doi: 10.1126/science.959840.

Nutt, S. L. *et al.* (1999) 'Commitment to the B-lymphoid lineage depends on the transcription factor Pax5', *Nature*, 401, pp. 556–562. doi: 10.1038/44076.

Nutt, S. L. and Kee, B. L. (2007) 'The Transcriptional Regulation of B Cell Lineage Commitment', *Immunity*. doi: 10.1016/j.immuni.2007.05.010.

O'Byrne, S. I. *et al.* (2019) 'Trisomy 21 Driven Pro-Inflammatory Signalling in Fetal Bone Marrow May Play a Role in Perturbed B-Lymphopoiesis and Acute Lymphoblastic Leukemia of Down Syndrome', *Blood*, 134(Supplement_1), p. 1206. doi: 10.1182/blood-2019-130180.

Oberlin, E. *et al.* (2010) 'VE-cadherin expression allows identification of a new class of hematopoietic stem cells within human embryonic liver', *Blood*, 116(22), pp. 4444–4455. doi: 10.1182/blood-2010-03-272625.

Ohno, S. (1979) *Major Sex-Determining Genes*. Springer. Available at: https://www.springer.com/gp/book/9783642812637.

Olson, L. E. *et al.* (2007) 'Trisomy for the Down syndrome "critical region" is necessary but not sufficient for brain phenotypes of trisomic mice', *Human Molecular Genetics*, 16(7), pp. 774–782. doi: 10.1093/hmg/ddm022.

Osawa, M. Hanada, K. I. H. and Hiromitsu, N. (1996) 'Long-Term Lymphohematopoietic Reconstitution by a Single CD34-Low/Negative Hematopoietic Stem Cell', *Science*, 273(1995), pp. 242–246.

Otero, D. C., Anzelon, A. N. and Rickert, R. C. (2003) 'CD19 function in early and late B cell development: I. Maintenance of follicular and marginal zone B cells requires CD19-dependent survival signals.', *Journal of immunology (Baltimore, Md. : 1950)*. doi: 10.4049/jimmunol.170.1.73.

Palis, J. and Yoder, M. C. (2001) 'Yolk-sac hematopoiesis: The first blood cells of mouse and man', *Experimental Hematology*. doi: 10.1016/S0301-472X(01)00669-5.

Papayannopoulou, T. and Scadden, D. T. (2008) 'Stem-cell ecology and stem cells in motion', *Blood*, 111(8), pp. 3923–3930. doi: 10.1182/blood-2007-08-078147.

Parrish, Y. K. *et al.* (2009) 'IL-7 Dependence in Human B Lymphopoiesis Increases During Progression of Ontogeny from Cord Blood to Bone Marrow', *Journal of Immunology*, 182(7), pp. 4255–4266. doi: 10.1080/21622671.2017.1297253.

Paul, F. *et al.* (2015) 'Transcriptional Heterogeneity and Lineage Commitment in Myeloid Progenitors', *Cell*. Elsevier Inc., 163(7), pp. 1663–1677. doi: 10.1016/j.cell.2015.11.013.

Perluigi, M. and Butterfield, D. A. (2012) 'Oxidative stress and down syndrome: A route toward Alzheimer-like dementia', *Current Gerontology and Geriatrics Research*, 2012. doi: 10.1155/2012/724904.

Pfau, S. J. *et al.* (2016) 'Aneuploidy impairs hematopoietic stem cell fitness and is selected against in regenerating tissues in vivo', *Genes and Development*, 30(12), pp. 1395–1408. doi: 10.1101/gad.278820.116.

Pisco, A. O. and Huang, S. (2015) 'Non-genetic cancer cell plasticity and therapy-induced stemness in tumour relapse: "What does not kill me strengthens me"', *British Journal of Cancer*. Nature Publishing Group, 112(11), pp. 1725–1732. doi: 10.1038/bjc.2015.146.

Polacov *et al.* (2018) 'Transient Myeloproliferative Disorder in Neonates with Down syndrome: Case Report and Review', *Journal of Down Syndrome & Chromosome Abnormalities*, 04(01), pp. 1–4. doi: 10.4172/2472-1115.1000126.

Pongubala, J. M. R. *et al.* (2008) 'Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5', *Nature Immunology*, 9(2), pp. 203–215. doi: 10.1038/ni1555.

Porse, B. T. *et al.* (2001) 'E2F repression by C/EBPα is required for adipogenesis and granulopoiesis in vivo', *Cell*, 107(2), pp. 247–258. doi: 10.1016/S0092-8674(01)00516-5.

Quách, T. D. *et al.* (2016) ' Human B-1 and B-2 B Cells Develop from Lin − CD34 + CD38 lo Stem Cells ', *The Journal of Immunology*, 197(10), pp. 3950–3958. doi: 10.4049/jimmunol.1600630.

Quesenberry, P. *et al.* (2001) 'Physical and physiological plasticity of hematopoietic stem cells', *Blood Cells, Molecules, and Diseases*, 27(5), pp. 934–937. doi: 10.1006/bcmd.2001.0460.

Rabin, K. R. and Whitlock, J. A. (2009) 'Malignancy in Children with Trisomy 21', *The Oncologist*. doi: 10.1634/theoncologist.2008-0217.

Rhodes, J. *et al.* (2005) 'Interplay of pu.1 and Gata1 determines myelo-erythroid progenitor cell fate in zebrafish', *Developmental Cell*, 8(1), pp. 97–108. doi: 10.1016/j.devcel.2004.11.014.

Richard R. Hardy, Condie E. Carmack, S. A. S. and John D. Kemp, and K. H. (1991) 'Resolution and Characterization of Pro-B and Pre-Pro-B Cell Stages in Normal Mouse Bone Marrow', *Journal of Experimental Medicine*, 173(May).

Richard R. Hardy and Kyoko Hayakawa (2015) 'Perspectives on fetal-derived CD5+ B1 B cells', *Eur J Immunol*, 45(11), pp. 2978–2984. doi: 10.1016/j.physbeh.2017.03.040.

Rieger, M. a *et al.* (2009) 'HAeamtopoietic Cytokines Can instruct Lineage Choice', *Science*, 325(July), pp. 217–218.

Roberts, I. *et al.* (2013) 'The impact of trisomy 21 on foetal haematopoiesis', *Blood Cells, Molecules, and Diseases*. doi: 10.1016/j.bcmd.2013.07.008.

Roberts, I. and Izraeli, S. (2014) 'Haematopoietic development and leukaemia in Down syndrome', *British Journal of Haematology*, 167(5), pp. 587–599. doi: 10.1111/bjh.13096.

Ronaldson, K. *et al.* (2018) 'Advanced maturation of human cardiac tissue grown from pluripotent stem cells', *Nature*, 556(7700), pp. 1–34. doi: 10.1038/s41586-018-0016-3.Advanced.

V. Roschke, A. and R. Kirsch, I. (2012) 'Targeting Karyotypic Complexity and Chromosomal Instability of Cancer Cells', *Current Drug Targets*, 11(10), pp. 1341–1350. doi: 10.2174/1389450111007011341.

Rowe, R. G. and Daley, G. Q. (2019) 'Induced pluripotent stem cells in disease modelling and drug discovery', *Nature Reviews Genetics*. Springer US, 20(7), pp. 377–388. doi: 10.1038/s41576-019-0100-z.

Roy, A. *et al.* (2009) 'Acute megakaryoblastic leukaemia (AMKL) and transient myeloproliferative disorder (TMD) in Down syndrome: A multi-step model of myeloid leukaemogenesis', *British Journal of Haematology*, 147(1), pp. 3–12. doi: 10.1111/j.1365-2141.2009.07789.x.

Roy, A. *et al.* (2012) 'Perturbation of fetal liver hematopoietic stem and progenitor cell development by trisomy 21.', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1211405109.

Ruiz-Herguido, C. *et al.* (2012) 'Hematopoietic stem cell development requires transient Wnt/β-catenin activity', *Journal of Experimental Medicine*, 209(8), pp. 1457–1468. doi: 10.1084/jem.20120225.

Sabin, F. R. (1917) 'Preliminary note on the differentiation of angioblasts and the method by which they produce blood-vessels, blood-plasma and red blood-cells as seen in the living chick', *The Anatomical Record*, 13(4), pp. 199–204. doi: 10.1002/ar.1090130403.

Saida, S. *et al.* (2013) 'Clonal selection in xenografted TAM recapitulates the evolutionary process of myeloid leukemia in Down syndrome', *Blood*, 121(21), pp. 4377–4387. doi:

10.1182/blood-2012-12-474387.

Salvagiotto, M.G. Zhao, Y. Vodyanik, M. Ruotti, V. Stewart, R. and Marra, M. Thomson, J. Eaves, C., and Slukvin, I. (2008) 'Molecular Profiling Reveals Similarities and Differences Between Primitive Subsets of Hematopoietic Cells Generated In Vitro from Human Embryonic Stem Cells and In Vivo during Embryogenesis', *Experimental Hematology*, 36(10), pp. 1377–1389. doi: 10.1038/jid.2014.371.

Sanz, E. *et al.* (2010) 'Ordering human CD34+CD10-CD19+ pre/pro-B-cell and CD19- common lymphoid progenitor stages in two pro-B-cell development pathways', *Proceedings of the National Academy of Sciences of the United States of America*, 107(13), pp. 5925–5930. doi: 10.1073/pnas.0907942107.

Satgé, D. *et al.* (1997) 'An excess of testicular germ cell tumors in down's syndrome three case reports and a review of the literature', *Cancer*, 80(5), pp. 929–935. doi: 10.1002/(SICI)1097-0142(19970901)80:5<929::AID-CNCR14>3.3.CO;2-E.

Schepers, K. Pietras, E.M. Reynaud, D. Flach, J. Binnewies, M. Garg, T. Wagers, A.J. Hsiao, E.C. and Passegue, E. (2013) 'Myeloproliferative Neoplasia Remodels the Endosteal Bone Marrow Niche into a Self-Reinforcing Leukemic Niche', *Cell Stem Cell*, 13(1), pp. 285–299. doi: 10.1161/CIRCULATIONAHA.110.956839.

Schiffmana, J. D. *et al.* (2009) 'Molecular inversion probes reveal patterns of 9p21 deletion and copy number aberrations in childhood leukemia', *Cancer Genet Cytogenet*, 193(1), pp. 9–18. doi: 10.1161/CIRCULATIONAHA.110.956839.

Schwartzman, O. *et al.* (2017) 'Suppressors and activators of JAK-STAT signaling at diagnosis and relapse of acute lymphoblastic leukemia in Down syndrome', *Proceedings of the National Academy of Sciences of the United States of America*, 114(20), pp. E4030–E4039. doi: 10.1073/pnas.1702489114.

Shapiro, B. L. (1999) 'The Down syndrome critical region.', *Journal of neural transmission. Supplementum*.

Shirai, C. L. *et al.* (2015) 'Mutant U2AF1 Expression Alters Hematopoiesis and Pre-mRNA Splicing In Vivo', *Cancer Cell*. Elsevier Inc., 27(5), pp. 631–643. doi: 10.1016/j.ccell.2015.04.008.

Shochat, C. *et al.* (2011) 'Gain-of-function mutations in interleukin-7 receptor-α (IL7R) in childhood acute lymphoblastic leukemias.', *The Journal of experimental medicine*. doi: 10.1084/jem.201105802011512c.

Siminovitch, L., McCulloch, E. A. and Till, J. E. (1963) 'The distribution of colony-forming cells among spleen colonies', *Journal of Cellular and Comparative Physiology*, 62(3), pp. 327–336. doi: 10.1002/jcp.1030620313.

Slukvin, I. I. (2013) 'Hematopoietic specification from human pluripotent stem cells: current advances and challenges toward de novo generation of hematopoietic stem cells', *American Society of Haematology*, 122(25), pp. 4035–4047. doi: 10.1182/blood-2013-07-474825.patients.

Somasundaram, R. *et al.* (2015) 'Transcription factor networks in B-cell differentiation link development to acute lymphoid leukemia', *Blood*, 126(2), pp. 144–152. doi: 10.1182/blood-2014-12-575688.

Spangrude, G. J., Heimfeld, S. and Weissman, I. L. (1988) 'Purification and characterization of mouse hematopoietic stem cells', *Science*, pp. 58–62.

Stankiewicz, M. J. and Crispino, J. D. (2009) 'ETS2 and ERG promote megakaryopoiesis and synergize with alterations in GATA-1 to immortalize hematopoietic progenitor cells', *Blood*. doi: 10.1182/blood-2008-08-174813.

Stark, B. *et al.* (2002) 'Acquired trisomy 21 and distinct clonal evolution in acute

megakaryoblastic leukaemia in young monozygotic twins', *British Journal of Haematology*, 118(4), pp. 1082–1086. doi: 10.1046/j.1365-2141.2002.03756.x.

Subramanian, A. *et al.* (2005) 'Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles', *Proceedings of the National Academy of Sciences of the United States of America*, 102(43), pp. 15545–15550. doi: 10.1073/pnas.0506580102.

Sugimura, R. *et al.* (2017) 'Haematopoietic stem and progenitor cells from human pluripotent stem cells', *Nature*. Nature Publishing Group, 545(7655), pp. 432–438. doi: 10.1038/nature22370.

Sullivan, K. D. *et al.* (2016) 'Trisomy 21 consistently activates the interferon response', *eLife*, 5(JULY). doi: 10.7554/eLife.16220.

Suzuki, N. *et al.* (2013) 'Generation of engraftable hematopoietic stem cells from induced pluripotent stem cells by way of teratoma formation', *Molecular Therapy*, 21(7), pp. 1424–1431. doi: 10.1038/mt.2013.71.

Taichman, R. S., Reilly, M. J. and Emerson, S. G. (1996) 'Human osteoblasts support human hematopoietic progenitor cells in in vitro bone marrow cultures', *Blood*, 87(2), pp. 518–524. doi: 10.1182/blood.v87.2.518.bloodjournal872518.

Takahashi, K. and Yamanaka, S. (2006) 'Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors', *Cell*. doi: 10.1016/j.cell.2006.07.024.

Tarleton, H. P. and Lemischka, I. R. (2010) 'Delayed differentiation in embryonic stem cells and mesodermal progenitors in the absence of CtBP2', *Mechanisms of Development*. Elsevier Ireland Ltd, 127(1–2), pp. 107–119. doi: 10.1016/j.mod.2009.10.002.

Teichweyde, N., Horn, P. A. and Klump, H. (2017) 'HOXB4 Increases Runx1 Expression to

Promote the de novo Formation of Multipotent Hematopoietic Cells', *Transfusion Medicine and Hemotherapy*, 44(3), pp. 128–134. doi: 10.1159/000477130.

Thompson, B. J. *et al.* (2015) 'DYRK1A controls the transition from proliferation to quiescence during lymphoid development by destabilizing Cyclin D3', *Journal of Experimental Medicine*, 212(6), pp. 953–970. doi: 10.1084/jem.20150002.

Tie, R. *et al.* (2019) 'Interleukin-6 signaling regulates hematopoietic stem cell emergence', *Experimental and Molecular Medicine*. Springer US, 51(10). doi: 10.1038/s12276-019-0320-5.

Tunstall-Pedoe, O. *et al.* (2008) 'Abnormalities in the myeloid progenitor compartment in Down syndrome fetal liver precede acquisition of GATA1 mutations', *Blood*, 112(12), pp. 4507–4511. doi: 10.1182/blood-2008-04-152967.

Uenishi, G. *et al.* (2014) 'Tenascin C promotes hematoendothelial development and T lymphoid commitment from human pluripotent stem cells in chemically defined conditions', *Stem Cell Reports*. The Authors, 3(6), pp. 1073–1084. doi: 10.1016/j.stemcr.2014.09.014.

Vesely, C. *et al.* (2017) 'Genomic and transcriptional landscape of P2RY8-CRLF2-positive childhood acute lymphoblastic leukemia', *Leukemia*. doi: 10.1038/leu.2016.365.

De Vita, S. *et al.* (2010) 'Trisomic dose of several chromosome 21 genes perturbs haematopoietic stem and progenitor cell differentiation in Down's syndrome', *Oncogene*. doi: 10.1038/onc.2010.351.

Vodyanik, M. A. *et al.* (2005) 'Human embryonic stem cell-derived CD34+ cells: Efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential', *Blood*. doi: 10.1182/blood-2004-04-1649.

Vodyanik, M. A., Thomson, J. A. and Slukvin, I. I. (2006) 'Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures', *Blood*,

108(6), pp. 2095–2105. doi: 10.1182/blood-2006-02-003327.

Voisin, V. *et al.* (2010) 'Gene profiling of the erythro- and megakaryoblastic leukaemias induced by the Graffi murine retrovirus', *BMC Medical Genomics*, 3. doi: 10.1186/1755-8794-3-2.

Voßhenrich, C. A. J. *et al.* (2003) 'Thymic stromal-derived lymphopoietin distinguishes fetal from adult B cell development', *Nature Immunology*, 4(8), pp. 773–779. doi: 10.1038/ni956.

Wamaitha, S. E. *et al.* (2020) 'IGF1-mediated human embryonic stem cell self-renewal recapitulates the embryonic niche', *Nature Communications*. Springer US, 11(1), pp. 1–16. doi: 10.1038/s41467-020-14629-x.

Wang, L. *et al.* (2005) 'Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression', *Journal of Experimental Medicine*, 201(10), pp. 1603–1614. doi: 10.1084/jem.20041888.

Wang, W. *et al.* (2017) 'Reduced hematopoietic stem cell frequency predicts outcome in acute myeloid leukemia', *Haematologica*, 102(9), pp. 1567–1577. doi: 10.3324/haematol.2016.163584.

Wang, X. *et al.* (2009) 'Inhibition of caspase-mediated anoikis is critical for basic fibroblast growth factor-sustained culture of human pluripotent stem cells', *Journal of Biological Chemistry*, 284(49), pp. 34054–34064. doi: 10.1074/jbc.M109.052290.

Webb, S. *et al.* (1999) 'Mechanisms of deficient cardiac septation in the mouse with trisomy 16', *Circulation Research*, 84(8), pp. 897–905. doi: 10.1161/01.RES.84.8.897.

Wechsler, J. *et al.* (2002) 'Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome', *Nature Genetics*. Nat Genet, 32(1), pp. 148–152. doi: 10.1038/ng955.

Wickham, H. Chang, W. Henry, L. Pedersen, T.L. Takahashi, K. Wilke, C.Woo, K. Yutani, H. Dunnington, D. (2016) *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New

York. Available at: https://ggplot2.tidyverse.org.

Wiemels, J. L. *et al.* (2000) 'Microclustering of TEL-AML1 translocation breakpoints in childhood acute lymphoblastic leukemia', *Genes Chromosomes and Cancer*, 29(3), pp. 219–228. doi: 10.1002/1098-2264(2000)9999:9999<::AID-GCC1028>3.0.CO;2-D.

Wilder, P. T. *et al.* (2006) 'Recognition of the tumor suppressor protein p53 and other protein targets by the calcium-binding protein S100B', *Biochimica et Biophysica Acta - Molecular Cell Research*, 1763(11), pp. 1284–1297. doi: 10.1016/j.bbamcr.2006.08.024.

Wilson, A. and Trumpp, A. (2006) 'Bone-marrow haematopoietic-stem-cell niches', *Nature Reviews Immunology*, 6(2), pp. 93–106. doi: 10.1038/nri1779.

Wolman, M. D. I. J. (1962) 'Parallel responses to chemotherapy in identical twin infants with concordant leukemia', *The Journal of Pediatrics*, 60(1), pp. 91–95.

Wright, M. H. *et al.* (2008) 'Brca1 breast tumors contain distinct CD44+/CD24-and CD133+cells with cancer stem cell characteristics', *Breast Cancer Research*, 10(1), pp. 1–16. doi: 10.1186/bcr1855.

Xavier, A. C., Ge, Y. and Taub, J. W. (2009) 'Down syndrome and malignancies: A unique clinical relationship - A paper from the 2008 William Beaumont Hospital symposium on molecular pathology', *Journal of Molecular Diagnostics*. doi: 10.2353/jmoldx.2009.080132.

Xie, H. *et al.* (2004) 'Stepwise Reprogramming of B Cells into Macrophages', *Cell*, 117, pp. 663–676.

Xie Ting and Allan C. Spradling (1998) 'decapentaplegicIs Essential for the Maintenanceand Division of Germline Stem Cells in the Drosophila Ovary', *Cell*, 94, pp. 251–260. doi: 10.1002/047120918x.emb0359.

Xu, R. *et al.* (2019) 'OLIG2 Drives Abnormal Neurodevelopmental Phenotypes in Human iPSC-Based Organoid and Chimeric Mouse Models of Down Syndrome', *Cell Stem Cell*.

Elsevier Inc., 24(6), pp. 908-926.e8. doi: 10.1016/j.stem.2019.04.014.

Yoder, M. C., Hiatt, K. and Mukherjee, P. (1997) 'In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus', *Proceedings of the National Academy of Sciences of the United States of America*, 94(13), pp. 6776–6780. doi: 10.1073/pnas.94.13.6776.

Yoshida, K. *et al.* (2011) 'Frequent pathway mutations of splicing machinery in myelodysplasia', *Nature*. Nature Publishing Group, 478(7367), pp. 64–69. doi: 10.1038/nature10496.

Yu, G. *et al.* (2012) 'ClusterProfiler: An R package for comparing biological themes among gene clusters', *OMICS A Journal of Integrative Biology*, 16(5), pp. 284–287. doi: 10.1089/omi.2011.0118.

Zhang, C. C. and Lodish, H. F. (2004) 'Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells', *Blood*, 103(7), pp. 2513–2521. doi: 10.1182/blood-2003-08-2955.

Zhang, M. *et al.* (2018) 'Hoxb5 reprograms B cells into functional T lymphocytes', *Nat Immunol*, 19(3), pp. 279–290. doi: 10.1038/s41590-018-0046-x.Hoxb5.

Zhou, B. *et al.* (2005) 'Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice', *Nature Immunology*, 6(10), pp. 1047–1053. doi: 10.1038/ni1247.

Zhou, Y. *et al.* (2015) 'Lin28b promotes fetal B lymphopoiesis through the transcription factor Arid3a', *Journal of Experimental Medicine*, 212(4), pp. 569–580. doi: 10.1084/jem.20141510.

Zhou, Y. *et al.* (2019) 'Overexpression of GATA2 Enhances Development and Maintenance of Human Embryonic Stem Cell-Derived Hematopoietic Stem Cell-like Progenitors', *Stem Cell Reports*. ElsevierCompany., 13(1), pp. 31–47. doi: 10.1016/j.stemcr.2019.05.007.

Zriwil, A. *et al.* (2016) 'Macrophage colony-stimulating factor receptor marks and regulates a fetal myeloid-primed B-cell progenitor in mice', *Blood*, 128(2), pp. 217–226. doi: 10.1182/blood-2016-01-693887.