University of the West of England
BRISTOL

THE EFFECT OF EXOGENOUS *IN VITRO* EXPRESSION OF WILD TYPE AND MUTANT JAK2 IN ERYTHROBLAST PROGENITORS

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A thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol for the degree of Professional Doctorate in Biomedical Sciences.

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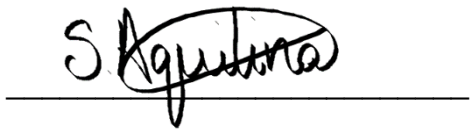
To my family and fiancé...

for their constant love, support and encouragement.

Authorship Declaration

I, Sephora Aquilina, hereby certify that this thesis has been composed by myself, that it is a record of my own work except where stated otherwise, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.



Abstract

Red blood cell (RBC) transfusion has become a routine and indispensable procedure for many clinical purposes. As there is no appropriate alternative, the *in vitro* manufacture of RBCs is a potential means to ensure an adequate and safe supply of blood products. Various protocols are being employed to grow erythroblasts in culture and it is the aim of this research to understand the cellular changes associated with the expression of exogenous mutant *JAK2* on cultured haematopoietic cells, mimicking the hyperproliferative capacity in polycythaemia vera disease.

The human *JAK2* coding sequence was amplified by PCR and inserted into a linearised pIRES2 vector to create the *JAK2*-pIRES2 construct. Another two constructs were obtained, namely wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3 constructs. Transient transfections were performed in $CD34^{pos}$ cells harvested from human buffy coats using the Amaxa® Nucleofection protocol. The expression of Jak2 was confirmed by flow cytometry through the detection of *JAK2* mRNA with the target probe. The expansion of transfected cells under erythroblast proliferative medium (including erythropoietin, stem cell factor and dexamethasone) was similar to control cells, suggesting that overexpression of Jak2 in $CD34^{pos}$ cells followed by culturing in expansion medium, does not result in over-expansion of erythroblasts.

RNA sequencing analysis compared mut*JAK2*-transfected $CD34^{pos}$ cells to wt*JAK2*-transfected $CD34^{pos}$ cells providing a differential gene expression profile. The top differentially expressed genes encode ribosomal proteins and translation factors indicating that $CD34^{pos}$ cells that were transfected with mut*JAK2* resulted in enhanced translation initiation, a well-known mechanism of proliferation induction. Through Ingenuity Pathway Analysis it was observed that NPM1 was overexpressed, that PI3K/PKB/mTOR pathway was activated and that the differentially expressed genes identified in this study are regulated by MYC, erythropoietin, kit ligand and dexamethasone, concluding that mut*JAK2* expression prepares the cells for proliferation, differentiation and survival.

This research is the first to study the differential gene expression of mutant Jak2 against wild-type Jak2 expressing cells and is the first study to show an association between the *JAK2*-V617F mutation and expression of wild-type *NPM1* in the context of driving over-expansion of haematopoietic cells. Of interest, differential gene expression resulted in a list of genes that are regulated by the factors used in the erythroblast proliferative medium, namely erythropoietin, stem cell factor and dexamethasone. Further studies are required to understand the maturity stage (beyond $CD34^{pos}$) at which this differential gene expression exerts a function on erythroblast proliferation.

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List of Abbreviations

4E-BP	eIF4E-binding protein
5637-CM	5637-Conditioned medium
αMEM	α minimal essential medium
A	adenine
AML	acute myeloid leukaemia
ANOVA	one-way analysis of variance
APC	allophycocyanin
APS	ammonium persulfate
bDNA	branched DNA
BFU-E	burst-forming unit-erythroid
BLAST	Basic Local Alignment Search Tool
BME	basal medium eagle
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CD	cluster of differentiation
cDNA	complementary DNA
CFU-E	colony-forming unit-erythroid
CFU-GEMM	colony-forming unit granulocyte, erythroid, monocyte, megakaryocyte
CL	left-hand evaluation cursor
CMP	common myeloid progenitor
CMV	cytomegalovirus
CNT	counts
CO_2	carbon dioxide
CPDA	citrate phosphate dextrose adenine
CR	right-hand evaluation cursor
ctl	control
deH_2O	deionised water
Dex	dexamethasone
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate

E. coli	*Escherichia coli*
EDM	erythroid differentiation medium
EDN1	endothelin 1
EDTA	ethylene-diamine-tetra-acetic acid
eEF1	eukaryotic elongation factor-1
eEF1A1	eukaryotic translation elongation factor 1, alpha-1
eEF1G	eukaryotic translation elongation factor 1, gamma
eIF	eukaryotic initiation factor
Epo	erythropoietin
EpoR	erythropoietin receptor
ET	Essential thrombocythemia
Et Br	ethidium bromide
FBS	foetal bovine serum
$FeNO_3$	ferric nitrate
FERM	4-point-1, erzin, radixin, moesin
$FeSO_4$	ferrous sulphate
FFP	fresh frozen plasma
FI	fold increase
FISH	fluorescent *in situ* hybridisation
FITC	fluorescein isothiocyanate
Flt-3 L	Flt-3 ligand
FSC	forward scatter
G	guanine
gDNA	genomic DNA
GFP	green fluorescent protein
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	Good Manufacturing Practice
GR	glucocorticoid receptor
HEP	human erythroid progenitor cell
hESC	human embryonic stem cell
HSC	haematopoietic stem cell
IGF-I/II	insulin-like growth factor-I/II
IL-1β	interleukin-1 beta
IL-3	interleukin-3

IMDM	Iscove's modified Dulbecco's medium
IMF	idiopathic myelofibrosis
IPA	Ingenuity Pathway Analysis
iPSC	induced pluripotent stem cell
IRES	internal ribosome entry site
JAK2	Janus kinase 2
JH	Jak homology
KITLG	kit ligand
KLH	keyhole limpet hemocyanin
LB	Luria-Bertani
LCM	long-term serum-free culture medium
m7G	methyl-7-guanidine
MCS	multiple cloning site
MDM	modified Dulbecco's medium
MEP	megakaryocytic-erythroid progenitors
$MgCl_2$	magnesium chloride
MNC	mononuclear cell
mRNA	messenger RNA
MSCV	murine stem cell virus
mTOR	mammalian target of rapamycin
mut	mutant
MYC	V-Myc avian myelocytomatosis viral oncogene homolog
MYCN	V-Myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
NIR	near-infrared
NL	left-hand normalisation cursor
NPM1	Nucleophosmin 1
NR	right-hand normalisation cursor
NTC	no template control
ORF	open reading frame
p70S6K	p70S6 kinase
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDK1/2	phosphoinositide-dependent kinase 1/2
PE	phycoerythrin

PE-Cy7	phycoerythrin cyanine 7
PI3K	phosphatidylinositol-3 kinase
PIC	preinitiation complex
PIP2/3	phosphatidylinositol (3,4,5-triphosphate)
PKB	protein kinase B
PLB	passive lysis buffer
PTEN	phosphatase and tensin homologue
PV	Polycythaemia vera
RBC	red blood cell
RCC	red cell concentrate
Rheb	RAS-homologue enriched in brain
RNA	ribonucleic acid
RPKM	reads per kb per million reads
RPL41	ribosomal protein L41
rpm	revolutions per minute
RPM	reads per-million
RPMI	Roswell Park Memorial Institute
RPS	ribosomal proteins
RPS18	ribosomal protein S18
rRNA	ribosomal RNA
SAG-M	saline, adenine, glucose, mannitol
SCF	stem cell factor
SDS	sodium dodecyl sulphate
SFEM	serum-free expansion medium
SH2	SRC homology 2
SOC	super optimal broth with catabolite repression
SSC	side scatter
STAT	signal transducer and activator of transcription
T	thymine
TAC	tetrameric antibody complex
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBS-T	TBS with Tween20
TEMED	tetramethylethylenediamine
Tf	transferrin

TK	tyrosine kinase
TNFα	tumour necrosis factor alpha
TP53	tumour protein p53
Tpo	thrombopoietin
TRALI	transfusion-related acute lung injury
tRNA	transfer RNA
Tsc1/2	tuberous sclerosis protein
Tyk2	tyrosine kinase 2
U	uracil
V	Volts
VEGF	vascular endothelial growth factor
VIA	% viability
w/v	weight per volume
wt	wild-type

CHAPTER 1

INTRODUCTION

1.1 Blood donation

Red blood cell (RBC) transfusion has become a routine and indispensable procedure for many clinical purposes. Each year there are approximately 112.5 million blood donations globally and it is reported that there are around 13,000 blood centres in 176 countries (WHO, 2016). Overall, the blood supply in industrialised countries is adequate, however several developing countries have a shortage of transfusable blood products (Migliacco *et al.*, 2012; Whitsett, Vaglio and Grazzini, 2012). The whole blood donation rate per 1000-population is an indicator for the general availability of blood in a country. The median blood donation rate in high-income countries is 33.1 donations per 1000 individuals, while this rate is lower in middle-income and low-income countries: on average 11.7 donations per 1000 individuals and 4.6 donations per 1000 individuals respectively (Figure 1.1) (WHO, 2016). In the United States it is estimated that 37% of the population are eligible blood donors, however less than 10% donate annually (America's Blood Centers, 2012). The United Kingdom and Malta report similar blood donation levels of only 4% (NHS, 2012; National Blood Transfusion Service, 2012).

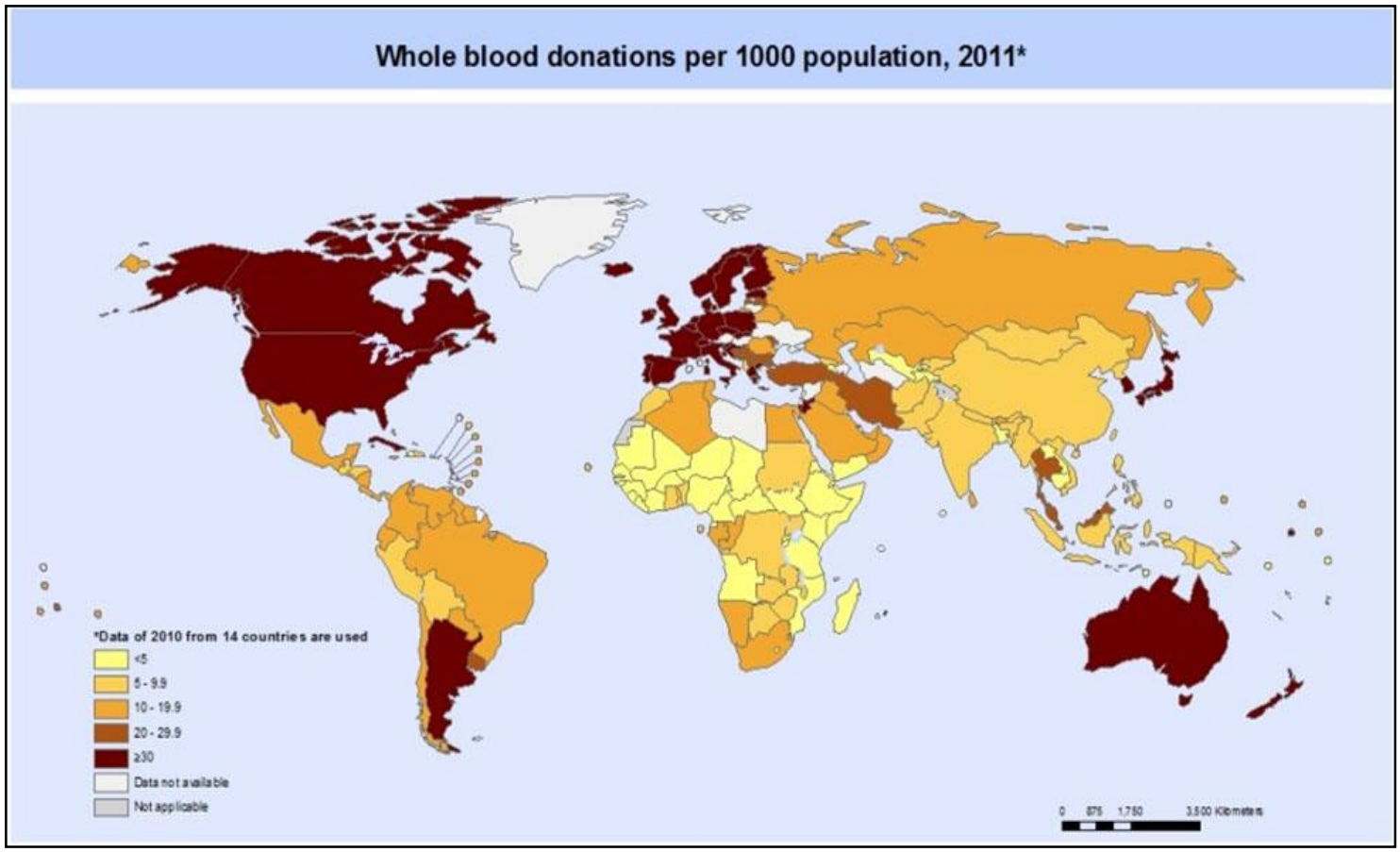


Figure 1.1: Whole blood donations per 1000 population. The whole blood donation rate per 1000-population is an indicator for the general availability of blood in a country. Data source: World Health Organisation. Map production: Blood Transfusion Safety World Health Organisation (WHO, 2014). (Used with permission from the WHO Permissions team).

While at present time, the blood supply in developed countries is adequate, there is growing concern that in the future the blood supply may become inadequate (Whitsett, Vaglio and Grazzini, 2012; Riley, Schwei and McCullough, 2007). The number of conditions and situations for which donors are temporarily or permanently deferred is increasing. This is due to increased rates of diseases (such as acquired immune deficiency syndrome (CDC, 2010) and malaria (Nchinda, 1998)), the emergence of new diseases and pathogens (such as Creutzfeld-Jakob disease, West Nile Virus (Alter, Stramer and Dodd, 2007) and Zika Virus (Grard *et al.,* 2014)), disease-spread to new geographical areas (such as Dengue fever (Migliaccio *et al.*, 2012)) and an increase in the intake of prescription drugs by donors. Paralleling to the decrease in blood donors is the ageing population with increasing transfusion needs for 65+ patients (Rogers *et al.*, 2011; Ali, Auvinen and Rautonen, 2010; Baek *et al.*, 2008). In fact, 76% of blood transfusions in Denmark are given to patients over 65 years of age (Rousseau, Giarratana and Douay, 2014). Man-made or natural disasters and extreme weather conditions can also disrupt routine blood collection leading to sporadic shortages of blood as the limited storage time of RBCs (which is 42 days (Liumbruno *et al.*, 2009)) makes it difficult to have an abundant stock to be prepared in case of an emergency.

Every blood establishment should strive to maintain a safe and adequate blood supply. However, screening for infectious diseases is not fail-safe because occasionally tests to detect them yield false-negative results (Busch *et al.*, 2000). Healthy blood donors who may be asymptomatic, can transmit common viral infections that can cause significant harm to at-risk patients, for example neonates, immuno-compromised patients and pregnant women may develop

serious complications when transfused with cytomegalovirus (CMV) - positive blood (Ziemann *et al.*, 2010). Additionally, frequently transfused patients (such as patients with haemoglobinopathies) can become immunised to some RBC antigens, and they can also develop a very severe complication of transfusion which is transfusion-related acute lung injury (TRALI) where a factor in the transfused blood (often thought to be an anti-HLA antibody) acts against the leukocytes of the patient (Nakamura, 2008) resulting in major morbidity and death. It is estimated that 1 to 3% of the total population of transfused patients worldwide is immunised to blood group antigens (Whitsett, Vaglio and Grazzini, 2012; Lapillone *et al*., 2010). These adverse reactions make it very difficult to find compatible RBC concentrates.

There is also a chronic shortage of blood for some patient groups (Zimring *et al.*, 2011) such as in the USA where more than 40% of sickle cell anaemia patients of African descent develop immune reactions when transfused with blood from Caucasian donors (Migliaccio *et al.*, 2012). Certain blood types are unique to specific racial and ethnic groups, such as the rare blood group U negative which is only found amongst individuals of African or Caribbean descent (Migliaccio *et al.*, 2012). U negative phenotyped blood is essential to patients lacking the U antigen. In Japan RBCs with an AB Negative blood group is always lacking since individuals with this RBC phenotype are rare (Nakamura, 2008).

There are quite a few patients that have other rare red cell phenotypes and it is estimated that there are 165 different rare phenotypes/genotypes (Lapillonne *et al.*, 2010). A rare blood donor phenotype is one who has high-frequency-antigen-negative blood or multiple-common-antigen-negative blood (Flickinger, Petrone

and Church, 2004). To enable the supply of compatible blood for patients with a particular rare red cell phenotype in an efficient manner, Rare Blood Banks have been set up. Donors having rare phenotype blood are recruited and their red cells are processed and stored frozen for an extended period of time for future use. The American Rare Donor Program holds a database of rare donor phenotypes together with frozen stock of these rare red cells. The European Database and Bank of Frozen Blood of Rare Groups operates a similar program. The Malta National Blood Transfusion Service has started this Rare Blood Bank in 2016 where O Negative and O Positive donors who have a rare red cell phenotype are identified through analysis of previous antigen profiling test results. The donors are recruited and their red cells are processed and stored frozen at -80^{o}C for 10 years.

Nevertheless, even Rare Blood Banks encounter difficulty in providing compatible RBC concentrates for some particular patients. As there is no appropriate alternative to RBC transfusion, the *in vitro* manufacture of RBCs is a potential means to ensure an adequate and safe supply of blood products. A successful *in vitro* culture system will enable the production of RBC units from an individual to be used for self-transfusion and it will also allow the banking of certain rare red cell phenotype combinations which would enable the production of RBCs to be given to patients requiring them within a minimal time.

1.2 Erythropoiesis

The haematopoietic system consists of different cell types including red blood cells that function as oxygen carriers, white blood cells that have a role in fighting infections, and platelets that help prevent bleeding (Rörby, 2014). While haematopoiesis refers to the production of all the blood cells, erythropoiesis is the process of proliferation and differentiation of haematopoietic stem cells (HSCs) to mature RBCs. Erythrocytes have an average life-span of 120 days and they represent the most common cell type in adult blood. Human blood contains approximately 5 x 10^6 erythrocytes per microlitre (normal range 4.7 x 10^6 to 6.1 x 10^6 erythrocytes per microlitre for males and 4.2 x 10^6 to 5.4 x 10^6 erythrocytes per microlitre for females) (Dzierzak and Philipsen, 2013). About 2 x 10^{11} new erythrocytes are produced daily in the bone marrow (Nieldez-Nguyen *et al.*, 2002). This process involves a set of complex interactions and it occurs mainly in the bone marrow where the cell surface glycoprotein CD34 is expressed on the immature compartment of early progenitor cells (Tirelli *et al.,* 2011). CD34 is an adhesion molecule which is expressed on haematopoietic progenitor cells that functions in early haematopoiesis by mediating the attachment of stem cells to the bone marrow extracellular matrix or directly to stromal cells (NCBI, 2016a).

The production of erythroid cells begins from the multipotent HSCs, which are primitive stem cells that can differentiate into all cells of the haematopoietic system (Rörby, 2014). These cells undergo long-term self-renewal cell divisions which lead to the production of more HSCs, preventing the depletion of the HSC pool (Rörby, 2014; Koury, 2011). HSCs also differentiate into other cells and form all lineages of the lympho-haematopoietic cells, terminating in the production of mature cells of the blood and lymphoid tissues (Weissman and Shizuru, 2008)

(Figure 1.2). The haematopoietic system is divided into two lineages: the HSC differentiates into distinct progenitor populations, either common myeloid progenitor (CMP) or common lymphoid progenitor (CLP), giving rise to myeloid or lymphoid restricted lineages respectively (Rörby, 2014). This process is driven by various transcription factors, growth factors and expression of specific receptors, which ultimately regulate the cells' commitment into a specific lineage.

The differentiation into the erythroid lineage starts with the HSCs (which also have self-renewal capacity) which differentiate into CMPs and then into megakaryocytic-erythroid progenitors (MEP) (Koury, 2011). As soon as the MEPs differentiate into burst-forming unit-erythroid (BFU-E) cells, the sequence becomes restricted to the erythroid lineage for the eventual production of reticulocytes (Figure 1.3). The BFU-E cells proliferate slowly and differentiate into rapidly dividing colony-forming unit-erythroid (CFU-E) cells. These cells divide three to five times over two to three days as they differentiate into pro-erythroblasts and erythroblasts where they undergo many substantial changes including a decrease in cell size, chromatin condensation and haemoglobinisation. Eventually, this leads up to their enucleation and expulsion of other organelles to form reticulocytes (Hattangadi *et al.,* 2011) (Figure 1.3).

In humans, blood formation begins in the yolk sac and then progresses to the liver, thymus and spleen before finally establishing in the bone marrow, which in adults is the permanent site for haematopoiesis (Ho *et al.,* 2015; Rörby, 2014). The first stages of erythrocyte differentiation take place in these sites, but eventually the reticulocyte is released into the bloodstream where it matures into an erythrocyte after one to two days.

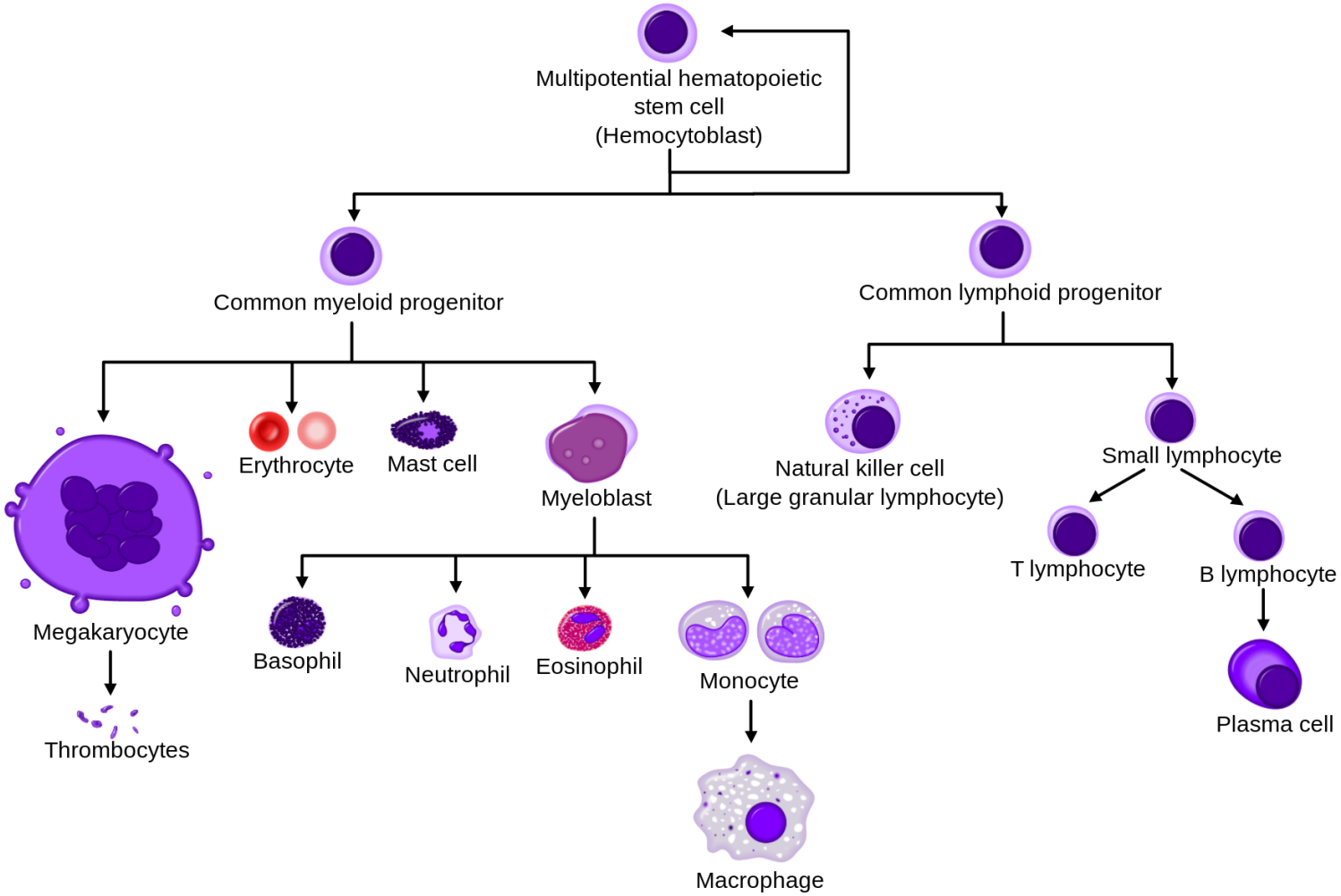


Figure 1.2: The development of different blood cells from haematopoietic stem cell to mature cells. (Häggström, 2009). (Permission granted by public domain).

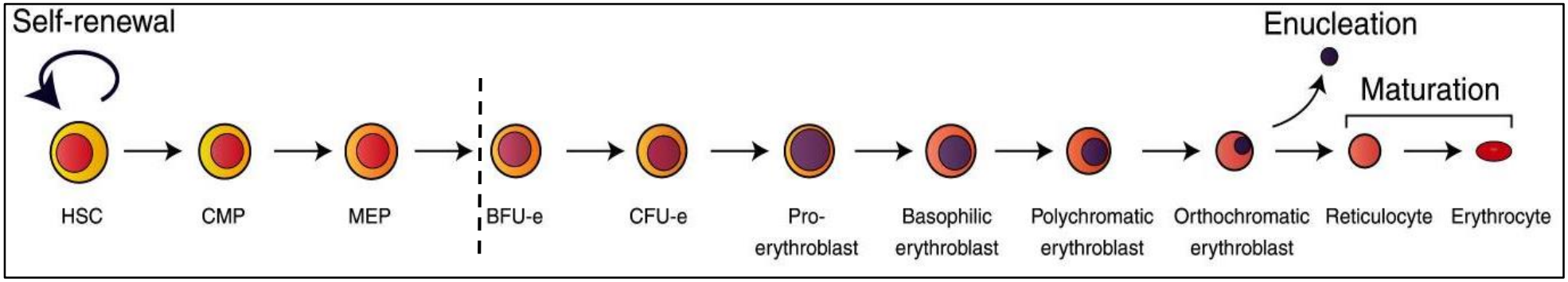


Figure 1.3: Differentiation of erythroid cells. Stages after the dashed line are committed to erythroid differentiation, while stages before the dashed line have the potential to develop into non-erythroid lineages. [HSC: haematopoietic stem cell; CMP: common myeloid progenitor; MEP: megakaryocytic-erythroid progenitor; BFU-E: burst-forming unit-erythroid; CFU-E: colony-forming unit-erythroid]. (Adapted from Dzierzak and Philipsen, 2013). (Used with permission from Cold Spring Harbor Laboratory Press).

A progenitor cell in a specific stage has a lower proliferative potential than the cell preceding it and it was believed that, apart from the HSC, none of the subsequent progenitor cells have the potential for long-term self-renewal. However, studies have established that the pro-erythroblast also has a capacity for self-renewal *in vitro,* which suggests another possible source for culturing clinical-grade RBCs (Leberbauer *et al.*, 2005; von Lindern *et al.*, 1999; Wessely *et al.*, 1999). The human erythroid progenitor cells (HEPs) as well as HSCs can be obtained from bone marrow, cord blood or from peripheral blood.

Most of the mechanisms underlying erythroid differentiation and maturation, both at cellular and molecular levels, still remain unclear. In particular, enucleation, where the nucleus is extruded from the cells to produce reticulocytes, is still poorly understood (Xi *et al.,* 2013). Hence, the culturing of erythroid cells *in vitro* is also invaluable for investigating RBC development and diseases of the erythroid lineage including testing the effects of various pharmacological drugs, especially where only small amounts of blood may be available for study.

1.3 Culturing of red blood cells *in vitro*

Several studies have shown that human haematopoietic stem cells and progenitor cells depend on various factors to be able to proliferate and differentiate (Leberbauer *et al.*, 2005). These include lineage-nonspecific early-acting and lineage-specific late-acting cytokines, e.g. stem cell factor (SCF) belongs to the former (Wessely *et al.*, 1999) and erythropoietin (Epo) belongs to the latter group (Carotta *et al.*, 2004; Sato *et al.*, 2000).

RBC production requires a balance between the opposing effects of proliferation and differentiation. While proliferation-promoting factors maintain the renewal capacity of erythroid progenitor cells, differentiation-inducing factors are required for the successful terminal maturation and production of RBCs (Leberbauer *et al.*, 2005). The two most important cytokines are Epo and SCF. *In vivo,* when there is a decrease in oxygen in the circulation, the kidneys secrete the hormone Epo, which stimulates the proliferation and differentiation of HEPs into RBCs (Sherwood, Klandorf and Yancey, 2005). Epo activates anti-apoptotic proteins to protect HEPs from apoptosis and it also stimulates haemoglobin synthesis and terminal differentiation into RBCs (Dolznig *et al.,* 2002). SCF, which is constantly produced by endothelial cells and by fibroblasts, interacts with other cytokines to preserve the viability of HSCs and progenitor cells by inducing survival signals. SCF acts on HSCs and stimulates them to enter the cell cycle thereby promoting proliferation and differentiation (Rörby, 2014; Yoshihara *et al.,* 2007; Muta *et al.,* 1994). Inflammatory stimuli such as interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNFα) enhance the production of SCF as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte

colony-stimulating factor (G-CSF) leading to the production of white blood cells and granulocytes respectively (Broudy, 1997). Studies have showed that SCF in combination with interleukin-3 (IL-3) and other cytokines, enhances the production of BFU-E and colony-forming unit granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM) substantially (Broudy, 1997). A variety of other factors, such as insulin-like growth factor I (IGF-I), insulin, and glucocorticoids, also have a supportive effect on the development of RBCs.

Erythroid expansion requires the cooperation of Epo and SCF (von Lindern, Schmidt and Beug, 2004; Wessely *et al.*, 1999). Through binding and activating their respective receptors, EpoR and c-kit, an intracellular cascade of biochemical events is triggered, which, in addition to permitting proliferation and differentiation, results in the transduction of a survival signal to developing erythroid progenitors (von Lindern, Schmidt and Beug, 2004; Somervaille, Linch and Khwaja, 2001). The main signal transduced by the erythropoietin receptor is the JAK/STAT pathway, while c-kit signals activate the PI3K/PKB/mTOR pathway (Figure 1.4). Studies by Grech *et al.* (2008), James *et al.* (2005) and Levine *et al.* (2005) have shown that these pathways have a major role in promoting erythroid progenitor expansion.

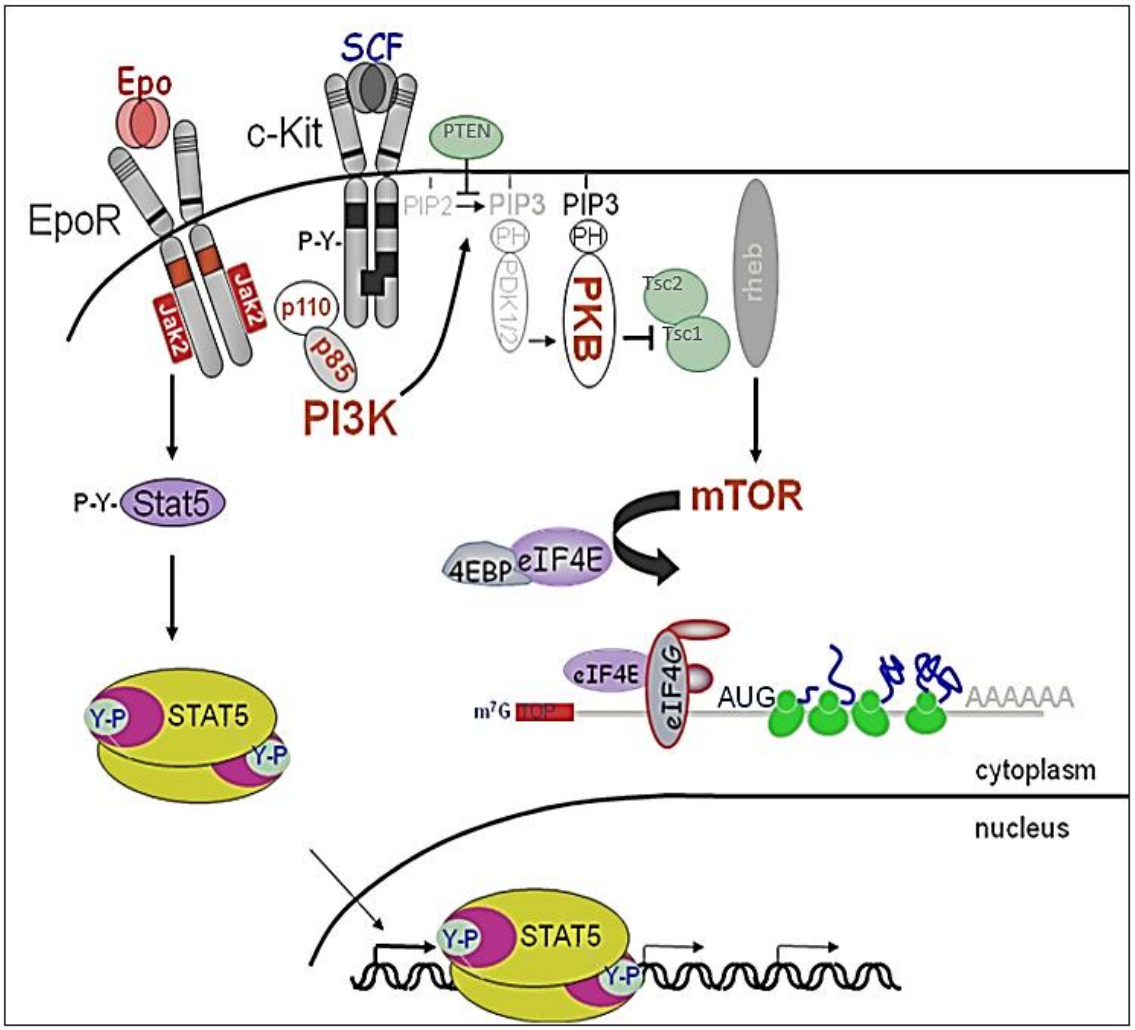


Figure 1.4: The JAK/STAT and PI3K/PKB/mTOR pathways. Epo binds to EpoR to activate the JAK/STAT pathway and SCF binds to c-kit activating the PI3K/PKB/mTOR pathway, resulting in the transduction of survival signals including initiation of translation through the release of eukaryotic initiation factor 4E (eIF4E). [Epo: erythropoietin; EpoR: erythropoietin receptor; Jak2: Janus kinase 2; STAT: signal transducer and activator of transcription; SCF: stem cell factor; PI3K: phosphotidylinositol-3 kinase; PTEN: phosphatase and tensin homologue; PIP2/3: phosphatidylinositol (3,4,5-triphosphate); PDK1/2: phosphoinositide-dependent kinase 1 and 2; PKB: protein kinase B; mTOR: mammalian target of rapamycin; Tsc1/Tsc2: tuberous sclerosis protein; Rheb: RAS-homologue enriched in brain; 4EBP: 4E-binding protein; eIF4E/eIF4G: eukaryotic initiation factor 4 E/G; m7G: methyl-7-guanidine]. (Adapted from Grech *et al.,* 2008). (Used with permission from the author Grech).

Epo is a lineage-specific haematopoietic growth factor required for survival, proliferation and differentiation of committed erythroid progenitor cells (Goodnough, Skikne and Brugnara, 2000). EpoR is composed of two identical subunits and when Epo binds to it, a conformational change occurs. The two subunits dimerise and Janus kinase 2 (Jak2) is recruited to the receptor and binds to the cytoplasmic domain of EpoR catalysing phosphorylation of tyrosine. As a result, signal transducer and activator of transcription (STAT) molecules are recruited and they also become phosphorylated. Eventually these molecules homodimerise and translocate to the nucleus where they act as transcription factors (McLornan, Percy and McMullin, 2006). STAT5 factors (STAT5A and STAT5B) are the most prominent STAT proteins activated by EpoR (Richmond, Chohan and Barber, 2005) (Figure 1.4).

When SCF binds to c-kit, phosphatidylinositol-3 kinase (PI3K) is recruited to the receptor where it binds through its regulatory subunit p85. The inhibitory interaction with p110 subunit is relieved resulting in PI3K activation. PI3K phosphorylates membrane lipids PIP2 to generate PIP3. These form an anchor for the PH-domain containing kinases PDK1 (phosphoinositide-dependent kinase 1) and PKB (protein kinase B). PIP3 is dephosphorylated by the tumour suppressor phosphatase and tensin homologue (PTEN), which silences the PI3K-pathway. At the membrane PDK1 phosphorylates PKB, which in turn phosphorylates the tuberous sclerosis tumour suppressor genes Tsc1 and Tsc2. Upon phosphorylation these genes release the GTPase Rheb (RAS-homologue enriched in brain) to activate mTOR (mammalian target of rapamycin). Activation of mTOR results in phosphorylation of p70S6 kinase (p70S6K) and eIF4E-binding protein (4E-BP). Upon phosphorylation, 4EBP releases the mRNA cap-binding

translation initiation factor 4E (eIF4E) that binds to eIF4G and forms eIF4F complex. eIF4F unwinds the 5' proximal region of mRNA and mediates the attachment of 43S complex to it (Grech *et al.,* 2008) (Figure 1.4).

eIF2 binds GTP and tRNA to form a complex and associates with 40S ribosomal unit to form the 43S preinitiation complex (PIC). This PIC then binds mRNA that has previously been unwound by the eIF4F complex. The 43S PIC and the eIF4F proteins form a new 48S complex on the mRNA which starts searching along the mRNA for an AUG start codon. Then eIF2 hydrolysis GTP to GDP and dissociates from the mRNA, permitting the binding of the 60S ribosomal unit and elongation of the polypeptide chain (Poulin and Sonenberg, 2013). Jak2 also interacts with the PI3K/PKB/mTOR pathway by activating PI3K and PKB after it becomes phosphorylated (McLornan, Percy and McMullin, 2006).

Studies have established that by adding the synthetic glucocorticoid hormone dexamethasone (Dex) to the culture medium containing Epo and SCF, HEPs proliferate greatly *in vitro* (Leberbauer *et al.*, 2005; von Lindern *et al.*, 1999; Panzenböck *et al.*, 1998), which permits the production of a very large number of progenitor cells. When the glucocorticoid receptor (GR) is activated by Dex, there is a cooperation with activated EpoR and c-kit to retain the erythroid cells in an immature state, thus inducing long-term proliferation (Nakamura, 2008; von Lindern *et al.*, 1999). Hence, glucocorticoids promote proliferation and inhibit differentiation of erythroid progenitor cells (Miharada *et al.,* 2006; Kolbus *et al.,* 2003). Current studies are investigating the mechanism by which Dex cooperates with Epo and SCF to maintain long-term proliferation of erythroid progenitor cells.

While both Epo and SCF are needed to promote survival and proliferation in erythroid progenitors, Epo alone leads to terminal differentiation into erythrocytes (Carotta *et al.*, 2004; Panzenböck *et al.*, 1998). Epo also regulates the anti-apoptotic protein Bcl-XL, which in turn regulates red cell numbers by inhibiting apoptosis (Dolznig *et al.*, 2002). In a series of experiments Dolznig *et al.* (2002) showed that transfection of Bcl-XL did not alter the proliferative capacity of erythroid progenitor cells cultured using Epo, SCF and Dex. Transfection is the process by which nucleic acids are introduced into mammalian cells. In their experiments, Dolznig *et al.* (2002) infected their erythroblasts with a retroviral vector (Murine Stem Cell Virus (MSCV)) expressing human Bcl-XL, linked to a green fluorescent protein (GFP) gene via an internal ribosome entry site (IRES) (MSCV-GFP-IRES-Bcl-XL). In this experiment the cells underwent terminal differentiation in the complete absence of Epo. This is due to the exogenous Bcl-XL that protects the erythroid progenitors from apoptosis, allowing the terminal differentiation of the committed erythroblasts.

The above knowledge is important to define the signals required for proliferation and survival of erythroid progenitors. These signals include the JAK/STAT and the PI3K/PKB/mTOR pathways and protection of apoptosis via the anti-apoptotic effector, Bcl-XL. Understanding the regulation of erythroid progenitor expansion provides the knowhow to define cultural requirements to expand such progenitors *in vitro* for potential transfusion utilisation.

1.3.1 Development of culture methods

A well characterised model exists which can efficiently mass culture pure, immature erythroid progenitors from mouse embryonic stem cells (Carotta *et al.*,

2004). While culturing of erythroblasts has already been achieved *in vitro* using a murine model (Hiroyama *et al.*, 2008; Chen, Lewis and Kaufman, 2003; Orkin, 1998; von Lindern *et al.*, 1999) production of erythrocytes from human blood is currently still under research and has not yet proven practical for routine therapeutic applications.

Several techniques have been described for the production of erythrocytes in liquid cultures (Heideveld *et al.,* 2015; Migliaccio *et al.,* 2010; van den Akker *et al.,* 2010; Fujimi *et al.,* 2008; Miharada *et al.,* 2006; Giarratana *et al.,* 2005; Leberbauer *et al.,* 2005; Migliaccio *et al.,* 2002; Neildez-Nguyen *et al.,* 2002; Panzenböck *et al.,* 1998; Fibach *et al.*, 1989). Although culture conditions differ in each protocol, SCF, Epo, Dex, and transferrin are commonly present, usually supplemented by insulin or Insulin-like Growth Factor-I (IGF-I) or a lipid-mixture (Dzierzak and Philipsen, 2013). IGF-I is 50 to 70% identical to insulin and both assist Epo in the maturation of the erythroid cells (Muta *et al.,* 1994). Through various studies, it was established that the optimum settings for expansion of erythroid cells *in vitro* is obtained when cells are first cultured in proliferation medium and then transferred to another medium that promotes erythroid maturation.

Studies to expand erythroid cells *in vitro* commenced in 1989 when Fibach and colleagues developed a two-step liquid culture. During the first seven days mononuclear cells from adult blood were cultured in α minimal essential medium (αMEM) supplemented with 10% 5637-Conditioned Medium (5637-CM) and Foetal Bovine Serum (FBS) and then transferred to αMEM supplemented with FBS, Bovine Serum Albumin (BSA), β-mercaptoethanol, L-glutamine, Dex and

Epo until day 18. This culture yielded a 7.5 fold increase (FI) of cells. The FI is used for normalisation purposes and it is the ratio between the number of cells observed on a specific day, usually between day 10 and 13, and the number of cells at the start of the culture on day 0 (Migliaccio *et al.,* 2011). Subsequent studies focused on producing more efficient culture methods, and the conditioned medium was replaced with human SCF and IL-3 (Migliaccio *et al.,* 1992). Epo was invariably used in all liquid cultures as it was well known that Epo stimulates proliferation and differentiation of erythroid cells.

Great hopes were raised in 2002 when Neildez-Nguyen and colleagues developed a three-phase serum-free culture method that lead to a FI of 2×10^5. They used Long-term serum-free Culture Medium (LCM) supplemented with BSA, iron-saturated human transferrin (Tf), ferrous sulphate ($FeSO_4$), ferric nitrate ($FeNO_3$), insulin, lipids, and hydrocortisone. In phase 1 from day 0 to day 7, $CD34^{pos}$ cells derived from cord blood were additionally supplemented with Flt-3 Ligand (FLt-3 L), thrombopoietin (Tpo), and SCF in order to promote HSC proliferation. In phase 2 from day 8 to day 14, the enhanced serum-free medium was supplemented with SCF, Epo, and IGF-I, in order to promote proliferation of the erythroid progenitors, and in phase 3 (up to 21 days) LCM was supplemented with Epo and IGF-I to promote terminal erythroid differentiation. This culture method led to a successful expansion of HSCs, but erythroid cells failed to mature fully *in vitro* as enucleation was observed in only 4% of cells. Subsequent research focused on obtaining mature erythrocytes.

Three years later in 2005, Leberbauer *et al.* described a two-phase serum-free culture protocol that led to a 1×10^9-fold expansion for up to 60 days. In phase 1

from day 0 to day 14, MNCs derived from cord blood were cultivated in serum-free medium supplemented with SCF, Epo, Dex, lipids and IGF-1 in order to expand erythroid progenitor cells. In phase 2, terminal differentiation was induced by culturing the erythroblasts in serum-free medium supplemented with Epo, insulin, a Dex antagonist, and iron-saturated human transferrin. This protocol achieved long-term proliferation of erythroid progenitors, however it had poor control of final maturation into RBCs and only limited enucleation was observed.

In the same year, Giarratana and colleagues developed a three-step protocol that lead to a 1.95 x 10^6 FI with 100% terminal differentiation, generating fully mature RBCs possessing all the characteristics of *in vivo* RBCs. Cord blood-derived $CD34^{pos}$ cells were first cultured for eight days in enhanced LCM (similar to that described by Neildez-Nguyen *et al.* (2002)) supplemented with SCF, Epo, and IL-3 to allow them to proliferate and differentiate into erythroid progenitors. For the next three days the cells were co-cultured on a murine stromal cell line (MS5) or on human mesenchymal cells with the addition of Epo. In phase 3 cells were incubated on a simple stroma alone devoid of factors for another ten days. By using the stromal cell lines, a microenvironment is produced that supports the growth and maturation of the $CD34^{pos}$ cells. Bone marrow stromal cells produce specific extracellular matrix proteins and regulatory factors that have major roles in the regulation of haematopoiesis (Verfaillie, 1993). Using the cell line MS5 is a drawback since it is derived from mice, hence human mesenchymal cells are preferred since they are derived from normal adult bone marrow. However, this still poses limitations for the scaling-up of the protocol.

Similarly, in 2006, Miharada *et al.* developed a four-step protocol that lead to a 0.72 x 10^6 FI differentiation, without using stromal cells. However, this method lead to a less efficient enucleation with a rate of 77.5%. Cord blood-derived $CD34^{pos}$ cells were first cultured for six days in erythroid differentiation medium (EDM) supplemented with SCF, Epo, IL-3, vascular endothelial growth factor (VEGF) and IGF-II. In phase 2 the cells were cultured from day 7 to day 10 in EDM supplemented with SCF and Epo only. Then from day 11 to day 16, cells were cultured in the same medium but containing a lower concentration of Epo. In phase 4 the cells were cultured for four days in enucleation medium (Iscove's modified Dulbecco's medium (IMDM) supplemented with Plasmanate®, mannitol, adenine, disodium hydrogenphosphate dodecahydrate, and mifepristone) to promote enucleation of erythroblasts and terminal maturation into erythrocytes.

The highest yield with an efficient enucleation rate was obtained by Fujimi *et al.* (2008) who developed a four-phase culture system that also involved culturing on stromal cells and terminal differentiation in enucleation medium. This protocol produced a 3.63 x 10^6 FI and 99.4% enucleation. Cord blood $CD34^{pos}$ cells were first cultured on human stromal cells in serum-free medium supplemented with SCF, Flt-3/Flk-2 ligand, and Tpo, from day 0 to day 14, in order to expand haematopoietic progenitor stem cells. In phase 2 the cells were cultured in serum-free medium supplemented with BSA, transferrin, SCF, IL-3 and Epo, from day 15 to day 28, in order to promote proliferation of erythroid cells. In the third phase, from day 29 to day 34, erythroblasts were co-cultured with CD34-derived macrophages in the presence of BSA, transferrin and Epo to promote expansion and differentiation of the erythroid cells. Then the cells were detached from the macrophages and cultured for four days in enucleation medium (described

previously by Miharada *et al.,* 2006) to promote enucleation. It has been shown in other experiments that enucleation is induced by the interaction of erythroid precursors with macrophages (Hanspal, Smockova and Uong, 1998; Hanspal and Hanspal, 1994).

These *in vitro* cultures for the production of RBCs present some difficulties including low yield, inefficient enucleation and a lengthy process. They also involve the use of culture components and supplements that can be quite expensive. These are limiting factors in the large-scale *in vitro* production of RBCs. Hence, studies should focus on developing a cost-effective protocol for a culture system that utilises a minimal amount of culture components and supplements but can still produce large numbers of functional RBCs in the shortest time possible.

1.3.2 Source of stem cells

The yield of a culture system also depends on the source of the HSCs for the production process. Erythroid progenitor cells are present in the $CD34^{pos}$ cell fraction of the MNC layer of adult blood and cord blood. MNCs and $CD34^{pos}$ cells derived from adult blood or cord blood were invariably used as a source of erythroid progenitors in various studies. These studies reveal that cord blood-derived $CD34^{pos}$ cells and adult blood-derived $CD34^{pos}$ cells yield more erythroblasts (10^5 – 10^6 FI and 29 – 10^5 FI respectively) than MNCs from cord blood and adult blood (300 – 1000 FI and 2 – 150 FI respectively) (Migliaccio *et al.*, 2011; Giarratana *et al.*, 2005). If it is taken in consideration that $CD34^{pos}$ cells represent a small fraction of MNCs, calculations indicate that MNCs from both cord blood and adult blood produce on average 10 to 100 times more

erythroblasts than the corresponding $CD34^{pos}$ cells (Migliaccio *et al.*, 2011). Interestingly, van den Akker *et al.* (2010) demonstrated that by using total peripheral blood MNCs or $CD34^{neg}$ cells, the yield of erythroblasts was significantly higher than by utilising only the $CD34^{pos}$ cells purified from the same amount of MNCs. They have shown that the $CD34^{neg}$ cells enter a $CD34^{pos}$ phase and then progress through the normal haematopoietic pathway to become reticulocytes. This is supported by Goodell (1999) where it is proposed that the true HSC is $CD34^{neg}$.

Adult human peripheral blood is quite simple to obtain and therefore constitutes an easy source of HSCs. The disadvantage of using stem cells from peripheral blood is that they have limited expansion capacity compared to those from cord blood. However, they are easier to obtain and they display mature forms of haemoglobin, whereas those from cord blood only express foetal haemoglobin (Lu *et al.*, 2009). Nevertheless, studies demonstrated that while erythroblasts generated *in vitro* from cord blood express mainly foetal haemoglobin, when injected *in vivo* using mice these cells generate RBCs that express adult haemoglobin (Fujimi *et al.*, 2008; Neildez-Nguyen *et al.*, 2002).

Other stem cell resources include induced pluripotent stem cells (iPSCs) and human embryonic stem cell (hESC) lines (Migliaccio *et al.*, 2012; Whitsett, Vaglio and Grazzini, 2012). RBCs have been successfully generated *in vitro* from these stem cells whose expansion potential is infinite (Whitsett, Vaglio and Grazzini, 2012). However, limitations to using these stem cells include ethical as well as safety concerns. Since these cells are generated *in vitro*, there may be risks of introducing mutations and hence they can be potentially tumorigenic (Migliaccio

et al., 2012). The use of hESC also poses a moral dilemma because to obtain these stem cells, the early embryo has to be destroyed which means destroying potential human life. Moreover, these culture protocols are too complicated to be used for large-scale production and cell yields are much less than those obtained when using cord blood (Rousseau, Giarratana and Douay, 2014).

1.4 Janus kinase 2

Janus kinase 2 belongs to the Janus kinase family which consists of four intracellular non-receptor tyrosine kinases, namely Janus kinase 1 (Jak1), Janus kinase 2 (Jak2), Janus kinase 3 (Jak3), and tyrosine kinase 2 (Tyk2) (McLornan, Percy and McMullin, 2006). Initially they were named “just another kinase” as they were discovered in 1989 during a large PCR-based screen of kinases (Wilks, 1989). Eventually, they were published as Janus kinases as a reference to the two-faced Roman god, Janus, due to the fact that JAKs possess two similar ‘active’ and ‘inactive’ domains. Kinase activity is exerted by one domain, while the other domain provides a negative regulation on the kinase activity (McLornan, Percy and McMullin, 2006). These kinases are ubiquitously expressed, except Jak3 that is restricted to haematopoietic cells (Zouein, Duhé and Booz, 2011).

The *JAK2* gene is 3399 base pairs (bp) long and is found on the short arm (p) of chromosome 9 at position 24 (9p24) (Figure 1.5). It encodes a protein tyrosine kinase, consisting of 1132 amino acids, that is involved in a specific subset of cytokine receptor signalling pathways resulting in the growth and proliferation of cells. Jak2 is a non-receptor kinase that has a modular structure composed of seven Jak homology (JH) domains (Figure 1.6). JH1 is an active tyrosine kinase domain, JH2 is an inactive pseudokinase domain that negatively regulates the activity of the JH1 kinase domain, the JH3 and JH4 domains have homology with SRC homology 2 domain (SH2), and the amino terminal domains JH4 to JH7, collectively called FERM (4-point-1, Erzin, Radixin, Moesin) are involved in interaction with cytokine receptors (McLornan, Percy and McMullin, 2006) (Figure 1.6).

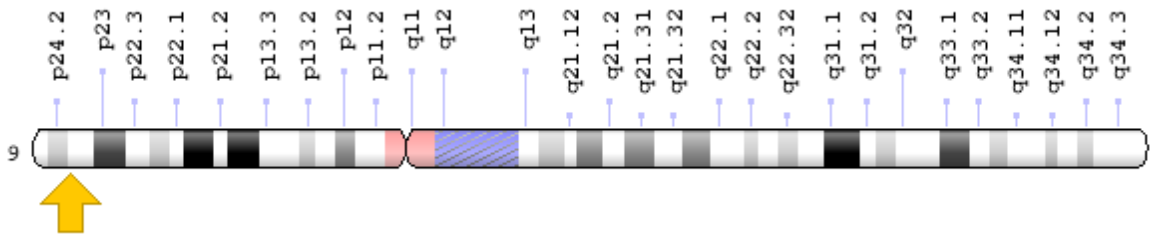


Figure 1.5: Chromosomal location of the *JAK2* gene. The *JAK2* gene is found on the short arm (p) of chromosome 9 at position 24 (9p24). (Image obtained from Genetics Home Reference, 2014). (Permission to use granted by public domain).

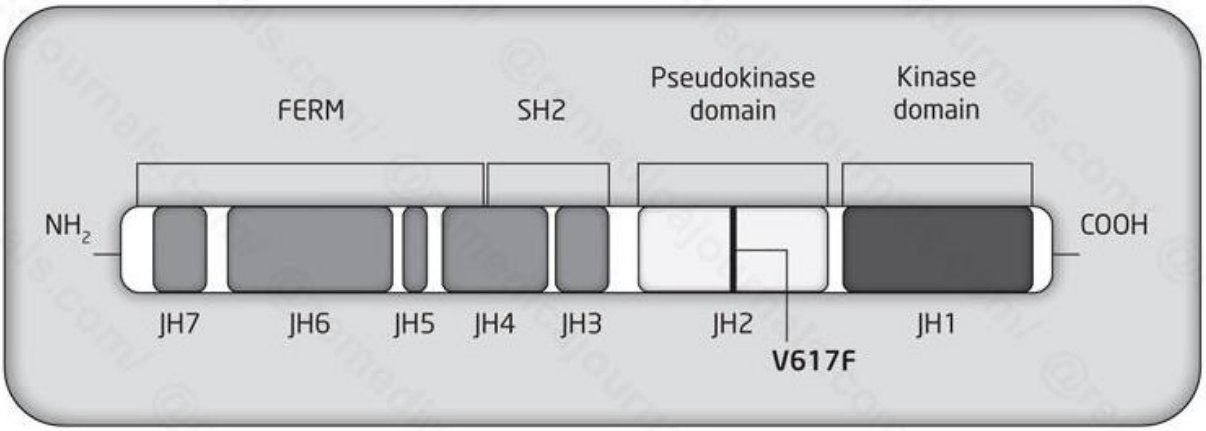


Figure 1.6: Jak2 domains. Diagram of Jak2 domains including the approximate location of the V617F mutation. [FERM: 4-point-1, Erzin, Radixin, Moesin; SH2: SRC homology 2; JH1-7: Jak homology 1-7; NH_2: amino terminal; COOH: carboxyl terminal]. (Vannucchi *et al.,* 2012). (Reproduced with permission from Remedica).

Jak2 that is found in the cytoplasm associates with type I receptors (such as erythropoietin, growth hormone, prolactin, and thrombopoietin) or type II receptors (such as interferon and various interleukins) by binding through the FERM domain. Once a ligand binds to the cell surface receptor, Jak2 is recruited to the cytoplasmic tail of the receptor initiating the JAK/STAT pathway (described above) resulting in the activation of gene transcription (McLornan, Percy and McMullin, 2006). The Jak2 protein is especially important for erythropoiesis as it controls the production of blood cells from HSCs. When Epo binds to EpoR, Jak2 is recruited and the JAK/STAT pathway is initiated, resulting in the transcription of several genes involved in the modulation of erythropoiesis (Huang, Constantinescu and Lodish, 2001). Results show that Jak2-deficient murine embryonic stem cells did not survive due to the absence of erythropoiesis because progenitor cells did not respond to Epo, Tpo and IL-3 (Neubauer *et al.,* 1998; Parganas *et al.,* 1998).

The JH2 domain has several crucial regulatory functions. By interacting with JH1, it auto-inhibits Jak2 function in the absence of ligand-binding (McLornan, Percy and McMullin, 2006). Hence an alteration in this domain would result in dysregulation of this auto-inhibition. Studies have found that one single base guanine to thymine (G → T) substitution in *JAK2* exon 14 results in a valine to phenylalanine substitution at codon 617 (V617F mutation) within the JH2 autoinhibitory domain (Levine *et al.*, 2005). This leads to the production of Jak2 protein that is constantly activated. In the absence of Epo, mutated Jak2 activates spontaneously STAT-mediated transcription leading to an increased production of RBCs.

This constitutively active *JAK2* mutant is the molecular basis of the myeloproliferative disease, polycythaemia vera (PV) (Baxter *et al.*, 2005; James *et al.*, 2005; Levine *et al.*, 2005) although the cause of the mutation in the *JAK2* gene is not known. The *JAK2 V617F* mutation has been detected in at least 90% of patients with PV, as well as in up to 70% of patients with essential thrombocythemia (ET), and up to 50% of patients with chronic idiopathic myelofibrosis (IMF) (Dupont *et al.*, 2007). While several studies suggest that the *JAK2 V617F* mutation directly causes erythrocytosis in patients with PV, its implication in ET and IMF pathogenesis has not been clearly identified yet (Kim *et al.,* 2013).

PV is a non-inherited chronic myeloproliferative disorder that is characterised by an increased production of RBCs as well as granulocytes and platelets (Hricik *et al.,* 2013; Dai, Chung and Krantz, 2005; Green, 1996). These cells accumulate in the bone marrow and in the blood stream, thus increasing the blood volume causing it to become thicker and more viscous than normal. As a result, the chance of blood clot formation is increased, which can block blood flow through arteries and veins, possibly resulting in a heart attack or a stroke. The incidence of PV is estimated to be 2.3 per 100,000 persons per year with a median patient age of 60 years and a slight predominance (1.2:1) in males (Stuart and Viera, 2004). The majority of people with PV are asymptomatic, however symptoms can develop over time and are related to the hyperviscosity and abnormally high number of blood cells. These include headaches, blurred vision, fatigue, weakness, dizziness, itchy skin, and night sweats. Splenomegaly, hypertension and a red complexion with reddening of the palms of the hand and soles of the feet, ear lobes, mucous membranes and the eyes due to the high numbers of

RBCs in the circulation can also be commonly observed. PV can be diagnosed through a full blood count (high red cell count, increase in haemoglobin and haematocrit and a raised white cell count and platelet count), *JAK2* mutation testing (presence of V617F mutation) and bone marrow examination (active with abnormally high number of cells) (NHLBI, 2014).

PV patients also have an increased number of HSCs and exhibit Epo-independent erythroid maturation. This is supported by various studies that show that erythroid progenitor cells isolated from patients with PV proliferate and differentiate in culture in the complete absence of Epo (Dupont *et al.*, 2007; James *et al.,* 2005; Levine *et al.,* 2005; Ugo *et al*., 2004; Correa, Eskinazi and Axelrad, 1994; Fisher *et al*., 1994). However, other studies suggest that some PV patients' erythroid progenitors show a hypersensitive response to Epo, resulting also in an increased number of RBCs (Dai, Chung and Krantz, 2005; Levine *et al.,* 2005; Casedevall *et al.,* 1982); while a study performed by Laubach *et al.* (2009) concluded that erythroblasts derived from PV patients did not survive and proliferate when cultured in the absence of Epo. In reality, the molecular mechanism underlying Epo hypersensitivity and independency is not completely understood (Bogeska and Pahl, 2013; Laubach *et al.,* 2009).

In conclusion, the underlying mutation involved in PV disease leads to the production of Jak2 that is constantly activated resulting in increased numbers of RBCs. This observation lead to the development of this research whereby it was hypothesised that by introducing the V617F mutation in HSCs this would mimic PV disease resulting in overproduction of RBCs.

1.5 Aim and objectives

1.5.1 Hypotheses

There are three main hypotheses for this study:

- Constitutive erythropoietin receptor signalling induced by exogenous *JAK2* expression results in enhanced proliferation resulting in mass-production of erythroid cells.
- Transfection of *JAK2* mutant in $CD34^{pos}$ cells provides a transcriptional program that enhances selection and proliferation of erythroid progenitors resulting in increased numbers of cells.
- Transfection of *JAK*2 mutant results in a differential gene expression when compared to transfected wild-type *JAK2* in $CD34^{pos}$ cells derived from human blood.

1.5.2 Aim

In this project, the aim is to study the effect of exogenous *JAK2* (mutant and wild-type) on cultured haematopoietic cells. The main goal is to understand the expression signatures that are different when comparing $CD34^{pos}$ cells that overexpress wild-type Jak2 and $CD34^{pos}$ cells overexpressing mutant Jak2 which is associated with polycythaemia vera disease.

1.5.3 Objectives

1. Design *JAK2* expression constructs for transfection experiments,
2. Overexpression of *JAK2* (wild-type and mutant) in $CD34^{pos}$ cells,
3. Study differential gene expression following transfection with wild-type *JAK2* or mutant *JAK2* construct.

4. Immunophenotyping to characterise the cells produced by this *in vitro* expansion process.

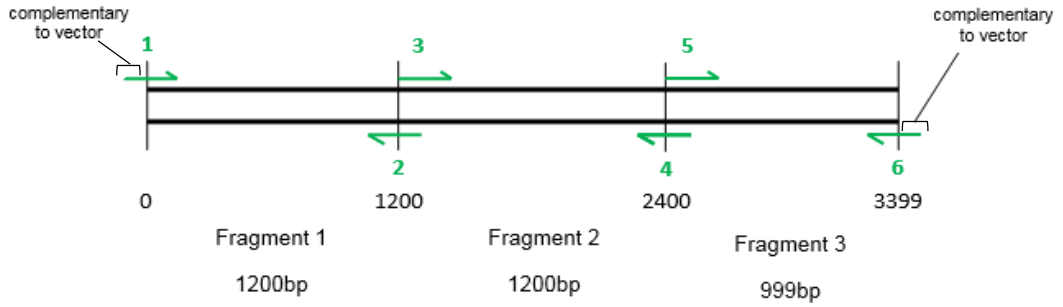
CHAPTER 2

METHODOLOGY

2.1 Primer design for the human *JAK2* gene

The coding sequence (start to stop codon) for the human *JAK2* gene is 3399 base pairs (bp) long. This coding sequence was divided into three fragments and three pairs of primers were designed (a pair for each fragment) using the Geneious software (Figure 2.1). By using the In-Fusion® Cloning technique (Clontech, USA) multiple fragments can be joined together, as long as they share 15 bases of homology at each end. In addition, to ligate the *JAK2* gene fragments with the vector, the primers corresponding to the start and end of the *JAK2* coding sequence have to comprise a 15bp overlap complementary to the vector; the vector used during cloning is pIRES2-AcGFP1 (Clontech, USA) (Appendix A). Hence, restriction enzymes which have unique restriction sites in the multiple cloning site (MCS) of the vector were selected. Two restriction enzymes, *Nhe*I and *Eco*RI, were selected to design the forward primers, while another restriction enzyme, *Sal*I, was selected for the design of the reverse primer (Figure 2.2). The restriction sites of the enzymes were identified (*Nhe*I cuts after 591bp, *Eco*RI cuts after 629bp, and *Sal*I cuts after 639bp) and the primers were designed accordingly. Some of the primers were also tagged with sequences derived from c-*myc*. These sequences encode short peptides, creating an epitope (9E10) allowing recognition of the ectopic expression of Jak2 using an anti-Myc antibody (Santa Cruz Biotechnology, Germany). The sequence of the primers is shown in Table 2.1.

A



B

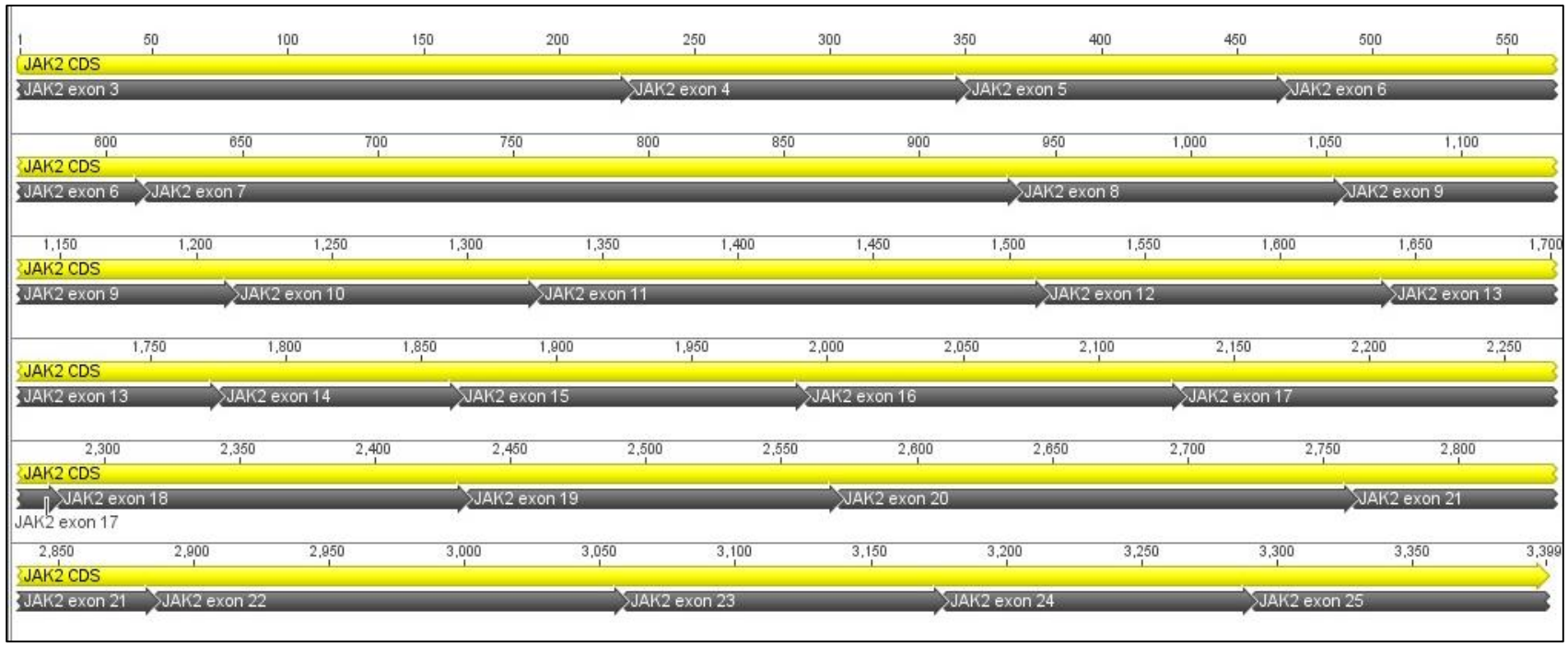


Figure 2.1: Position of primers and the *JAK2* coding sequence. (A) Designed primers are shown in green. Two primers (a forward and a reverse primer) were designed for each fragment. Fragment 1 flanked by primers 1 and 2, fragment 2 flanked by primers 3 and 4, fragment 3 flanked by primers 5 and 6. **(B)** The 3399 bp *JAK2* coding sequence is depicted in yellow, while the exons are depicted in grey [Image obtained using Geneious software].

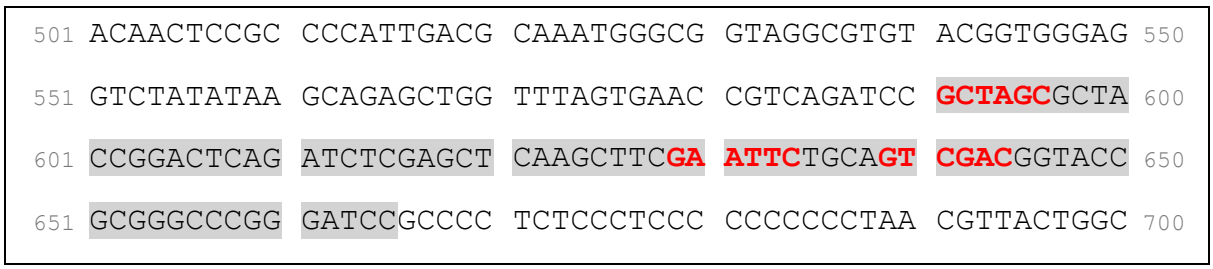


Figure 2.2: Sequence of pIRES2-AcGFP1 vector. The nucleotides show part of the sequence of the vector from 501 to 700 bp. The MCS (591 – 665 bp) is highlighted in grey, while the restriction sites of the enzymes *Nhe*I, *Eco*RI and *Sal*I are shown in red: 591-596: *Nhe*I (G▼CTAGC); 629-634: *Eco*RI (G▼AATTC); 639-644: *Sal*I (G▼TCGAC). The MCS is flanked by cytomegalovirus (CMV) promoter (520 – 589) and internal ribosome entry site (IRES) (666 – 1250). [A: adenine; T: thymine, G: guanine; C: cytosine].

Table 2.1: Designed primers. Primers sequences were designed using the Geneious software. Green nucleotides depict the c-*myc* tag. The start codon **ATG** is highlighted in bold. The position of these primers is denoted in green in Figure 2.1A. [A: adenine; T: thymine, G: guanine; C: cytosine].

No	Primer name	Primer sequence 5'-3'
1a	Jak2 F (*Nhe*I@AUG)	CGTCAGATCCGCTAGC**ATG**GGAATGGCCTGC
1b	Jak2 F (*Eco*RI@AUG)	CTCAAGCTTCGAATTC**ATG**GGAATGGCCTGCCTTACG
2	Jak2 R (Frag 1 1215-1185)	GAAATTGGGCCATGACAGTTGCTTTGTATA
3	Jak2 F (Frag 2 1200-1230)	TCATGGCCCAATTTCGATGGATTTTGCCAT
4	Jak2 R (Frag 2 2415-2385)	AGATCTCGTATGATGGCTCTGAAAGAAGGC
5	Jak2 F (Frag 3 2400-2430)	CATCATACGAGATCTTAACAGTTTGTTTAC
6	Jak2 R (*Sal*I@TCA)	CCGCGGTACCGTCGACTCATCCAGCCATGTTATCCC
1aT	Jak2 F (*Nhe*I@AUG) + tag	CGTCAGATCCGCTAGC**ATG**GAACAAAAACTTATTTCTGAAGAAGATCTGGGAATGGCCTGC
1bT	Jak2 F (*Eco*RI@AUG) + tag	CTCAAGCTTCGAATT**ATG**GAACAAAAACTTATTTCTGAAGAAGATCTGGGAATGGCCTGC
6T	Jak2 R (*Sal*I@TCA) + tag	CCGCGGTACCGTCGATCACAGATCTTCTTCAGAAATAAGTTTTTGTTCTCCAGCCATGTT

The primers were sequence searched using the Basic Local Alignment Search Tool (BLAST) of the Geneious software. This enables to query a sequence database to find entries that are similar to the query sequence. Geneious uses the NCBI BLAST service to perform such searches. The designed primers were ordered through Bioneer (Korea).

2.2 Polymerase chain reaction

2.2.1 Optimisation of standard polymerase chain reaction

A series of experiments were carried out in order to optimise the polymerase chain reaction (PCR) for *JAK2*. A gradient PCR was performed using sequence specific primers (Table 2.1) to establish the optimum annealing temperature of the primers. Each 25µl PCR reaction contained 5µl of 5x Ultra Buffer (which contains 15mM $MgCl_2$, 5mM dNTPs, enhancers and stabilisers), 0.25µl PCRBIO Ultra Polymerase (5 U/µl) (PCR Biosystems, UK), 1µl of 10µM forward primer and 1µl of 10µM reverse primer, 1µl complementary DNA (cDNA) template (<100ng) (such as U937 cDNA or human erythroblast cDNA) and 16.75µl PCR grade distilled water. For the no template control (NTC), cDNA was replaced with distilled water. Cycling was performed using TProfessional Thermocycler (Biometra, Germany) with the conditions as described in Table 2.2.

Table 2.2: Thermal cycles for standard PCR

Cycles	Step	Temperature (ºC)	Time (minutes)
1	Initial Denaturation	95ºC	1:00
35	Denaturation	95ºC	0:15
	Annealing	55 - 65ºC	0:15
	Extension	72ºC	1:30
1	Final Extension	72ºC	10:00
-	Pause	4ºC	--

2.2.2 High-fidelity polymerase chain reaction

Since sequence accuracy is crucial, high-fidelity PCR was performed, where a DNA polymerase with a low error rate was utilised thus resulting in a high degree of accuracy in the replication of the DNA of interest.

Each 25µl PCR reaction contained 5µl of 5x PCRBIO Reaction Buffer (which contains 15mM $MgCl_2$, 5mM dNTPs, enhancers and stabilisers), 0.25µl PCRBIO HiFi Polymerase (2 U/µl) (PCR Biosystems, UK), 1µl of 10µM forward primer and 1µl of 10µM reverse primer, 1µl cDNA template (<100ng) (such as U937 cDNA or human erythroblast cDNA) and 16.75µl PCR grade distilled water. For the NTC, cDNA was replaced with distilled water. Cycling was performed using TProfessional Thermocycler (Biometra, Germany) under the conditions described in Table 2.3.

Table 2.3: Thermal cycles for high-fidelity PCR

Cycles	Step	Temperature (°C)	Time (minutes)
1	Initial Denaturation	95°C	1:00
30	Denaturation	95°C	0:15
	Annealing	55 - 65°C	0:15
	Extension	72°C	1:45
1	Final Extension	72°C	10:00
-	Pause	4°C	--

2.2.3 Polymerase chain reaction using Platinum® Taq DNA Polymerase

Platinum® *Taq* DNA Polymerase is a DNA polymerase containing an antibody that blocks the polymerase's activity at ambient temperatures. Its activity is restored after the denaturation step at 94ºC, thus providing an automatic 'hot start' for the DNA polymerase. This 'hot start' in PCR provides increased sensitivity, specificity and yield (Chou *et al*., 1992). Each 25µl PCR reaction contained 2.5µl of 10x PCR Buffer, 0.5µl of 10mM dNTP mixture, 0.75µl of 50mM $MgCl_2$, 0.1µl Platinum® *Taq* DNA Polymerase (Invitrogen, USA), 0.5µl of 10µM forward primer and 0.5µl of 10µM reverse primer, 1µl template DNA (<500ng) and 19.15µl PCR grade distilled water. For the NTC, DNA was replaced with distilled water. Cycling was performed using TProfessional Thermocycler (Biometra, Germany) under the conditions described in Table 2.4.

Table 2.4: Thermal cycles for Platinum® *Taq* DNA PCR

Cycles	Step	Temperature (ºC)	Time (minutes)
1	Initial Denaturation	94ºC	1:00
30	Denaturation	94ºC	0:30
	Annealing	56.8ºC	0:30
	Extension	72ºC	1:00
1	Final Extension	72ºC	7:00
-	Pause	4ºC	--

2.2.4 Agarose gel electrophoresis

After PCR, 10µl of each amplified product was electrophoresed in 1% weight per volume (w/v) agarose gel with 1µg/ml ethidium bromide (EtBr) (Fisher BioReagents, USA) alongside DNA molecular markers (O'RangeRuler 500bp DNA ladder, Thermo Scientific USA; Solis BioDyne 1kb DNA ladder, Medibena Austria (Appendix B)) and visualised under a transilluminator. Agarose powder (Sigma Aldrich, USA) was added to 1x Tris-acetate-EDTA (TAE) buffer (composed of 40mM Tris (pH 7.6), 20mM acetic acid and 1mM EDTA) and melted in a microwave oven. EtBr was added (1µl for every 10ml of gel) and mixed gently to avoid formation of air bubbles. Then the gel was poured into the gel mould containing combs and allowed to solidify. TAE buffer (1x) was also poured into the electrophoresis chamber to about 2mm above the gel surface. Since the PCR products were colourless, 10µl were mixed with 2µl 6x Orange DNA loading dye (Thermo Scientific, USA) and loaded in the gel slots. Electrophoresis was carried out at 80 Volts (V) for 40 minutes. After the run, the PCR products were visualised under the UV transilluminator (UVP, USA).

2.2.5 Gel extraction and DNA purification

In order to use the DNA for cloning experiments, PCR products were purified to remove excess nucleotides and primers using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). After electrophoresis, the DNA was visualised under the UV transilluminator and the band of interest was excised, transferred to a microcentrifuge tube and weighed. The band was dissolved in a solution containing 4.5M guanidine isothiocyanate and 0.5M potassium acetate (pH 5.0) (membrane binding solution) by adding it at a ratio of 10µl of solution per

10mg of agarose gel slice. The mixture was vortexed and incubated at 60^{o}C for 10 minutes with intermittent vortexing until the gel slice was completely dissolved.

An SV minicolumn was placed in a collection tube and the dissolved mixture was transferred to the SV minicolumn assembly and incubated for 1 minute at room temperature. Then the SV minicolumn assembly was centrifuged in a microcentrifuge at 16000 x g for 1 minute. The flow-through was discarded and the minicolumn was reinserted into the collection tube.

The column was washed by adding 700µl of membrane wash solution, previously diluted with 95% ethanol, to the SV minicolumn. The assembly was centrifuged for 1 minute at 16000 x g and then the flow-through was discarded and the minicolumn was reinserted into the collection tube. The wash was repeated with 500µl of membrane wash solution and centrifuged for 5 minutes at 16000 x g. The collection tube was emptied and the column assembly was recentrifuged for 1 minute with the microcentrifuge lid open to allow evaporation of any residual ethanol.

The SV minicolumn was carefully transferred to a clean 1.5ml microcentrifuge tube. To elute DNA 30µl of nuclease-free water was added directly to the centre of the column. After incubation at room temperature for 1 minute, the column was centrifuged for 1 minute at 16000 x g. Finally, the minicolumn was discarded and the microcentrifuge tube containing the eluted DNA was stored at 4^{o}C or -20^{o}C until further use.

2.2.6 Assessment of DNA quantity and purity

DNA concentration was measured using NanoDrop™ 2000 spectrophotometer (Thermo Scientific, USA). This spectrophotometer measures 0.5 to 2 µl samples with high accuracy and reproducibility. The NanoDrop™ technology is an innovative sample retention system that uses the surface tension to hold and measure micro volume samples.

The samples are measured between two optical pedestals. An appropriate volume of sample is pipetted directly onto the lower measurement pedestal that has a fibre optic cable (the receiving fibre) embedded. When the sampling arm is lowered and a spectral measurement is initiated using the software on the PC, a second fibre optic (the source fibre) is brought into contact with the liquid sample causing the liquid to bridge the gap between the ends of the two fibres due to surface tension, creating a liquid column which forms a vertical optical path. The light source is provided by a xenon flash lamp and the spectrometer utilises a linear charged couple detector array to analyse the light passing through the sample (Thermo Scientific, 2009). For nucleic acid quantification, the NanoDrop uses a modification of the Beer-Lambert equation:

$$c = (A * \varepsilon) / b$$

c = nucleic acid concentration in ng/µl

A = absorbance in AU

ε = wavelength-dependent extinction coefficient in ng-cm/µl

b = path length in cm

The ratio of absorbance at 260nm and 280nm ($A_{260/280}$) was used to assess the purity of DNA. A ratio of around 1.8 is generally accepted as “pure” for DNA and indicates the absence of contamination. If the ratio is lower, it indicates the presence of contaminants that absorb strongly at or near 280nm, such as protein, and phenol. The ratio of absorbance at 260nm and 230nm ($A_{260/230}$) provided a secondary measure of nucleic acid purity. The 260/230 values for a “pure” nucleic acid are often higher than the respective 260/280 values and are commonly in the range of 1.8 to 2.2. If the ratio is lower, this indicates the presence of co-purified contaminants.

2.3 Cloning of the *JAK2* sequence

2.3.1 Double digest with EcoRI and SalI enzymes

To clone the *JAK2* open reading frame (ORF), the vector was linearised using restriction enzymes and the insert ligated in the correct orientation. The vector used was pIRES2 (Appendix A) and another vector pDBLeu was used as a control to assess the digest reaction. The linearised vector was generated using restriction enzymes *Eco*RI and *Sal*I (double digest) (Promega, USA).

Each 20µl reaction contained 2µl restriction enzyme 10x buffer D, 0.2µl acetylated BSA (10µg/µl), 0.5µl restriction enzyme *Eco*RI (10 U/µl) and 0.5µl restriction enzyme *Sal*I (10 U/µl) (Promega, USA), 1µg/µl of vector (volume depends on the concentration of the vector), topped up to 20µl with sterile deionised water. The reactions were then incubated at 37^{o}C for 3 hours.

To check if the digest was successful, the mixtures were loaded in 1% (w/v) agarose gel and electrophoresed for 40 minutes at 80V (as described in *2.2.4*). Then the gel was visualised under the transilluminator and the band corresponding to the linearised vector was excised from the gel and purified (as described in *2.2.5*). The concentration of vector DNA was quantified using NanoDrop 2000 spectrophotometer (as described in *2.2.6*).

2.3.2 Ligation of the JAK2 open reading frame into pIRES2 vector

To ligate the *JAK2* ORF into the linearised pIRES2 vector, the In-Fusion® ligation reaction was set up (Clontech, USA) by adding 50 to 100 ng of purified PCR fragment and 50 to 100 ng of purified linearised vector to 2µl of 5x In-Fusion HD

enzyme premix and topping up the reaction to 10µl with deionised water. A negative and a positive control reaction were also set up. The negative control reaction omitted the addition of the PCR fragment, while for the positive reaction a 2kb control insert and pUC19 control vector provided with the kit were used. The reactions were incubated for 15 minutes at 50^{o}C and then stored at -20^{o}C until the transformation procedure (as described in *2.3.3*).

2.3.3 Transformation using Stellar^{TM} competent cells

Once the *JAK2* ORF was ligated with pIRES2 vector, this construct was transformed into Stellar^{TM} competent cells (Clontech, USA). These cells consist of an *Escherichia coli* (*E. coli)* HST08 strain that provides high transformation efficiency. The cells were thawed in an ice bath and then mixed gently to ensure even distribution. The ligated mixture (1µl) was added to 50µl of competent cells and placed on ice for 30 minutes. The cells were heat shocked for exactly 60 seconds at 42^{o}C and then placed on ice for one to two minutes. SOC medium (Super Optimal broth with Catabolite repression) warmed to 37^{o}C was added to bring the final volume to 500µl and then the tubes were incubated by shaking for one hour at 37^{o}C.

After incubation, 150µl of each culture was plated on selective medium. Since pIRES2 vector is resistant to kanamycin, Luria-Bertani (LB) agar containing 30µg/ml kanamycin was used (Preparation of LB agar: Appendix C). Finally the plates were incubated overnight at 37^{o}C.

2.3.4 Purification of plasmid DNA

The day after transforming the cells, a number of individual isolated colonies were picked from each experimental plate and grown in LB broth containing kanamycin (50µg/ml) (Preparation of LB broth: Appendix C) overnight at 37^{o}C in a shaking incubator.

After growing the colonies in LB broth, the plasmid DNA was purified using QIAprep® Miniprep kit (QIAGEN, Germany). A volume of 1ml from each culture was pipetted in a microcentrifuge tube and centrifuged at 6800 x g for 3 minutes. The pelleted bacterial cells were first lysed by resuspending them in 250µl Buffer P1 and vortexing until no cell clumps remain. Then 250µl Buffer P2 was added and mixed thoroughly by inverting the tubes 4 to 6 times. After 350µl Buffer N3 was added, the tubes were mixed immediately and thoroughly by inverting 4 to 6 times to avoid localised precipitation. Then the tubes were centrifuged for 10 minutes at 17900 x g.

The supernatants were applied to the QIAprep spin columns by decanting or pipetting and centrifuged for 60 seconds at 17900 x g. The flow-through was discarded and the columns were washed by adding 0.5ml Buffer PB. After another centrifugation for 60 seconds at 17900 x g, the flow-through was discarded and the column was washed by adding 0.75ml Buffer PE. After another centrifugation for 60 seconds at 17900 x g, the flow-through was discarded and the columns were centrifuged at 17900 x g for an additional one minute to remove residual wash buffer.

The QIAprep column was placed in a clean microcentrifuge tube. To elute DNA, 50µl Buffer EB (10mM Tris-Cl, pH 8.5) or 50µl of distilled water was added to the centre of each QIAprep spin column, left to stand for one minute and then centrifuged at 17900 x g for one minute. The concentration of eluted DNA was assessed using NanoDrop 2000 spectrophotometer.

2.3.5 DNA sequencing

To establish which minipreps contained the plasmids with *JAK2*, a PCR using primers Jak2 F (Frag 2 1200-1230) and Jak2 R (Frag 2 2415-2385) and 1:100 dilutions of the minipreps as templates was performed (as described in *2.2.3*). The PCR products were loaded in 1% (w/v) agarose gel and electrophoresed at 80V for 40 minutes. The gel was visualised under the transilluminator and a band at 1200bp corresponding to fragment 2 of the *JAK2* gene confirmed the presence of a plasmid with *JAK2*. These purified plasmid DNA were prepared for sequencing. For every reaction, 9µl of the plasmids having concentrations between 100 and 500 ng/µl and 12.5µl of 5µM primer was required. The primers used for sequencing are shown in Table 2.5.

Table 2.5: Primers used to sequence *JAK2* constructs.

No	Primer name	Primer sequence 5' – 3'
1	pIRES2-5-MCS	CGTGTACGGTGGGAGGTC
2	Jak2 R (Frag 1 1215-1185)	GAAATTGGGCCATGACAGTTGCTTTGTATA
3	Jak2 F (Frag 2 1200-1230)	TCATGGCCCAATTTCGATGGATTTTGCCAT
4	Jak2 R (Frag 2 2415-2385)	AGATCTCGTATGATGGCTCTGAAAGAAGGC
5	Jak2 F (Frag 3 2400-2430)	CATCATACGAGATCTTAACAGTTTGTTTAC
6	Jak2 R (*Sal*I@TCA)	CCGCGGTACCGTCGACTCATCCAGCCATG TTATCCC
7	Jak2 seq F1 (918-937)	TGAGACACTGACAGAACAGG
8	Jak2 seq R2 (2764-2746)	GTGTGCTACAGTGCTGGTC
9	Jak2 seq F3 (3073-3090)	TCACTGACAGAGAGCAAG

The DNA and primers were pipetted in a 96-well PCR microplate and sent to McGill University and Genome Quebec Innovation Centre, Canada to be sequenced. DNA sequencing was performed by the Sanger Sequencing (also called dideoxy sequencing) method using the Applied Biosystems 3730xl DNA analyser technology. During this fully automated process, the DNA is denatured and primers anneal at one end of the sequence on the template strand. The DNA polymerase extends the primer, using the template strand to guide incorporation of nucleotides. Periodically, a dideoxynucleotide is incorporated into the growing DNA strand. Because it is missing the 3' hydroxyl group, the dideoxynucleotide prevents the DNA chain from being extended further. In addition, each dideoxynucleotide has a different colour label and, consequently, each terminated DNA chain is coloured according to the nucleotide at its end. Then the chains are separated by length by capillary electrophoresis so that individual chains of increasing length can be identified by their colour. A laser at the bottom of the capillary excites the fluorescent labels as they come out of the capillary. The fluorescent colour then tells the computer which base is represented and the computer records each base, one by one, on a graph called an electropherogram (McGill University, 2015). The sequence of bases obtained from the various primers were then aligned together and compared to the *JAK2* coding sequence to check for any mutations in the original DNA.

2.3.6 JAK2 constructs

The *JAK2*-pIRES2 construct, was used in various transfection experiments (described below). The vector pIRES2 has an AcGFP1 coding region which produces a green fluorescent protein (GFP) and hence cells transfected with a plasmid containing pIRES2 exhibit fluorescence. Although the transcript containing the *JAK2*-pIRES2-AcGFP1 was expressed as indicated by the

successful fluorescence in the transfected cells, sequencing showed the presence of non-synonymous variants that were reconfirmed by sequencing using different primers.

Due to these mutations, published human *JAK2* constructs were kindly provided by Dr. P.P. Sayeski from the University of Florida, College of Medicine and used in further experiments. The wild-type plasmid consists of pcDNA3 vector (Appendix A) encoding the full-length human Jak2 cDNA, while the mutant construct retains the V617F mutation in the Jak2 cDNA.

Amplification of the Jak2 cDNA was performed by Zhao *et al.* (2005) and the primers used were 5′-CCTCCCGCGACGGCAAATGTTCT-3′ and 5′-CTTTGT TCTGTAAATCTACTTTGGTCTCAG-3′ for initial PCR and 5′-TGCATGGGAATG GCCTGCCTTAC-3′ and 5′-CTTTCATCCAGCCATGTTATCCCTTA-3′ for nested PCR. PCR was performed with high fidelity DNA polymerase *Pfu*-ultra (Agilent Technologies, USA). The PCR products were purified from agarose gels and sequenced directly using the ABI 3730xl DNA analyser (Applied Biosystems, USA) at the Vanderbilt-Ingram Cancer Center, USA (Zhao *et al., 2005)*.

The V617F mutation was created by Zhao *et al.* (2005) using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, USA). Wild-type *JAK2* and the *JAK2*-V617F mutant were cloned into the pcDNA3 vector (Invitrogen, USA) by Zhao *et al.* (2005).

For this research, the wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3 constructs were transformed into Stellar^{TM} competent cells as described in section *2.3.3* with the

difference of using LB agar containing 50µg/ml ampicillin (Appendix C), since pcDNA3 vector contains the ampicillin resistance gene. Plasmid DNA was also purified as described in section *2.3.4*, but using LB broth with 100µg/ml ampicillin (Appendix C), and checked by performing a PCR and running a gel electrophoresis.

2.4 Blood samples collection

For this study, the blood of incomplete donations from the National Blood Transfusion Service (Malta) were used. Whenever a donor is donating blood and the process is stopped for any reason, that blood cannot be used for transfusion purposes and so it is discarded. An information sheet about the study was given to the donor and the study was also explained verbally. If the donor agreed to take part in the study, he/she was asked to sign an informed consent form, which was labelled by the same donation number of the blood. The Director of the National Blood Transfusion Service (Malta) has given permission to use these incomplete blood donations (Appendix D) and ethical approval was granted by the Research Ethics Committee in Malta. The ethical approval letter, together with the information sheet and the consent form, are attached in Appendix D.

A quadruple bag kit system is used for whole blood collection at the National Blood Transfusion Service. This consists of a mother bag containing Citrate Phosphate Dextrose Adenine Solution (CPDA) as anti-coagulant, an upper transfer bag for Fresh Frozen Plasma (FFP), a bottom bag containing the preservative SAG-M (named after its constituents, saline, adenine, glucose and mannitol) for the Leucocyte-depleted Red Cell Concentrate (RCC), an in-line filter and a transfer bag. A standard donation of whole blood (excluding anticoagulants) usually consists of a donation of 450ml ± 10% (i.e. 405 to 495ml). Donations of less than 405ml are classified as insufficient and hence discarded at the Blood Products Laboratory. Insufficient blood donations with volumes between 300ml and 404ml were selected for this study because if the volume is

lower, separation of the whole blood into several blood components would not be appropriate and the resulting buffy coat will not be concentrated enough.

The whole blood units were centrifuged at 4561 x g for 12 minutes 30 seconds at 22^{o}C using the Hettich Roto Silenta centrifuge (Hettich, Germany). After centrifugation the whole blood bags were placed onto the automatic separators (Compomat G5, Fresenius Kabi, Germany) and the appropriate program for whole blood separation was selected. These automatic expressors provide controlled separations of whole blood into blood components, i.e. plasma, leucocyte-depleted red cell concentrate and buffy coat. The bags containing the plasma and the red cell concentrate were discarded and the bag containing the buffy coat was used to extract mononuclear cells (MNCs) and isolate CD34^{pos} cells.

2.5 Harvesting and culturing of cells

2.5.1 Isolation of mononuclear cells from peripheral blood (buffy coat)

The tube of the buffy coat, as well as the scissors, were sterilised by wiping them with 70% alcohol prior to opening the buffy coat tube. Volumes of 15ml buffy coats were poured in two 50ml tubes, diluted 1:1 with recommended medium (Ca^{++} and Mg^{++} free phosphate buffered saline (PBS) (Thermo Fisher Scientific, USA) with 2% foetal bovine serum (FBS) (PAN Biotech, Germany) plus 1 mM ethylene-diamine-tetra-acetic acid (EDTA)) and mixed well. Sterile-filtered Histopaque®-1077 (Sigma-Aldrich, USA) having a density of 1.077g/ml (15ml), was poured in two other 50ml tubes and brought to room temperature. Histopaque®-1077 is a sterile, endotoxin tested solution (≤0.3EU/ml) of polysucrose (57g/l) and sodium diatrizoate (90g/l), adjusted to a density of 1.077g/ml, having a pH of 8.8 to 9.0. This density-gradient medium facilitates rapid recovery of viable lymphocytes and other MNCs from small volumes of whole blood.

Using a Pasteur pipette, the buffy coat / medium mixture was gently layered on top of the Histopaque®-1077. The tubes were closed and centrifuged at 400 x g for 30 minutes with the brake off at room temperature. During centrifugation, erythrocytes are aggregated by polysucrose present in the Histopaque®-1077 and sediment rapidly. Granulocytes become slightly hypertonic, which increases their sedimentation rate, resulting in pelleting at the bottom of the tube. Lymphocytes and other mononuclear cells remain at the plasma / Histopaque®-1077 interface.

After centrifugation, the upper layer was carefully aspirated with a Pasteur pipette to within a 0.5cm of the opaque interface containing the mononuclear cells. This upper layer was discarded. The opaque ring interface was transferred into a clean 50ml tube using a Pasteur pipette. The cells were washed by filling the tube to the 50ml mark with recommended medium and mixed gently. The tubes were centrifuged at 250 x g for 10 minutes with full brake. The low speed centrifugation removes the platelets.

The supernatant was aspirated and discarded. If the supernatant was not clear, the washing step was repeated. Then the cell pellets were resuspended in 1ml recommended medium and recombined.

2.5.2 Separation of $CD34^{pos}$ cells from mononuclear cells in human buffy coat

$CD34^{pos}$ cells were isolated using the "Human whole blood / buffy coat CD34 selection kit" by EasySep (StemCell Technologies, USA). The kit contains the EasySep® CD34 Positive Selection Cocktail and the EasySep® Magnetic Nanoparticles that label $CD34^{pos}$ cells for magnetic separation. These positive selection reagents are designed to positively select $CD34^{pos}$ cells from freshly prepared buffy coat.

The Positive Selection Cocktail contains monoclonal antibodies bound in bispecific tetrameric antibody complexes (TAC) that are directed against both dextran and the target cell surface antigen CD34. When they are added to the MNC sample, they bind to the target cells. Then Magnetic Nanoparticles, which consist of a suspension of magnetic dextran iron particles in water, are added

and bind to the TACs. Hence, target cells become labelled with dextran-coated magnetic nanoparticles using TACs (Figure 2.3). The small size of the magnetic dextran iron particles allows for efficient binding to the TAC-labelled cells and does not interfere with subsequent flow cytometric analysis. Magnetically labelled cells are then separated from unlabelled cells.

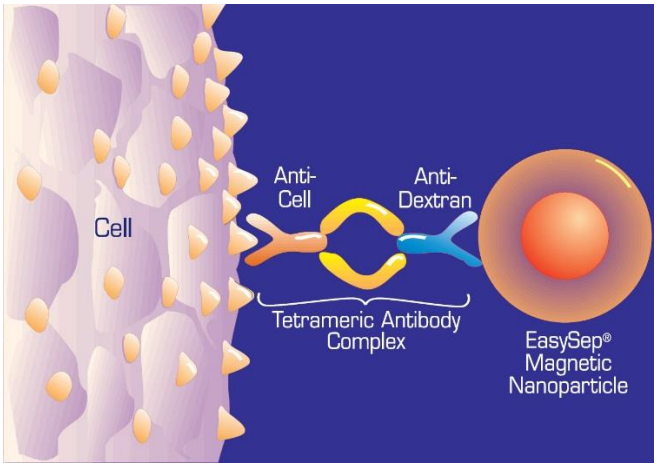


Figure 2.3: Schematic Drawing of EasySep® TAC Magnetic Labelling of Human Cells. (Image taken from StemCell Technologies, 2007). (Used with permission from StemCell Technologies).

The MNC sample was transferred to a Falcon™ 14ml (17 x 100 mm) polystyrene round-bottomed tube (Becton Dickinson, USA) in order to fit properly into the EasySep® Magnet. One part 1x EasySep® RBC lysis buffer was added to 1 part MNC sample and mixed well. EasySep® Positive Selection Cocktail was added at 40µl/ml of cell sample/lysis buffer mixture, mixed well and incubated at room temperature for 15 minutes. Then EasySep® Magnetic Nanoparticles were mixed well to ensure that they are in a uniform suspension by pipetting up and down vigorously more than five times. The nanoparticles were added at 40µl/ml of cell sample/lysis buffer mixture, mixed well and incubated at room temperature for 10 minutes.

The cell suspension was brought to a final volume of 10ml by adding the recommended medium. The cells were mixed well in the tube by gently pipetting up and down two to three times. The tube (without cap) was placed into the EasySep® Magnet and set aside for 10 minutes. Then “The Big Easy” Magnet was picked up and in one continuous motion the magnet and the tube were inverted, pouring off the supernatant fraction. The magnetically labelled cells remained inside the tube, held by the magnetic field of “The Big Easy” Magnet. The magnet and tube were left in inverted position for two to three seconds and then returned to upright position. The tube was removed from the magnet and 10ml recommended medium were added. The cell suspension was mixed gently by pipetting up and down two to three times and the tube was placed back in the magnet and set aside for five minutes. These washes were repeated for a total of one 10-minute and two 5-minute separations in the magnet (Figure 2.4).

Finally the tube was removed from the magnet and the $CD34^{pos}$ cells were resuspended in an appropriate amount of StemSpan™ Serum-Free Expansion Medium (SFEM) (StemCell Technologies, USA) supplemented with StemSpan™ cc100 cytokine cocktail (100x) (StemCell Technologies, USA). The cells were counted using an electronic cell counter (described in section *2.5.4*) and resuspended in an appropriate volume of medium at an appropriate concentration. The $CD34^{pos}$ cells were cultured in a 37°C incubator in an atmosphere of 5% CO_2 and over 95% humidity.

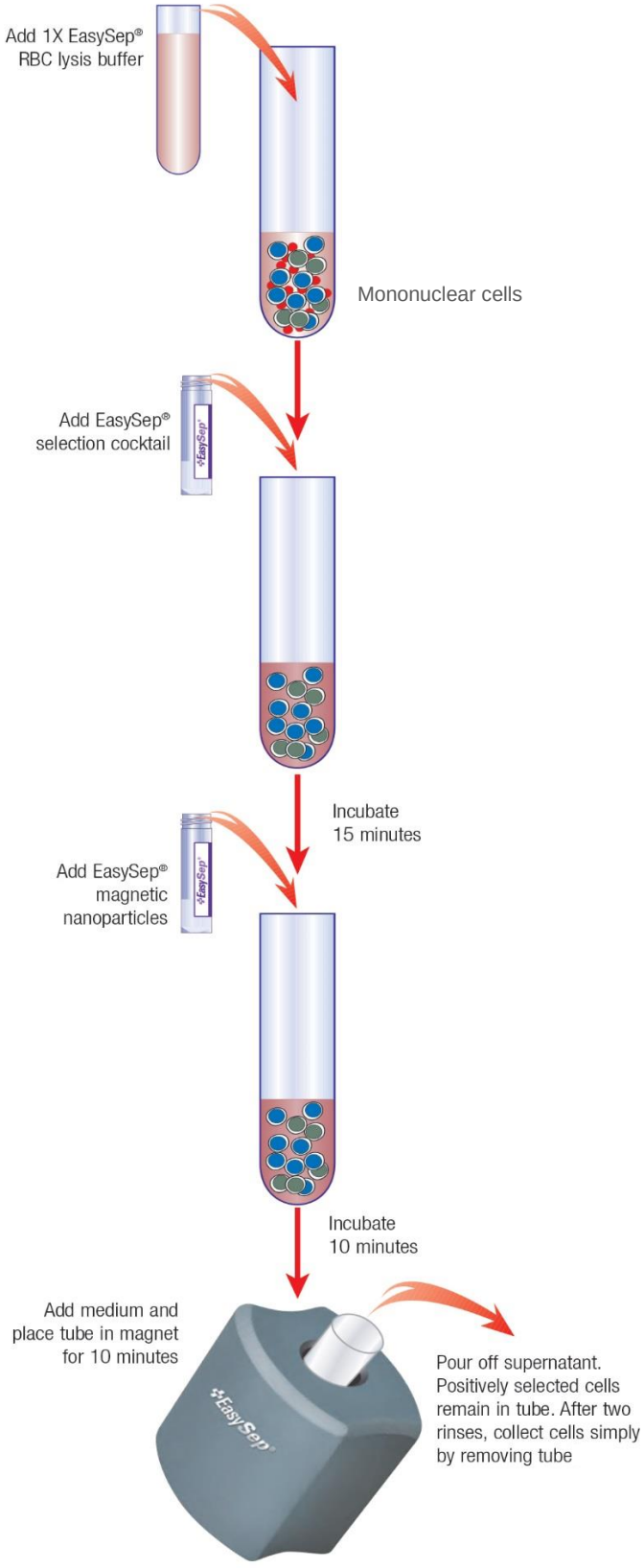


Figure 2.4: EasySep® protocol using the Silver "The Big Easy" EasySep® Magnet. (Image taken from StemCell Technologies, 2007). (Used with permission from StemCell Technologies).

2.5.3 Culturing of CD34^{pos} cells

StemSpan™ Serum-Free Expansion Medium (SFEM) (StemCell Technologies, USA) has been developed for the *in vitro* culture and expansion of human haematopoietic cells. This medium contains bovine serum albumin (BSA), recombinant human insulin, human iron-saturated transferrin, 2-mercaptoethanol and supplements in Iscove's Modified Dulbecco's Medium (IMDM). The sterile medium was stored at -20^{o}C and was thawed by placing it in a 37^{o}C water bath. When it was completely thawed, the medium was transferred in a Class II safety cabinet (SafeFAST Elite, Faster s.r.l, Italy) and wiped from outside using 70% alcohol. By means of aseptic techniques, the medium was supplemented with 1% cc100 cytokines (StemCell Technologies, USA), 100 Units per millilitre penicillin and 0.1mg per millilitre streptomycin (Pen/Strep PAN Biotech, Germany). The cc100 cytokine cocktail contains a combination of recombinant human cytokines formulated to support the proliferation of human haematopoietic progenitors. The antibiotics penicillin and streptomycin were added to the medium in order to prevent bacterial contamination (both gram-positive and gram-negative bacteria) of cell cultures. The medium was kept refrigerated at 4^{o}C.

CD34^{pos} cells were analysed using an electronic cell counter (described in section *2.5.4*) and maintained by adding fresh medium, keeping them at a concentration of around 1.4x10^{6}/ml.

2.5.4 Analysis of cells by CASY® cell counter

The cultured cells were followed-up by analysing them using CASY® cell counter and analyser (Roche, Germany). The CASY® cell counter assesses cell cultures both qualitatively and quantitatively. It combines the resistance measurement

principle, which is a proven particle measurement technique, with pulse area analysis, which is a modern method of signal evaluation. The cells to be measured are suspended in CASY®ton (Roche, Germany) which is an isotonic, iso-osmotic, particle-pure, odourless and colourless saline solution. It reduces the aggregation of the cells in the measuring cup and stabilises the cells for the duration of the measurement time. The defined conductivity guarantees consistent measurement results, while the physiological pH and osmolarity ensure the preservation of the cell size (Roche, 2010a).

A 100µl aliquot is taken from the cell suspension for counting and placed in a CASY® cup. The aliquot is diluted with 10ml CASY®ton. The CASY® cup is mixed gently and loaded on the CASY® analyser. The cells are aspirated through a precision measuring pore at a constant flow speed. During the measurement process, a pulsed low voltage field with 1 MHz is applied to the measuring pore through two platinum electrodes. The electrolyte-filled measuring pore represents a defined electrical resistance and as the cells pass through the measuring pore individually, they displace a quantity of electrolyte corresponding to their volume. Since intact cells can be considered as isolators, an increased level of resistance is achieved over the measuring pore. This resistance is a dimension for the volume of the cells. By contrast, the membrane of dead cells no longer acts as an electrical barrier, and are thus recorded by the size of their cell nucleus (Figure 2.5). Since the volume of the cell nucleus is significantly smaller than the volume of the entire cell, debris, dead cells and viable cells can be clearly distinguished (Roche, 2010a).

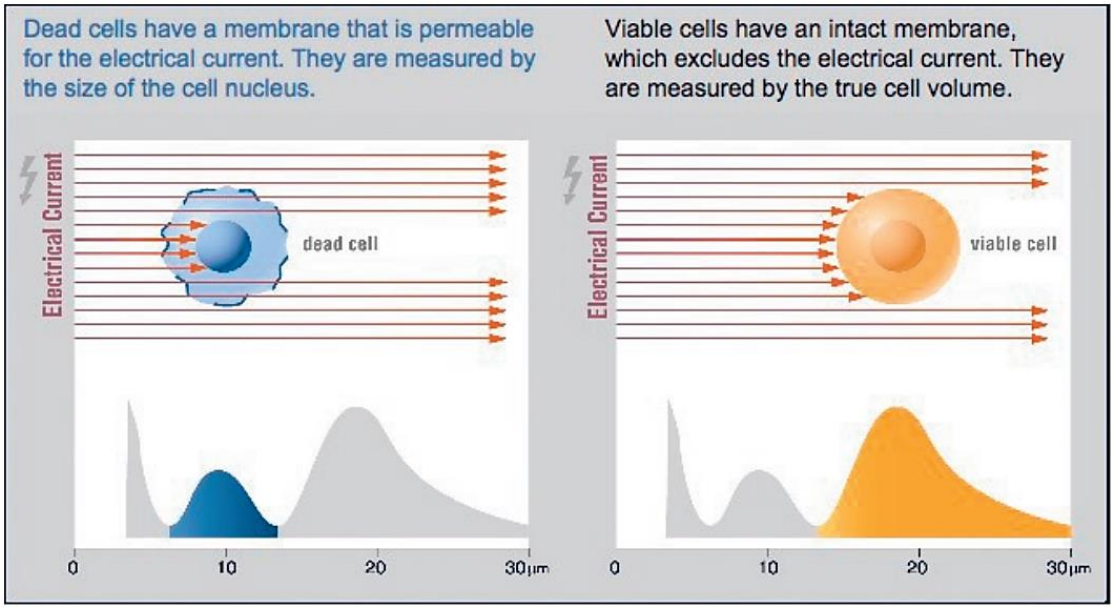


Figure 2.5: Viability measurement by CASY® cell analyser through electrical current exclusion. The status of the cell membrane affects the electrical signal generated when a cell is passing through the measuring pore. As dead cells have a permeable membrane, they are measured by the size of their nucleus, while viable cells have an intact membrane and thus are measured by the cell volume (Image taken from CASY Model TT – Cell Counter and Analyzer Handbook, Roche, 2010a). (Used with permission from OLS, OMNI Life Science).

The measuring signal is scanned by the analyser at high frequency. The amplitude and the shape of the measuring signal are recorded by the analyser. CASY® calculates the integral of the measuring signal from the individual measurements. The calculated signal areas are evaluated by a standardized method and accumulated in a multi-channel analyser (pulse area analysis) that provides a unique, high resolution recording of the cell size distribution. A diameter-linear size distribution is calculated from a linear-volume, original distribution.

A graph is displayed showing the recorded size distribution together with measured results (Figure 2.6). All measuring and output parameters can be displayed and adjusted through the menu control. Parameters for viable cells (/ml), percentage viability (%), mean diameter (µm) and peak diameter (µm) of cells were recorded for every analysis performed.

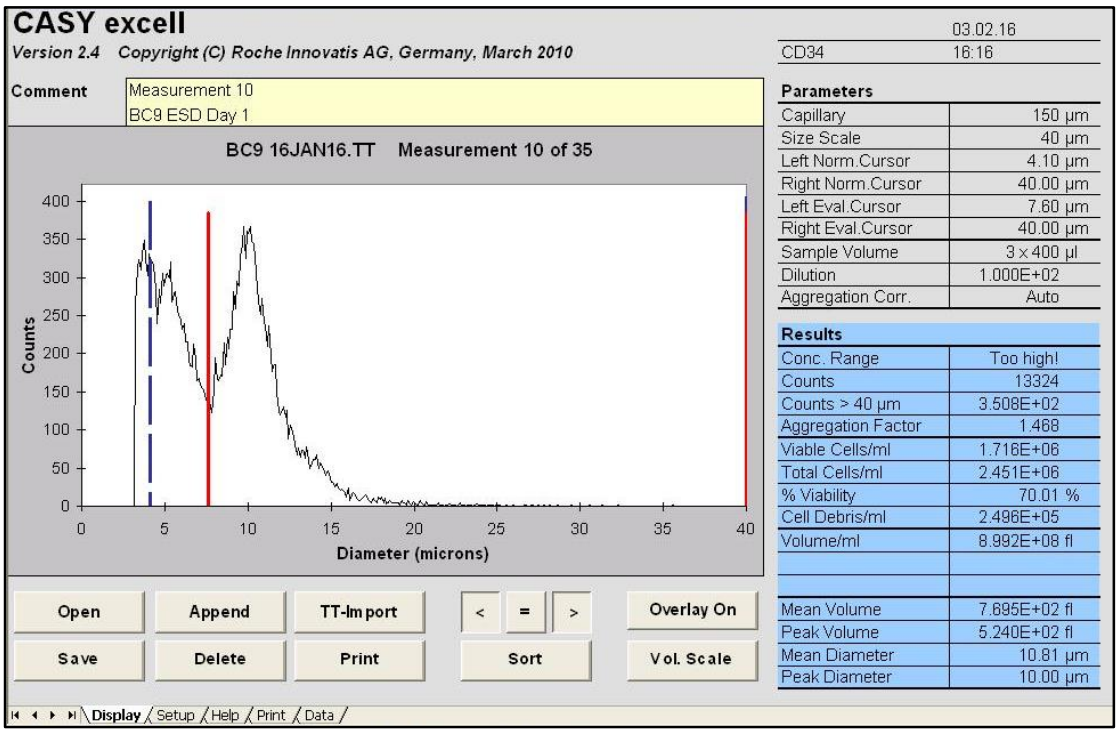


Figure 2.6: CASY® report of a cell culture. The graph displays the size distribution of cultured CD34^{pos} cells. To the left of the left-hand normalisation cursor appearing as a blue dotted line (< 4.10 µm), there is the debris peak. Between the left-hand normalisation cursor and the left-hand evaluation cursor shown by a red solid line (4.10 µm to 7.60 µm), there is a peak for the dead cells. To the right of the left-hand evaluation cursor, the peak for the viable cells begins followed by cell aggregates. On the right of the graph the numerical evaluation of the measured cell culture is displayed.

2.5.4.1 Setting up a CASY® program

To obtain accurate measurements, the measuring range and cursor positions were adjusted according to the cells to be measured. To be able to compare the samples and evaluate the data correctly, all samples were measured using identical settings.

To specify the position of the left-hand evaluation cursor (CL) and the left-hand normalisation cursor (NL), a culture with high viability was measured first. On the recorded size distribution display, the left-hand evaluation cursor was positioned at the point where the viable population begins. The debris peak (at the lower measuring limit) was located and the left-hand normalisation cursor was positioned where the debris peak ends (Figure 2.7). The changes of the cursor position were saved.

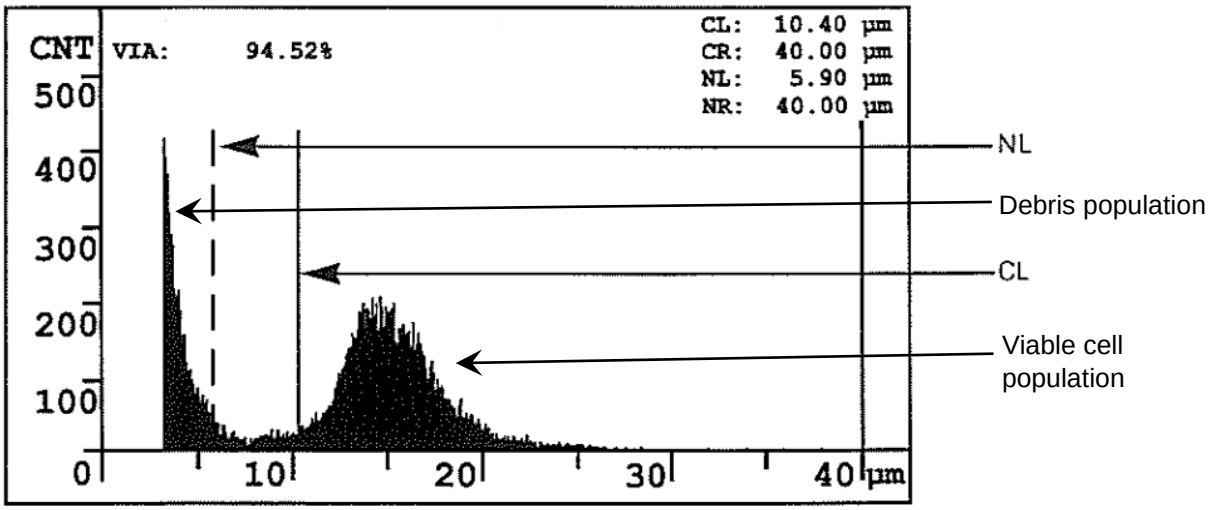


Figure 2.7: Setting up the left-hand evaluation cursor and the left-hand normalisation cursor. [CNT: counts; VIA: % viability; CL: left-hand evaluation cursor; CR: right-hand evaluation cursor; NL: left-hand normalisation cursor; NR: right-hand normalisation cursor]. (Image taken from CASY® Model TT instruction book, Roche, 2010b). (Used with permission from OLS, OMNI Life Science).

Secondly, a sample with dead cells was prepared by adding 800µl of CASY®blue (an alcoholic diluent) to 100µl of the same cell suspension used for the measurement of viable cells. The sample was mixed by pipetting up and down for five times and then incubated for two minutes at room temperature. Then 9 ml CASY®ton was added to the CASY®blue / cells mixture and mixed by inverting the CASY® cup with the lid on for three times. After one minute the CASY® cup was mixed again for one time and then measured on the CASY® cell analyser. Since the cells were killed by CASY®blue, they were only measured by the size of their nuclei. Hence the main peak of the recorded size distribution shifts to the left as most of the main population displayed in the measurement of viable cells completely shifted to the left of the left-hand evaluation cursor (Figure 2.8).

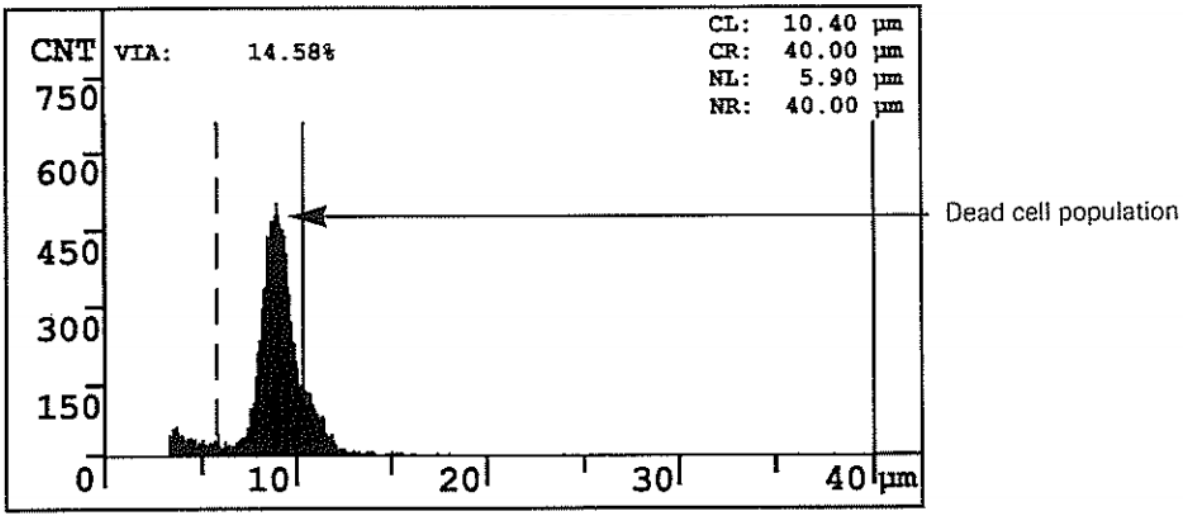


Figure 2.8: CASY® graph showing a dead cell population killed by CASY®blue. [CNT: counts; VIA: % viability; CL: left-hand evaluation cursor; CR: right-hand evaluation cursor; NL: left-hand normalisation cursor; NR: right-hand normalisation cursor]. (Image taken from CASY® Model TT instruction book, Roche, 2010b). (Used with permission from OLS, OMNI Life Science).

Finally, to be able to define correct cursor settings, a sample containing all necessary populations, i.e. debris, dead cells and viable cells was produced by preparing a mixture from the existing control samples of dead and viable cells to get a ratio of 70% viable cells and 30% dead cells. This was done by adding 7ml of the viable cells suspension to 3ml of dead cells suspension, mixing well and measuring using the CASY® cell analyser. Based on the recorded size distribution, the position of the normalisation and evaluation cursors were defined (Figure 2.9). For the measurement of the erythroid progenitors, the left-hand evaluation cursor was positioned exactly between the viable and the dead cell peak, i.e. at 7.60µm; the left-hand normalisation cursor was positioned exactly between the debris and the dead cell peak, i.e. at 4.10µm; and both right hand cursors (evaluation and normalisation cursors) were positioned at the end of the scale, i.e. at 40µm, to guarantee that all cell aggregates are included in the calculations.

For calculating the viability of the cell suspension, the two pairs of cursors that define specific size ranges, are also used to calculate the percentage of viable cells. The normalisation cursor range define the total cell number, while the evaluation cursor range includes the viable population only. Based on these settings the CASY® cell counter calculates the viability in %.

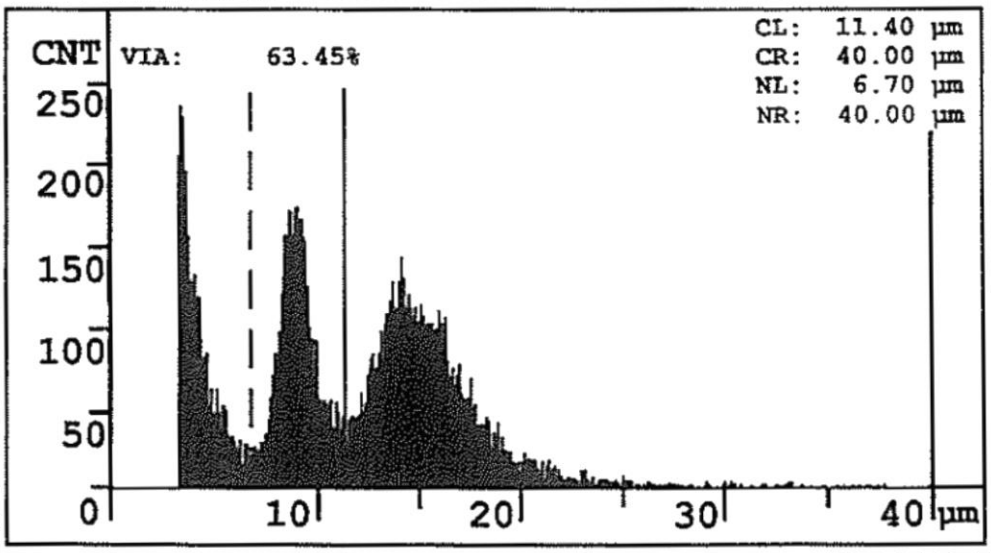


Figure 2.9: An example of a cell population measured by CASY®. The peaks represent debris, dead cells and viable cells. [CNT: counts; VIA: % viability; CL: left-hand evaluation cursor; CR: right-hand evaluation cursor; NL: left-hand normalisation cursor; NR: right-hand normalisation cursor] (Image taken from CASY® Model TT instruction book, Roche, 2010b). (Used with permission from OLS, OMNI Life Science).

2.5.5 Immunophenotypic analysis of cells

The $CD34^{pos}$ cell cultures were antigenically profiled for CD34 and CD133 expression by standard flow cytometric techniques. The cells were suspended in buffer and stained by the two antibodies. Two controls were prepared with every run, i.e. an unstained sample control and an isotype control.

First the cell number was determined using the CASY® cell counter and analyser. An appropriate volume was taken in order to have at least 2 x 10^5 cells per tube. The cell suspension was centrifuged at 300 x g for 10 minutes. The supernatant was aspirated completely and the cells were then resuspended in 100µl of buffer (consisting of PBS with 1% BSA).

Ten microlitres of CD34-FITC antibody (Clone: AC136; Isotype: mouse IgG2aκ; Isotype control: mouse IgG2a – isotype control antibodies) (Miltenyi-Biotec, Germany) and 10µl of CD133-PE antibody (Clone: 293C3; Isotype: mouse IgG2bκ; Isotype control: mouse IgG2b – isotype control antibodies) (Miltenyi-Biotec, Germany) were added to the sample tube, while 10µl of mouse IgG2a-FITC isotype control antibody (Miltenyi-Biotec, Germany) and 10µl of mouse IgG2b-PE isotype control antibody (Miltenyi-Biotec, Germany) were added to the isotype control tube. The mouse IgG2a isotype control antibody is specific for the hapten NP (4-hydroxy-3-nitro-phenyl) acetyl, which is not expressed on cells or cell lines, while the mouse IgG2b isotype control antibodies are specific for KLH (keyhole limpet hemocyanin), a protein which is not expressed on human cells. Therefore these antibodies are used as a negative control, to distinguish specific from non-specific binding due to interactions of the fluorochromes with the cell surface and to assess the level of background staining during flow cytometry analysis. No antibodies were added to the unstained sample control tube.

The tubes were mixed well by vortexing briefly and incubated for 10 minutes in the dark at 2 to 8^{o}C. Then the cells were washed by adding 1 ml of buffer and centrifuged at 300 x g for 10 minutes. The supernatant was aspirated completely and finally the cell pellets were resuspended in 1 ml of buffer for analysis by flow cytometry.

The cells were analysed by the FACS Calibur flow cytometer using Cell Quest software (Becton Dickinson Biosciences, USA) or by the FACS Canto II flow cytometer using FACS Diva Software (Becton Dickinson Biosciences, USA). The

unstained sample control was run first to obtain a graph of the forward scatter versus the side scatter of the cells enabling gating of the cells of interest. Then the isotype control was run so that a gate could be drawn appropriately, to eliminate any irrelevant fluorescence. The isotype control graph of CD133 versus CD34 permitted the optimal positioning of the quadrant, thus enabling analysis of the stained sample.

2.6 Transfection protocols

The *JAK2* constructs were transfected in cell lines with high transfection efficiency, to assess expression of the Jak2 protein, using various transfection protocols described below (sections *2.6.1, 2.6.2 and 2.6.3*). The $CD34^{pos}$ cells were transfected using the Nucleofection protocol of Amaxa (section *2.6.3*).

2.6.1 Magnefect-nano transfection protocol

This magnet-assisted transfection technology is a novel technique developed by nanoTherics (UK). It accurately and rapidly transfects biomaterials into cells by employing magnetic nanoparticles and oscillating magnetic fields.

Day 1: Cell Seeding

For this transfection protocol, GH3 cells and A549 cells were used, as they were being cultured in the laboratory. The adherent cells were washed twice with the same medium in order to remove dead cells that are slightly adherent. The medium was then removed from the flask and the cells were washed with 5ml PBS to remove any remaining medium that can render trypsin less efficient. After removing the PBS, 4ml of 5x trypsin was added and the flask was incubated at 37ºC for five to six minutes. After this time, the cells were viewed under the microscope to check if they have become unstuck. Then 4ml of Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, USA) containing 10% FBS (PAN Biotech, Germany), 100 U/ml penicillin and 0.1mg/ml streptomycin (PAN Biotech, Germany) were added (denoted as DMEM++). DMEM is a modification of Basal Medium Eagle (BME) that contains a four-fold higher concentration of

amino acids and vitamins, as well as additional supplementary components. The bottom of the flask was washed and the cells were collected in a 15ml tube.

The tube was centrifuged at 300 x g for five minutes. The supernatant was removed and the cells were resuspended in 8ml of DMEM++. The cells were counted using CASY® analyser. To transfect the cells it is important that the percentage viability is more than 90%. Since for the first experiment a 96-well plate was used, for every transfection reaction, 1 x 10^4 cells in 100µl of DMEM++ were seeded per well. The plate was then incubated at 37^{o}C in 5% CO_2 overnight.

Day 2: On the day of transfection

The neuroMAG particles were allowed to thaw at room temperature and vortexed before use. The total amount of DNA (i.e. *JAK2*-pIRES2 construct) required for all wells was resuspended in a volume of serum-free and supplement-free medium that is 5% of the total volume of the transfection medium required, i.e. in a 96-well plate, since the final transfection volume per well is 100µl, 100ng of DNA was resuspended in × µl supplement-free DMEM to a final volume of 5µl. Then 0.05µl neuroMAG was added for every reaction to the transfection mix and mixed by pipetting up and down for five times. The reactions were incubated for 15 minutes at room temperature in order to allow the formation of the neuroMAG-DNA complex. After the incubation, DMEM (+10% FBS) was added to the reactions, in order to obtain the total volume of 100µl required for each transfection reaction.

The plate containing the seeded cells on day 1 was removed from the incubator and the medium was aspirated from all the wells of the plate. Then the

neuroMAG-DNA complex mixtures were added to corresponding wells of the 96-well culture plate. The plate was placed on the Magnefect Nano II device (nanoTherics Ltd, UK) and placed in a CO_2 incubator. The parameters were set: Frequency = 2 Hz, Displacement = 0.2 mm, Time = 30 minutes. Finally the plate was removed from the device and incubated at 37^oC in 5% CO_2 for 24 to 48 hours.

Day 3 and Day 4:

The cells were viewed under a microscope and checked for fluorescence.

This protocol was repeated, using A549 cells only to transfect them with the *JAK2*-pIRES2 construct in a 6-well plate. Amounts of reagents and volumes were scaled up due to the greater surface area of a 6-well plate. Hence, 2.8 x 10^5 cells in 2.82ml of medium with supplements were seeded per well. On the second day, since the final transfection volume per well is 2.82ml, 2.82µg of DNA was resuspended in × µl supplement-free DMEM to a final volume of 141µl. Then 1.4µl neuroMAG was added for every reaction to the transfection mix. The same conditions were set on the Magnefect Nano II device (nanoTherics Ltd, UK) as for the 96-well plate and after 48 hours of incubation the cells were viewed under a microscope and checked for fluorescence. Cell lysates were prepared as per protocol and stored at -80^oC for Western Blotting.

The same protocol (using a 6-well plate) was performed, this time to transfect A549 cells with the constructs wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3. Since the constructs do not include a GFP gene, the cells do not fluoresce. In fact only cells transfected with pmaxGFP exhibited fluorescence. Cell lysates were prepared

after 48 hours for Western blotting. However, when the lysates where quantified, their protein concentration was quite low. Therefore the protocol was repeated, using a full 6-well plate for each of the wild-type and mutant constructs. Cell lysates were prepared after 48 hours for Western blotting.

2.6.2 FuGENE® HD transfection protocol

Another type of transfection methodology was investigated. FuGENE® HD Transfection Reagent is a novel, nonliposomal formulation designed to transfect DNA into a wide variety of cell lines with high efficiency and low toxicity.

Day 1: Cell Seeding

For this transfection protocol, a variety of cells were used (i.e. PC3 cells, GH3 cells and MCF7 cells) (Table 2.6). The adherent cells were washed twice with the same medium in order to remove dead cells that are slightly adherent. The medium was then removed from the flask and the cells were washed with 5ml PBS to remove any remaining medium that can render trypsin less efficient. After removing the PBS, trypsin was added and the flask was incubated at 37^{o}C for five to six minutes. After this time, the cells were viewed under the microscope to check if they have become unstuck. Then 4ml of appropriate medium (with supplements) were added. The bottom of the flask was washed and the cells were collected in a 15ml tube.

The tube was centrifuged at 300 x g for five minutes. The supernatant was removed and the cells were resuspended in 8ml of medium (with supplements). The cells were counted using CASY® analyser. To transfect the cells it is important that the percentage viability is more than 90%. The cells were plated at

a density of 80% per well. Seeding conditions varied according to the cell type and plate used and these were previously optimised by the laboratory research group (Saliba, 2014):

PC3 cells: 1.8 x 10^5 cells per well of a 24-well plate in 500µl of DMEM + 10% FBS;

GH3 cells: 2 x 10^5 cells per well of a 24-well plate in 500µl of DMEM + 10% FBS;

MCF7 cells: 1.8 x 10^5 cells per well of a 24-well plate in 500µl of RPMI +10% FBS;

MCF7 cells: 2.3 x 10^4 cells per well of a 96-well plate in 100µl of RPMI + 10% FBS.

The plate was then incubated at 37ºC in 5% CO_2 overnight.

Table 2.6: FuGENE® transfection experiments using different cell types and conditions.

Experiment	Cells	DNA	Plate	Cell lysate prepared
1	PC3	*JAK2*-pIRES2	24-well	✓
2	GH3	*JAK2*-pIRES2	24-well	✓
3	MCF7	*JAK2*-pIRES2	96-well	✓
4	MCF7	*JAK2*-pIRES2	24-well	✓
	MCF7	wt*JAK2*-pcDNA3	24-well	✓
	MCF7	mut*JAK2*-pcDNA3	24-well	✓

Day 2: On the day of transfection

When using a 24-well plate: A 0.020 µg/µl plasmid solution in a final volume of 26µl medium (without supplement) was prepared for each reaction. This was achieved by adding 0.50µg of DNA (i.e. *JAK2*-pIRES2 construct, wt*JAK2*-pcDNA3 construct and mut*JAK2*-pcDNA3 construct) in 26µl medium. Then 1.7µl of FuGENE® HD reagent was added to each reaction, mixed carefully by pipetting up and down for 15 times and incubated for 10 minutes at room temperature. The

volumes were scalable according to the amount of wells that were transfected. This protocol provided a Reagent : DNA ratio of 3:1 at 0.50µg DNA per well of a 24-well plate.

After the incubation, 25µl of the complex was added to the cells per well and mixed thoroughly. The plate was incubated at 37ºC in 5% CO_2 for 24 to 48 hours. After 6 to 8 hours, 10% FBS was added.

When using a 96-well plate: A 0.020 µg/µl plasmid solution in a final volume of 5.2µl medium (without supplement) was prepared for each reaction. This was achieved by adding 0.10µg of DNA in 5.2µl medium. Then 0.3µl of FuGENE® HD reagent was added to each reaction, mixed carefully by pipetting up and down for 15 times and incubated for 10 minutes at room temperature. The volumes were scalable according to the amount of wells that were transfected. This protocol provided a Reagent : DNA ratio of 3:1 at 0.10µg DNA per well of a 96-well plate.

After the incubation, 5µl of the complex was added to the cells per well and mixed thoroughly. The plate was incubated at 37ºC in 5% CO_2 for 24 to 48 hours. After 6 to 8 hours, 10% FBS was added.

Day 3 and Day 4:

The cells were viewed under a microscope and checked for fluorescence (where available). After 48 hours, cell lysates were prepared as per protocol and stored at -80ºC.

2.6.3 Amaxa® Nucleofection protocol

Various experiments were carried out in order to check the efficiency of this protocol, utilising K562 cells that were being cultured in the laboratory. As the Amaxa® Human $CD34^+$ Cell Nucleofector® kit (Lonza, Germany) provides an optimised protocol for human $CD34^{pos}$ cells, this method of transfection was also performed to transfect cultured $CD34^{pos}$ cells with the *JAK2* constructs.

Method:

A solution was prepared according to the number of reactions by adding Nucleofector® Solution to Supplement at a ratio of 4.5 : 1, i.e. for a single reaction 82µl of Nucleofector® Solution was added to 18µl of Supplement to make 100µl of total reaction volume. This was allowed to reach room temperature.

A 6-well plate was prepared by filling appropriate number of wells (according to the number of reactions) with 1.5ml of supplemented culture medium, i.e. Roswell Park Memorial Institute medium (RPMI) (Gibco, Life Technologies, USA) supplemented with 10% FBS. The plate was left to equilibrate at 37°C in a humidified 5% CO_2 incubator.

The cells were counted using CASY® and the required number of cells (1 to 5 x 10^6 cells per sample) were centrifuged at 200 x g for 10 minutes. The supernatant was removed completely and the cell pellet was resuspended carefully in 100µl pre-supplemented Nucleofector® Solution at room temperature. The cell suspension was combined with 1 to 5µg DNA or 2µg pmaxGFP® (as a positive

control). The cell / DNA suspension was transferred into a certified cuvette and the cuvette was closed with its cap.

The program U-008 was selected on the Nucleofector® II device. The cuvette with the cell / DNA suspension was inserted into the cuvette holder and the selected program was applied. Once the program was finished, the cuvette was taken out of the holder and 400µl of pre-equilibrated supplemented RPMI was added to the cuvette. Using the special pipette provided with the kit, the sample was aspirated gently and transferred into the 6-well plate (to obtain a final volume of 2ml medium per well / sample). The plate was then incubated at 37°C in 5% CO_2. After 24 to 48 hours the cells were viewed under a microscope and checked for fluorescence.

2.7 Western Blot analysis

2.7.1 Preparation of cell lysates for Western Blotting

Since adherent cells were used, the medium was removed from the wells and the cells were washed with PBS. After removing the PBS, trypsin was added and incubated at 37^{o}C for five minutes. The plate was viewed under a microscope to make sure that all the cells have been removed from the bottom of the plate. An appropriate medium was added and the cells were collected in microcentrifuge tubes. The tubes were centrifuged at 300 x g for five minutes and then the supernatant was discarded. Passive lysis buffer (PLB) (Promega, USA) 1x was prepared on ice by diluting it with PBS. About 30µl (enough to cover the cell pellet) was pipetted in each tube and left to incubate on ice for 30 minutes. Then the tubes were centrifuged at 4^{o}C at 20000 x g for 30 minutes so that nuclei will pellet. The supernatant was then collected in another microcentrifuge tube and stored at -80^{o}C until further use.

2.7.2 Protein quantification

The protein concentration of the cell lysates was determined by using the Bradford assay. In this manner, a known concentration of protein can be loaded on the gel. The Bradford assay involves the binding of the dye Brilliant Blue G to proteins in solution. The protein-dye complex causes a shift in the absorption maximum of the dye from 465 to 595nm. The amount of absorption is proportional to the protein present (Bradford, 1976). This blue protein-dye form is detected at 595nm using a spectrophotometer.

2.7.2.1 Preparation of Bovine Serum Albumin (BSA) standards

PLB 1x solution was prepared by adding 4ml distilled water to 1ml of 5x PLB (Promega, USA). BSA powder (Sigma-Aldrich, USA) was weighed (0.02g) and dissolved in 2ml 1x PLB to get a BSA stock of 10mg/ml. Another five BSA standards were prepared in microcentrifuge tubes according to Table 2.7.

Table 2.7: Preparation of BSA standards

Standard	Concentration	10mg/ml BSA	1x PLB
Standard 1	2000 µg/ml	200 µl	800 µl
Standard 2	1500 µg/ml	150 µl	850 µl
Standard 3	1000 µg/ml	100 µl	900 µl
Standard 4	500 µg/ml	500µl of Std 3	500 µl
Standard 5	250 µg/ml	500µl of Std 4	500 µl
Standard 6	125 µg/ml	500µl of Std 5	500 µl

2.7.2.2 Preparation of Bradford reagent

The Bradford reagent (Sigma-Aldrich, USA) requires no dilution. The standard 3.1 ml Bradford assay consists of mixing one part of the protein sample with 30 parts of the Bradford reagent. The sample may be a blank, a protein standard, or an unknown sample. The blank consists of buffer with no protein, the protein standard consists of a known concentration of protein, and the unknown sample is the solution to be assayed.

The Bradford reagent was mixed gently and an appropriate amount of volume was poured in a 15ml tube and brought to room temperature. In a 96-well plate, 150µl of Bradford reagent was pipetted in duplicate for each standard and sample. The mix appears brown, but when the protein is added it turns blue. Then 5µl of standard or sample was pipetted in the corresponding well and mixed by pipetting up and down. For the blank, 5µl of 1x PLB was added. The standards and samples were left to incubate at room temperature for at least five minutes and up to 45 minutes. The absorbance of the standards and samples must be recorded before the 60 minute time limit and within 10 minutes of each other.

First the absorbance of the standards was read using the NanoDrop™ 2000 spectrophotometer. The standards were measured in duplicate for each well, thus obtaining four readings. The average absorbance against protein concentration of each standard was plotted (Figure 2.10).

Standard	Concentration (µg/ml)	Average absorbance
Std1	2000	0.061
Std2	1500	0.06
Std3	1000	0.048
Std4	500	0.026
Std5	250	0.025
Std6	125	0.008

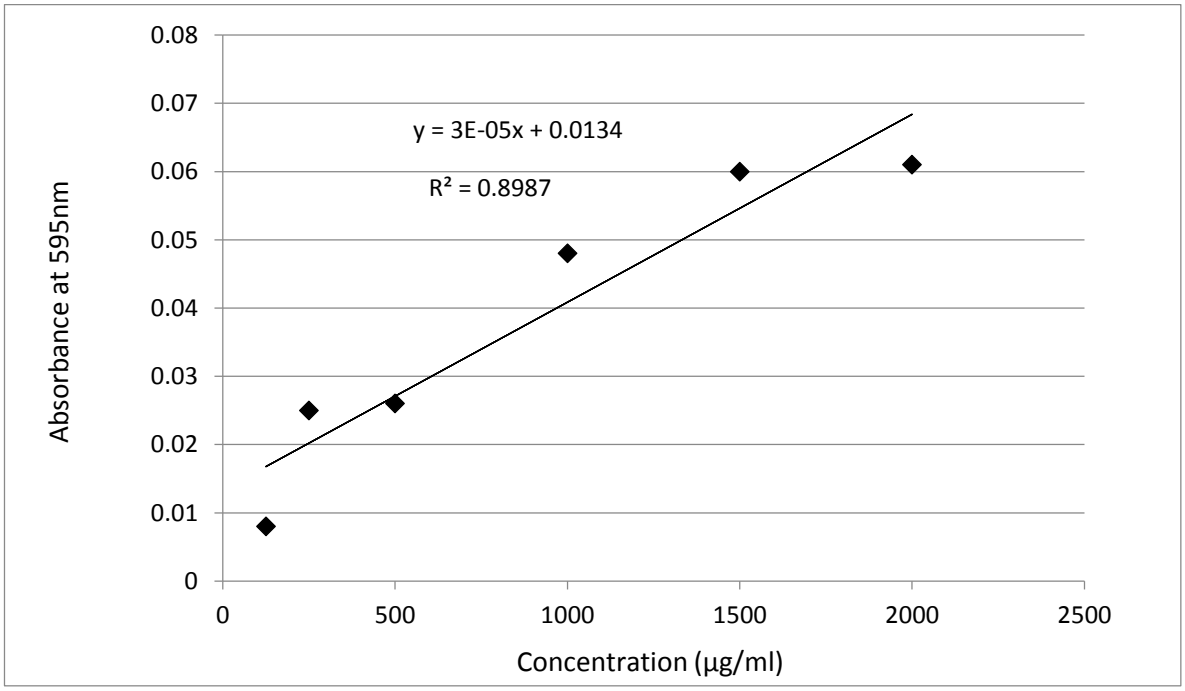


Figure 2.10: Example of a standard curve showing absorbance (595nm) against BSA standards protein concentration (µg/ml). A regression of 0.8987 shows that the standard curve produced using different concentrations of BSA solution is valid.

This reference standard curve was used to convert the absorbance of the samples to protein concentration in µg/µl. The average absorbance of the measured samples was calculated. The protein concentrations (x values) were determined using the values from the formula. Since y = mx + c; x = y-c/m (m and c values were obtained from the trendline of the graph). Finally the amount of protein that is required for Western Blotting was calculated in µl.

2.7.3 Western Blotting protocol

By means of gel electrophoresis, the mixture of proteins is separated based on molecular weight, and thus by type. These results are then transferred to a nitrocellulose membrane producing a band for each protein.

Two gels were prepared: a running gel and a stacking gel. The composition of the running (separating) gel depends on the size of the protein. The percentage of the gel determines the cross-linkage in the running gel composition. The greater the percentage, the greater the resistance to protein movement during electrophoresis. Therefore, for large proteins, a small percentage is used. A 7.5% running gel was prepared by combining 1.25ml acrylamide, 1.86ml 1M Tris pH8.8, 50µl 10% (w/v) sodium dodecyl sulphate (SDS), 1.9ml deionised water (deH_2O), 50µl ammonium persulfate (APS) and 5µl Tetramethylethylenediamine (TEMED) (Roth, Germany). This was poured in between the glass plates. The APS and TEMED together catalyse the polymerization of acrylamide. A layer of water-saturated butanol was added on top of the running gel to avoid the introduction of air bubbles and left to set for around 30 minutes. Once the running gel solidified, the butanol layer was poured off the gel and rinsed well with deH_2O.

The stacking gel was prepared at 5% by combining 0.25ml acrylamide, 0.2ml 1M Tris pH6.8, 7.5µl 10% (w/v) SDS, 1.15ml deH_2O, 25µl APS and 3.75µl TEMED. This was poured over the running gel and then the comb was placed carefully in place and left to solidify for 30 minutes. When the stacking gel solidified, the comb was removed carefully, thus exposing the loading wells. The sandwich was placed in the electrophoresis cell and secured with the retainers. Any air bubbles that were trapped in between the sandwich under the running gel were removed by gently tilting the cell to one side. The middle compartment of the electrophoresis cell and in front of the sandwich were filled with 1x Running buffer (10x Running buffer stock is composed of 15.18g Tris (25mM), 71.31g glycine (190mM), and 5g SDS (0.1%) dissolved in 500ml deH_2O and adjusted to pH8.8 using HCl. To prepare 1x Running buffer, 100ml 10x Running buffer was diluted to 1 litre with deH_2O).

Various concentrations of proteins were loaded, ranging from 11µg to 70µg. The protein concentration of the samples was calculated as described above (section *2.7.2*) and the volume needed for Western Blotting was worked out. This volume was mixed with Protein Loading Buffer (LI-COR, Biosciences, USA) at a 4x concentration. Before loading onto the wells, the samples were heated at 95^oC for about five minutes and then placed on ice, in order to denature the proteins.

Using a pipette, 7µl of the molecular weight standard marker ColourBurst™ Electrophoresis Marker (Sigma-Aldrich, USA) were loaded in the first well while the samples were loaded in the other wells. Then the leads from the power pack were connected to the electrophoresis cell and the voltage was set at 150V.

Electrophoresis was left to run until the dye front reached the bottom of the gel (about 50 minutes).

Three plastic dishes were filled with 1x Transfer buffer (10x Transfer buffer stock is composed of 18.391g Tris (30mM), 14.63g glycine (39mM), and 2g SDS (0.04%) dissolved in 500ml deH_2O and adjusted to pH 8.1 to 8.6. To prepare 1x Transfer buffer, 200ml methanol and 700ml deH_2O were added to 100ml 10x Transfer buffer stock). Four blotting papers (having the size of the gel) were soaked in one dish and a nitrocellulose membrane (cut at an appropriate size to cover the gel) was placed in another dish containing Transfer buffer for 10 minutes. When electrophoresis was finished, the glass sandwich was opened carefully. The stacking gel was removed and discarded, while the running gel was placed in the dish containing Transfer buffer to soak.

In the meantime, the Electroblotter system (Trans-Blot® SD Semi-Dry Transfer Cell, Bio-Rad, USA) was assembled (Figure 2.11). The surface of the graphite cover plates were first cleaned with deH_2O. Two blotting papers were placed and any air bubbles were removed. The membrane was placed in position using tweezers and then the gel was transferred on top of the membrane. Another two blotting papers were placed on top of the gel one by one, each time removing any air bubbles gently. Finally, some transfer buffer was poured over the last blotting paper.

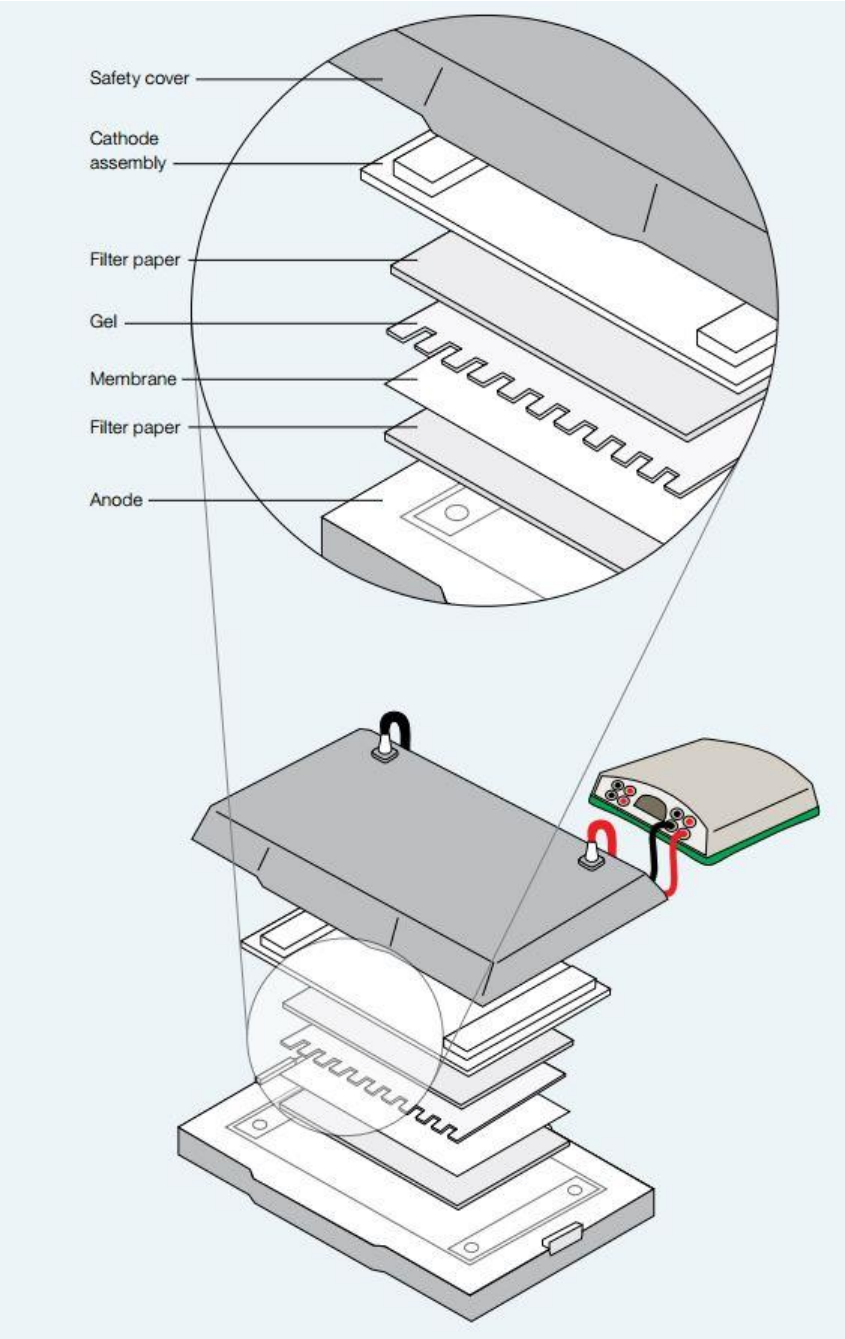


Figure 2.11: The electroblotter assembly. Two filter papers are placed on the anode plate, then the membrane is placed and then the gel is positioned carefully. Another two filter papers are placed on top and finally the cathode plate and safety cover are placed in position to close the electroblotter. (Image taken from Protein Blotting Guide, Bio-Rad Laboratories). (Used with permission from Bio-Rad Corporate Communications Representative).

The electroblotter was closed by placing the lid (cathode plate) very carefully over the blot and the leads were connected to the power pack. The power pack was switched on and the current was set at a constant 0.3A for two hours. When finished, the gel was removed and the membrane was transferred to a dish containing Ponceau red solution and left for five minutes on a rocking shaker. This dye marks the protein bands on the membrane and determines whether the transfer was successful. Then the membrane was washed with deH_2O several times to remove the red dye leaving behind the protein and the standard marker ladder which appear as red bands. The membrane was then left overnight in the Blocking solution on a rocking shaker at 4^oC. The Blocking solution was prepared by adding 3g milk powder in 30ml 1x Tris-buffered saline (TBS) with 0.1% Tween20 (TBS-T). The stock 10x TBS solution is composed of 30.3g Tris (50mM) and 43.8g NaCl dissolved in 500ml deH_2O and adjusted to pH 7.6 using HCl. To prepare 1x TBS, 100ml 10x TBS was diluted to 1 litre with deH_2O. For TBS-T 500µl of Tween20 was added to 500ml of 1x TBS).

Following overnight incubation, the membrane was then incubated with primary antibodies specific to the protein of interest. Rabbit polyclonal anti-human JAK2 antibody (ab39636 Abcam, USA) was used to detect the presence or absence of Jak2 in the samples. Mouse monoclonal anti-β-actin antibody (ab6276 Abcam, USA) was used as a loading control to detect β-actin since actins are highly conserved proteins that are involved in cell motility, structure and integrity and are ubiquitously expressed in all eukaryotic cells. Different dilutions of the antibodies were tested, both for Jak2 and β-actin, ranging from 1:15000 to 1:500. Antibody dilutions were prepared in 1x TBS-T (1x TBS with 0.1% Tween20) and the membrane was incubated with the two primary antibodies for two hours on a

rocking shaker. Since the two antibodies are from different species, they can be diluted in the same solution. However it is important that their corresponding secondary antibodies contain fluorochromes with different emission wavelengths. After the incubation, the membrane was washed three times for five minutes each with 1x TBS-T followed by another three washes for five minutes each in 1x TBS. Washes were performed with gentle shaking at room temperature so that the unbound antibodies are washed off leaving only the bound antibodies to the proteins of interest.

Secondary antibodies, labelled with infrared dyes, were prepared and diluted in 1x TBS-T. Different dilutions were tested ranging from 1:15000 to 1:5000. The fluorescent dyes have absorption and emission wavelengths in the NIR spectrum, between 680 and 800 nm (LI-COR Biosciences, 2010). Since anti-human JAK2 antibody is rabbit polyclonal, the secondary antibody goat anti-rabbit IRDye® 800CW (LI-COR, Biosciences, USA) was used and since anti-β-actin antibody is mouse monoclonal, the secondary antibody goat anti-mouse IRDye® 680RD (LI-COR, Biosciences, USA) was used. The membrane was incubated with the secondary antibodies on a rocking shaker for two hours in the dark. Following this, the membrane was washed three times for five minutes each with 1x TBS-T followed by another three washes for five minutes each in 1x TBS. Washes were performed with gentle shaking at room temperature.

Finally, the membrane was scanned using LiCor Odyssey Gel Imaging Scanner (LI-COR, Biosciences, USA) using the green and the red channels to detect bands for Jak2 at 125 kDa in the green channel, and bands for β-actin at 42 kDa in the red channel.

2.8 Detection of Jak2 in transfected CD34^{pos} cells using an *in situ* hybridisation assay

In addition to Western Blotting, the PrimeFlow™ RNA assay, which is a novel technique, was used to measure Jak2 expression together with surface markers as a measure of cell type. The PrimeFlow™ RNA assay is a fluorescent *in situ* hybridisation (FISH) assay that involves the innovative technology of the Affymetrix ViewRNA™ branched DNA (bDNA) technology together with flow cytometry. This makes possible the specific localisation and detection of up to three RNA targets and immunophenotyping for cell surface markers by using fluorochrome-conjugated antibodies simultaneously. This assay utilises branched DNA technology to amplify the detection of an RNA transcript by flow cytometry, rather than the target RNA itself.

During the hybridisation step, a gene specific oligonucleotide target probe set that contains 20 to 40 probe pairs, binds to the target RNA sequence. Three types of probe sets are currently available to allow detection of RNA labelled with Alexa Fluor® 647 (Type 1 probe sets), Alexa Fluor® 488 (Type 4 probe sets), or Alexa Fluor® 750 (Type 6 probe sets). In fact, the ViewRNA® probe human JAK2 is a Type 1 probe set specifically designed to hybridise to human *JAK2*, covering the region 2178 to 3399 bp of the *JAK2* coding sequence. Signal amplification takes place with the help of an individual probe pair designed to bind adjacent to each other. The signal is amplified through a series of sequential hybridisation steps. The pre-amplifier molecules hybridise to the target probes only when both sides of a respective probe pair have bound, granting an additional level of specificity. Multiple amplifier molecules then hybridise to their respective pre-amplifier, and

finally, multiple label probe oligonucleotides conjugated to a fluorescent dye hybridise to their corresponding amplifier molecules; when this signal amplification "tree" is assembled, there will be 400 label probe binding sites. When all target-specific oligonucleotide probes in the target probe set bind to the target RNA transcript, 8000 to 16000 fold amplification is achieved (Affymetrix, USA) (Figure 2.12). Once the cells have been processed using this assay, they can be analysed on a flow cytometer. In the experiments, transfected cells were first stained for cell-surface markers and then probed for *JAK2* RNA.

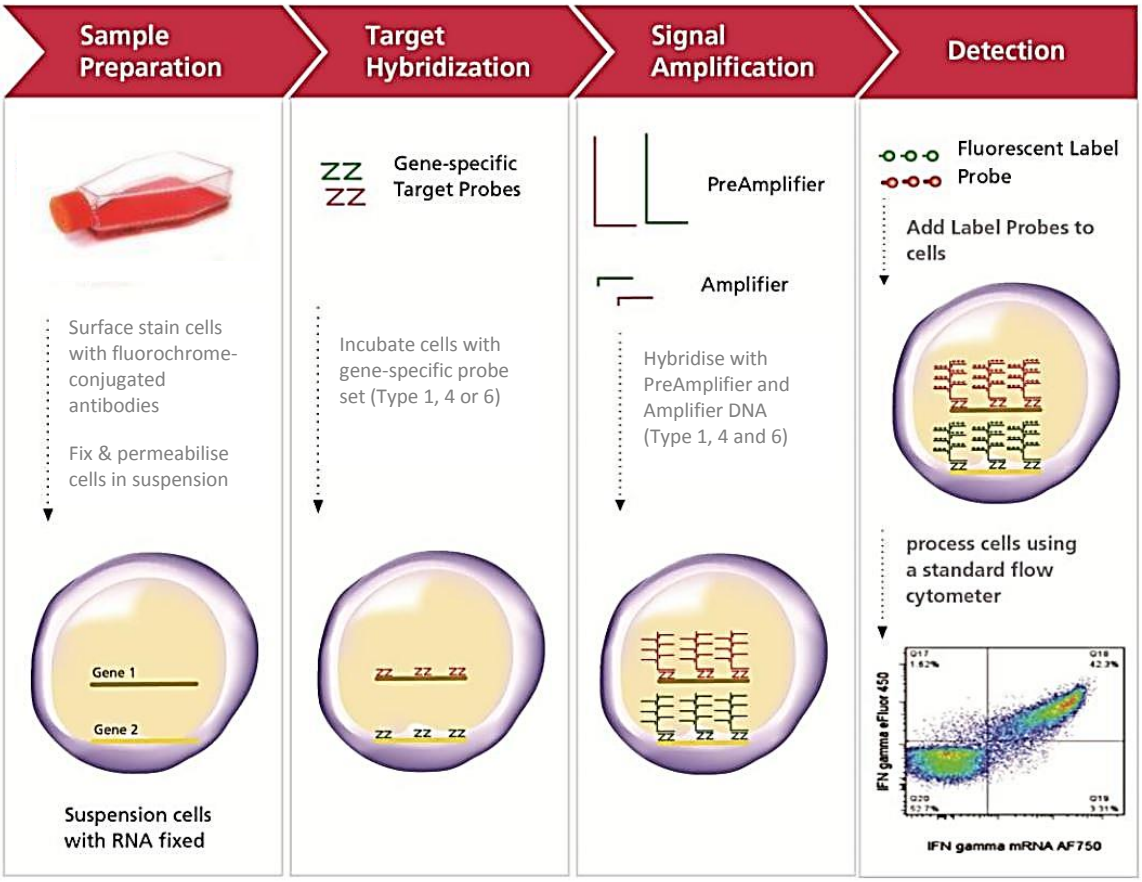


Figure 2.12: Detection of two RNA targets (brown and yellow, denoted as gene 1 and gene 2) using the PrimeFlow™ RNA assay, with only three of the 20-40 oligonucleotide target probe pairs per target RNA shown. (Image adapted from PrimeFlow™ RNA Assay User Manual and Protocol (Affymetrix, 2000)). (Used with permission from Thermo Fisher Scientific).

2.8.1 Transfection of CD34^{pos} cells with JAK2 constructs

First CD34^{pos} cells cultured in SFEM + 1% cc100 cytokines + 100 U/ml penicillin + 0.1mg/ml streptomycin were transfected with the plasmids using the Amaxa® Nucleofection protocol described above (section *2.6.3*). An appropriate number of cells (2 x 10^6 to 4 x 10^6 cells) were transfected with 2µg of wt*JAK2*-pcDNA3 and another appropriate number of cells (2 x 10^6 to 4 x 10^6 cells) were transfected with 2µg of mut*JAK2*-pcDNA3. The positive control consisted of cells transfected with pmaxGFP. Transfections were performed in triplicate and incubated in two 6-well plates at 37^{o}C in 5% CO_2. After 48 hours, one set of transfected cells had their medium changed to another medium suitable for CD34^{pos} cells to proliferate and mature into erythroblasts (Table 2.8 shows the composition of this ESD medium). The cells were analysed microscopically every two to three days and their growth was followed-up by analysing them using CASY® cell counter. Parameters for viable cells (/ml), percentage viability (%), mean diameter (µm) and peak diameter (µm) of cells were recorded. Another set of transfected cells were used for the PrimeFlow™ RNA assay, while the other set of transfected cells were used for RNA sequencing analysis.

Table 2.8: Preparation of the ESD medium

Component	Concentration	Volume (example)
Serum-free expansion medium (SFEM)	-	40 ml
Stem-cell factor	1 : 100 (100 ng/ml)	400 µl
Dexamethasone	1 : 1000 (1 µM)	40 µl
Erythropoietin (0.5 U/µl)	2 U/ml	160 µl
Lipid-mixture 1	1 : 250	160 µl
Penicillin/streptomycin	1 : 100	400 µl

2.8.2 The PrimeFlow™ RNA Assay

The assay workflow contains several steps: surface antibody staining, fixation and permeabilisation, followed by target probe hybridisation with RNA-specific probe sets, signal amplification using bDNA constructs, and detection by flow cytometry.

Day 1: Antibody staining; Fixation and permeabilisation; Target probe hybridisation.

The PrimeFlow™ RNA wash buffer was pre-warmed to room temperature. One set of the transfected cells (containing about $1x10^6$ cells) were aliquoted in Flow Cytometry staining buffer (PBS + 5% FBS). The cells were surface-stained with fluorochrome-conjugated antibodies CD34-FITC and CD133-PE (Miltenyi-Biotec, Germany) adding another tube containing cells only and stained with FITC and PE isotypes (mouse IgG2b isotype control antibodies conjugated to FITC or PE, Miltenyi-Biotec, Germany) were used. This staining was performed as described in section *2.5.5*.

Flow Cytometry staining buffer (1ml) was added to each sample, mixed by inversion and centrifuged at 600 x g for five minutes. Then the supernatant was discarded and the cells were resuspended in the residual volume.

Fixation buffer 1 was freshly prepared by mixing equal parts of Fixation buffer 1A and Fixation buffer 1B and mixed gently by inversion. This buffer was prepared in bulk to accommodate all the samples. Fixation buffer 1 (1ml) was added to each sample, mixed by inversion and then incubated for 30 minutes at 2 to 8^{o}C.

Then the samples were centrifuged at 800 x g for five minutes, the supernatant was discarded and the cells were resuspended in the residual volume.

Permeabilisation buffer with RNase inhibitors (1x) was prepared by diluting 10x Permeabilisation buffer to 1x with RNase-free water and then adding RNase inhibitor 1 at a 1/1000 dilution and RNase inhibitor 2 at a 1/100 dilution. This was mixed gently by inversion and kept at 2 to 8^{o}C. The buffer was prepared fresh and in bulk to accommodate all the samples. Permeabilisation buffer with RNase inhibitors 1x (1ml) was added to each sample, mixed by inversion and centrifuged at 800 x g for five minutes. Then the supernatant was discarded and the cells were resuspended in the residual volume. This wash was repeated for another two times.

Fixation buffer 2 (1x) was prepared by combining 125µl of Fixation buffer 2 (8x) with 875µl of wash buffer for every sample. This buffer was prepared fresh and in bulk to accommodate all the samples. A volume of 1ml of 1x Fixation buffer 2 was added to each sample, mixed by inversion and then incubated for 60 minutes in the dark at room temperature.

After incubation, the samples were centrifuged at 800 x g for five minutes. The supernatant was aspirated leaving 100µl, and the cells were resuspended in this residual volume by vortexing gently. Wash buffer (1ml) was added to each sample, mixed by inversion and centrifuged at 800 x g for five minutes. Then the supernatant was aspirated leaving 100µl, and the cells were resuspended in this residual volume by vortexing gently. The washing step was repeated. It is critical

that the residual volume is as close to 100µl as possible. The 1.5ml tubes provided with the kit had markings to assist.

The target probe i.e. ViewRNA® probe human JAK2 Type 1 (Affymetrix, USA) and the positive control probe were thawed at room temperature, while the target probe diluent was pre-warmed to 40^{o}C. The target probe and the positive control probe were diluted 1/20 in target probe diluent and mixed thoroughly by pipetting up and down. Diluted target probe or positive control probe (100µl) was added directly into the cell suspension for the appropriate samples, vortexed briefly to mix and then incubated for two hours at 40^{o}C. The samples were mixed by inversion after one hour.

After incubation, 1ml of wash buffer was added to each sample, mixed by inversion and centrifuged at 800 x g for five minutes. Then the supernatant was aspirated leaving 100µl, and the cells were resuspended in this residual volume by vortexing gently. Wash buffer with RNase inhibitor 1 was prepared by adding RNase inhibitor 1 to wash buffer at a 1/1000 dilution and mixed gently by inversion. This buffer was prepared fresh and in bulk to accommodate all the samples. Wash buffer with RNase inhibitor 1 (1ml) was added to each sample, mixed by inversion and centrifuged at 800 x g for five minutes. Then the supernatant was aspirated leaving 100µl, and the cells were resuspended in this residual volume by vortexing gently. The samples were stored overnight in the dark at 2 to 8^{o}C.

Day 2: Signal amplification

The samples and wash buffer were pre-warmed to room temperature, while PreAmp mix, Amp mix, and label probe diluent were pre-warmed to 40°C, and label probes were thawed on ice in the dark.

PreAmp mix (100µl) was added directly into the cell suspension for each sample, vortexed briefly to mix, and then incubated for 1.5 hours at 40°C. After incubation, 1ml of wash buffer was added to each sample, mixed by inversion and centrifuged at 800 x g for five minutes. Then the supernatant was aspirated leaving 100µl, and the cells were resuspended in this residual volume by vortexing gently. This wash with wash buffer was repeated two more times, for a total of three washes.

Amp mix (100µl) was added directly into the cell suspension for each sample, vortexed briefly to mix, and then incubated for 1.5 hours at 40°C. After incubation, 1ml of wash buffer was added to each sample, mixed by inversion and centrifuged at 800 x g for five minutes. Then the supernatant was aspirated leaving 100µl, and the cells were resuspended in this residual volume by vortexing gently. This wash was repeated for one other time.

Label probes were diluted 1/100 in label probe diluent in bulk to accommodate all samples. Diluted label probes (100µl) were added directly into the cell suspension for each sample, vortexed briefly to mix, and then incubated for one hour at 40°C. After incubation, 1ml of wash buffer was added to each sample, mixed by inversion and centrifuged at 800 x g for five minutes. Then the supernatant was aspirated leaving 100µl, and the cells were resuspended in this residual volume

by vortexing gently. This wash was repeated for one other time. Finally, 1ml of storage buffer was added to each sample and then the samples were transferred to 12x75 mm polystyrene tubes to be analysed on the flow cytometer.

Transfection of $CD34^{pos}$ cells with the *JAK2* constructs and follow-up growth analysis was performed twice, while analysis by PrimeFlow™ RNA assay was performed once.

2.9 RNA sequencing of JAK2^{pos} CD34^{pos} cells

2.9.1 Preparation of RNA

Cell cultures that were transfected with the constructs wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3 and whose Jak2 expression was confirmed with the FISH assay, were prepared for RNA sequencing. The cell cultures were centrifuged at 300 x g for five minutes. The supernatant was aspirated and 350µl of QIAzol lysis reagent (QIAGEN, Germany) was added to each pellet. This was mixed well by pipetting up and down for 25 times and then the cells were stored at -80^{o}C until the RNA extraction procedure. The QIAzol lysis reagent is a phenol/guanidine-based reagent that lyses the cells efficiently and provides higher yields of total RNA. The organic extraction step removes both proteins and DNA, improving the efficiency of further purification steps (QIAGEN, 2009).

2.9.2 Extraction of RNA

RNA was extracted from cell lysates prepared as described in *2.9.1*. using an in-house method combined with a well-established RNA purification technology using the RNeasy Mini Kit by QIAGEN (Germany). Before starting the procedure, all the required items and the benchtop were wiped with alcohol-wipes and RNaseZap™ (Sigma-Aldrich, USA) in order to eliminate any possible RNase contamination. The cell lysates were thawed on ice and then 140µl chloroform (Sigma-Aldrich, USA) was added and mixed well by pulse vortexing. The tubes were placed on the benchtop at room temperature for two minutes. Then they were centrifuged at 12000 x g at 4^{o}C for 15 minutes. After centrifugation, the centrifuge was heated up to room temperature (15 to 25^{o}C). After addition of chloroform, the homogenate is separated into aqueous and organic phases by

centrifugation. RNA partitions to the upper, aqueous phase while DNA partitions to the interphase and proteins to the lower, organic phase.

The upper aqueous phase was transferred to a new collection tube and placed on ice. Ethanol 100% (Sigma-Aldrich, USA) was added at a 1 : 1.5 volume and mixed thoroughly by pipetting up and down several times. By adding ethanol, RNA is precipitated from the aqueous phase.

Up to 700µl of the sample, including any precipitate that may have formed, was pipetted into an RNeasy Mini spin column in a 2ml collection tube. The lid was closed gently and the tube was centrifuged at 13000 x g for 15 seconds. The flow-through was discarded.

Then 700µl of Buffer RW1 was added to the RNeasy spin column, the lid was closed gently and the tube was centrifuged at 13000 x g for 15 seconds. The flow-through was discarded. Buffer RW1 contains a guanidine salt, as well as ethanol, and is used as a stringent washing buffer that efficiently removes biomolecules that are non-specifically bound to the silica membrane. At the same time, RNA molecules larger than 200 bases remain bound to the column.

Buffer RPE (500µl) was added to the RNeasy spin column, the lid was closed gently and the tube was centrifuged at 13000 x g for 15 seconds. The flow-through was discarded. Buffer RPE is a mild washing buffer containing ethanol. It removes traces of salts, which are still on the column due to buffers used earlier in the protocol.

Another 500µl of Buffer RPE was added to the RNeasy spin column, the lid was closed gently and the tube was centrifuged at 13000 x g for two minutes. The flow-through was discarded. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution as the residual ethanol may interfere with downstream reactions.

The RNeasy Mini spin column was placed into a new 2ml collection tube and centrifuged at full speed for one minute. The collection tube containing the flow-through was discarded. This step was performed to eliminate any possible carryover of Buffer RPE.

Then the RNeasy Mini spin column was transferred to a new 1.5ml collection tube and 20µl of RNase-free water was added directly onto the RNeasy Mini spin column membrane. The lid was closed gently and the tube was centrifuged at 13000 x g for one minute to elute the RNA. The RNeasy Mini spin column was removed and discarded. The collection tube was closed and kept on ice.

Finally, the RNA concentration was measured using the NanoDrop as described in section *2.2.6.* The ratio of absorbance at 260nm and 280nm ($A_{260/280}$) and the ratio of absorbance at 260nm and 230nm ($A_{260/230}$) were used to assess the purity of RNA. An $A_{260/280}$ ratio of around 2.0 and an $A_{260/230}$ ratio between 1.8 and 2.2 are generally accepted as “pure” for RNA. The extracted RNA was stored at -80^{o}C until further use.

2.9.3 Complementary DNA synthesis

RNA was transcribed to complementary DNA (cDNA) using the QuantiTect Reverse Transcription Kit by QIAGEN (Germany). The kit provides a fast and convenient procedure for efficient reverse transcription and effective genomic DNA (gDNA) elimination.

The RNA samples were thawed on ice, while the kit contents gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix were allowed to thaw at room temperature (15 to 25^{o}C). Each solution was mixed by flicking the tube, centrifuged briefly to collect residual liquid from the sides of the tube and then stored on ice.

The genomic DNA elimination reaction was prepared on ice by adding 2µl 7x gDNA Wipeout Buffer to 12µl RNA sample to get a total volume of 14µl. The reactions were incubated for two minutes at 42^{o}C and then placed immediately on ice.

The reverse-transcription mix was prepared on ice by adding 1µl Quantiscript Reverse Transcriptase, 4µl 5x Quantiscript RT Buffer, and 1µl RT Primer Mix to the entire genomic DNA elimination reaction. The reactions were incubated for 30 minutes at 42^{o}C and then for a further three minutes at 95^{o}C to inactivate Quantiscript Reverse Transcriptase. Finally, the reactions were stored at -80^{o}C and then sent to BGI Genomics (Hong Kong) for RNA sequencing.

2.9.4 RNA sequencing method

RNA sequencing was performed once, on $CD34^{pos}$ cells transfected with wt*JAK2*-pcDNA3 construct and $CD34^{pos}$ cells transfected with mut*JAK2*-pcDNA3 construct. This method was performed at BGI Genomics in Hong Kong. The original image data produced by the sequencer was transferred into sequences by base calling, resulting in the raw reads (raw data file) which were saved as ".bam" files. Before further analysis, low quality reads and adaptor sequences were identified and flagged. This data filtering was performed as a pre-processing step to obtain "clean read" for further analysis. Data filtering includes:

1. removal of adaptor sequences,
2. removal of reads when length is less than the set threshold,
3. calculate average quality of reads of 20 bases from 3' end until the average quality is larger than 9. The bases that negatively impact on the quality in the 3' end are removed.

The distribution of read lengths were plotted and in both samples sequenced, the majority of the reads' length was between 100bp and 150bp. The sequencing saturation analysis indicates that the number of detected genes is proportional to the increased number of reads and if the relationship flattens, detection of genes saturates. During the RNA sequencing experiment, mRNA is broken into short segments by chemical methods and then sequenced. The preference of reads to specific gene regions will affect subsequent bioinformatics analysis. The distribution of reads on the reference genes was used to evaluate the randomness of reads. Since the reference genes span different lengths, the reads position on a gene is standardised to a relative position, providing a ratio between reads on genes with different lengths. If the randomness is good, the reads on

every position would be evenly distributed, irrespective of the length of the transcript.

2.9.4.1 Quantification of gene expression

The expression level of a gene is determined by the number of reads uniquely mapped to the specific gene and the total number of uniquely mapped reads in the sample. The percentage of a gene covered by reads is called the gene coverage. This value is determined as the ratio of the base number in a gene covered by unique mapping reads to the total bases number of that gene.

The gene expression level is calculated by using the RPKM (reads per kb per million reads) method (Mortazavi *et al*., 2008). This metric attempts to normalise sequencing depth and gene length as follows:

1. The total reads in a sample is divided by 1,000,000 (per million scaling factor).
2. To normalise for sequencing depth, read counts are divided by the “per million” scaling factor. This gives the reads per-million (RPM).
3. The RPKM is calculated by dividing the RPM values by the length of the gene, in kilobases.

The following formula is used:

$$RPKM = \frac{10^6 C}{NL/10^3}$$

Where C is the number of reads that uniquely align with gene A, N is total number of reads that uniquely align with all genes, and L is the number of bases of gene A. The RPMK values can be directly used to compare the difference of gene

expression among samples. If there is more than one transcript for a gene, the longest one is used to calculate its expression level and coverage.

2.9.5 Identifying differentially expressed genes

Once a list of expressed genes was obtained for the two samples, the analysis of the differential expression of mut*JAK2*- versus wt*JAK2*- transfected cells was performed automatically using the programme AltAnalyze, an open-source software. It includes the screening of genes that are differentially expressed among the two samples analysed. AltAnalyze takes pre-aligned exon-junctions and/or exon coordinates and read counts for gene and alternative isoform-level analyses (junction and/or exon). This process is relatively easy, because after downloading and extracting the programme, only one set of basic files is required. A directory of RNA sequencing counts (junction and/or exon) from their alignment analysis results (.bam files produced as described in section *2.9.4*) was loaded into the programme. To analyse gene expression data, several basic expression statistics were calculated. These statistics were performed to compare mut*JAK2*- versus wt*JAK2*- transfected cells. Expression values were reported as non-log values for RNA sequencing analyses and statistics comprised of the following (AltAnalyze, 2017):

1. rawp: a one-way analysis of variance (ANOVA) p-value calculated for the pairwise comparison,
2. adjp: the Benjamini-Hochberg adjusted value of the rawp,
3. fold change: fold change is the non-$\log_2$ transformed fold value.

These statistics are intended for further data filtering and prioritization in order to assess putative transcription differences between genes. RPKM normalisation is

used for differential gene expression analysis and therefore gene expression is based on the RPKM of all expressed exons or junctions in the samples, taking into account the total read counts for expressed exons, their sequence length and the total number of reads per sample. Filtering thresholds for exons and junctions are separate variables, since the likelihood of obtaining a junction read count are typically lower than an exon read count, due to smaller sequence regions and algorithms available to identify both. To calculate a fold-change, the final gene-level counts are incremented by one, prior to calculating the RPKM. The total reads per gene is also given in order to filter for low versus high absolute expression (AltAnalyze, 2017).

There are five primary gene expression summary result files produced by AltAnalyze. These files contain raw data, summary statistics and/or comprehensive annotations (AltAnalyze, 2017):

1. DATASET file comprising of gene annotations, comparison and ANOVA statistics, raw expression values and counts,
2. GenMAPP file consisting of comparison statistics,
3. Summary statistics file that gives overview statistics for protein and non-coding genes, up- and down-regulated counts and microRNA binding site statistics,
4. Clustering input file that contains all differentially expressed genes based on user comparisons,
5. GO-Elite input files that are composed of lists of differentially expressed genes to be used as input for pathway over-representation.

2.9.6 Network analysis

After running AltAnalyze to identify differentially expressed genes, the resulting networks of genes were analysed using a gene expression pattern software called Ingenuity Pathway Analysis (IPA). IPA is a web-based software application that is used to identify the biological mechanisms, pathways, and functions most relevant to the experimental data sets or genes of interest. IPA is used to identify related proteins within a pathway or building pathway *de novo* from the proteins of interest which is helpful when studying differential expression of genes. Data analysis and interpretation with IPA builds on the comprehensive, manually curated content of the Ingenuity Knowledge Base against which the expression analysis data is evaluated. The Ingenuity Knowledge Base consists of a structured repository of biological and chemical findings curated from various sources including the literature. Literature findings are individually and manually selected, organised and modelled ontologically by trained curators, and include the direction of the effect from the upstream to downstream molecule in a molecular relationship when it is stated in the paper. The Ingenuity Knowledge Base currently contains approximately five million individual findings, most of which describe relationships between molecules or between molecules and diseases or biological functions. IPA is developed, maintained and updated continuously (Krämer *et al.,* 2014).

Using powerful algorithms combined with the literature, advanced analysis is performed to identify the most significant pathways and discover potential novel regulatory networks and causal relationships associated with the experimental data. The pathways depict artificial simplified models of a process within a cell or tissue. By analysing pathways, data from RNA sequencing is interpreted and this

allows finding distinct cell processes, diseases or signalling pathways that are statistically associated with selection of differentially expressed genes between the two samples (Garcia-Campos, Espinal-Enriquez and Hernandez-Lemus, 2015).

Before pathway analysis can be done, data from RNA sequencing is normalised and genes are ranked by differential expression with the help of ANOVA through AltAnalyze software. This data is loaded in IPA software and a report is produced that gives information on the differences between mut*JAK2*- and wt*JAK2*-transfected cells according to the p-value. Data such as the top canonical pathways, top upstream regulators and top networks are obtained.

2.10 Differentiation of erythroblasts

$CD34^{pos}$ cells isolated from human buffy coats were cultured in SFEM supplemented with cc100 cytokines to support their proliferation. After 16 days, the cells were then cultured in ESD medium (Table 2.8) to promote differentiation into erythroid cells. The cells were analysed microscopically every two to three days and maintained at a density of about 1.5 x 10^6 cells / ml. Their growth was measured using the CASY® cell analyser and data for number of viable cells, percentage viability, mean diameter and peak diameter were recorded.

When an adequate amount of erythroblasts were obtained, 2.1 x 10^6 cells were resuspended in 3ml fresh ESD medium to promote proliferation, while another 2.1 x 10^6 cells were resuspended in 3ml SFEM medium containing 5 U/ml erythropoietin, 0.5mg/ml holo-Transferrin (Sigma-Aldrich, USA), 100 U/ml penicillin and 0.1mg/ml streptomycin to promote differentiation of erythroblasts into reticulocytes and erythrocytes. Both cultures were analysed microscopically every two to three days, their growth was measured using the CASY® cell analyser and data for number of viable cells, percentage viability, mean diameter and peak diameter were recorded.

On day 2 and day 7 proliferating and differentiating cells were stained with anti-human CD71 (Transferrin Receptor) PE-Cyanine7 and anti-human CD235a (Glycophorin A) FITC (eBioscience, Affymetrix, USA). Isotype control was prepared for FITC only as no PE-Cy7 isotype control was available. This staining was performed as described in section *2.5.5*, with the exception that incubation with the antibodies was done in the dark at room temperature for 20 minutes.

Flow cytometry was performed on the FACS Canto II using FACS Diva Software (Becton Dickinson Biosciences, USA).

CHAPTER 3

RESULTS:

HUMAN *JAK2* CONSTRUCTS

3.1 Overview

The human *JAK2* coding sequence was amplified by PCR using forward and reverse primers designed by Geneious software having 15 bp extensions homologous to the ends of the pIRES2 vector. Then the *JAK2* ORF was cloned using In-Fusion® HD Cloning kit (Clontech, USA). During this cloning procedure the coding sequence was inserted into a linearised pIRES2 vector by means of the In-Fusion® Enzyme that fuses the PCR-generated sequence and linearised vector efficiently and precisely by recognising the base pair overlap at their ends. Then the *JAK2*-pIRES2 construct was transformed into competent cells and grown on selective medium. The plasmid DNA was extracted and purified from the colonies and screened for the presence of *JAK2* by PCR.

The DNA derived from colonies that included the target insert and had an optimum concentration were prepared for full sequencing. Some nucleotide changes were observed, so high-fidelity PCR was performed in order to obtain DNA with a high degree of accuracy during replication; however, this was unsuccessful. Due to these mutations and unsuccessful high-fidelity PCR, another two constructs were obtained, namely wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3 constructs.

Transfection experiments and Western Blot experiments were performed to assess the expression of the Jak2 protein by the constructs. These experiments showed that the *JAK2*-IRES-GFP transcript was successfully produced as the GFP protein was expressed by the transfected cells, i.e. fluorescence was observed in transfected cells. Expression of Jak2 by cells transfected with

wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3 constructs could not be evaluated in the same manner since the constructs do not include a GFP gene and hence the cells do not fluoresce.

Cell lysates of transfected cells were prepared for Western Blot analysis. Some experiments confirmed the presence of Jak2 through the production of faint bands at 125kDa. However, due to the fact that a lot of cells are required for Western Blotting and that probing Jak2 with an antibody was not satisfactorily visualised, it was decided that once $CD34^{pos}$ cells are transfected with the *JAK2* constructs, Jak2 expression is detected by an *in situ* hybridisation assay (Chapter 5) and not with Western Blot.

3.2 Amplification of the *JAK2* coding sequence

3.2.1 Polymerase chain reaction to amplify JAK2

Several PCRs were carried out to establish the optimum conditions for different sets of primers in order to amplify the *JAK2* coding sequence. Table 3.1 shows a summary of the experiments including the optimum annealing temperature of various primer pairs, while Figure 3.1 shows representative agarose gel electrophoresis results.

The full length coding sequence of *JAK2* was successfully amplified using various primer combinations. Hence optimisation of the *JAK2* fragments using the Jak2 F (*Eco*RI@AUG) & Jak2 R (Frag 1 1215-1185) primer set and the Jak2 F (Frag 3 2400-2430) & Jak2 R (*Sal*I@TCA) primer set, was not pursued further. The optimal primers to amplify the *JAK2* coding sequence were those that were designed to incorporate the *Eco*RI and *Sal*I restriction sites, with or without the c-*myc* tag at the N- or C-terminus.

Table 3.1: Polymerase chain reaction of *JAK2* using different primer sets. The optimal annealing temperature and the length of the product produced are given for each primer pair.

Forward primer	Reverse primer	Optimal annealing temperature	Band produced
Jak2 F (*Eco*RI@AUG)	Jak2 R (Frag 1 1215-1185)	N/A	No product obtained
Jak2 F (Frag 2 1200-1230)	Jak2 R (Frag 2 2415-2385)	56.8 ^{o}C	1200 bp
Jak2 F (Frag 3 2400-2430)	Jak2 R (*Sal*I@TCA)	N/A	No product obtained
Jak2 F (*Nhe*I@AUG)	Jak2 R (*Sal*I@TCA)	55 – 61^{o}C all temperatures	3399 bp
Jak2 F (*Nhe*I@AUG) + tag	Jak2 R (*Sal*I@TCA)	56.0^{o}C	3429 bp
Jak2 F (*Nhe*I@AUG)	Jak2 R (*Sal*I@TCA) + tag	N/A	No product obtained
Jak2 F (*Eco*RI@AUG)	Jak2 R (*Sal*I@TCA)	55 – 61^{o}C all temperatures	3399 bp
Jak2 F (*Eco*RI@AUG) + tag	Jak2 R (*Sal*I@TCA)	56.0^{o}C	3429 bp
Jak2 F (*Eco*RI@AUG)	Jak2 R (*Sal*I@TCA) + tag	56.0^{o}C	3429 bp

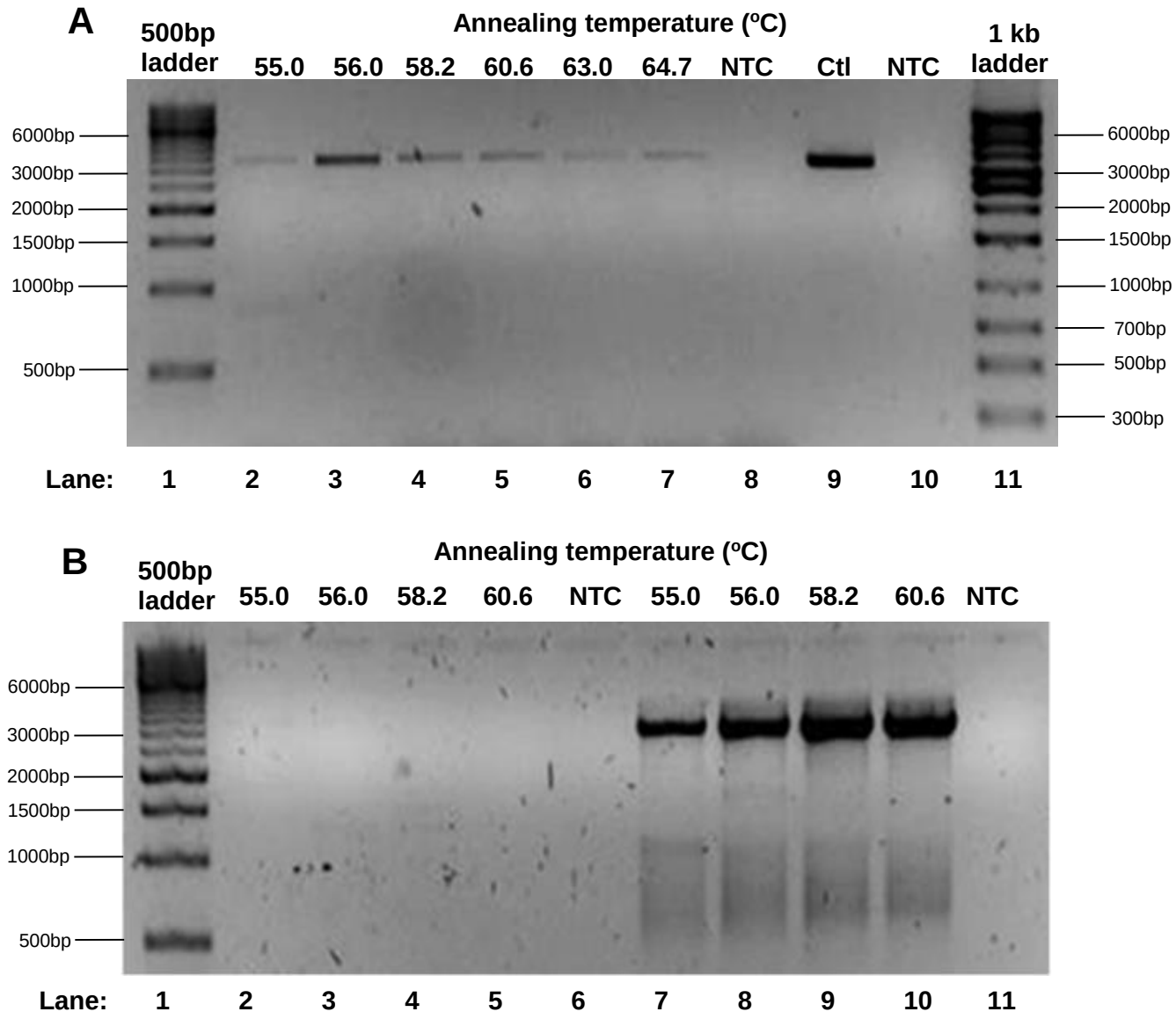


Figure 3.1: Agarose gel electrophoresis showing PCR product of *JAK2* coding sequence. Agarose gel electrophoresis after gradient PCR to produce *JAK2* coding sequence. **A:** Gel electrophoresis showing results for a gradient PCR using forward primer Jak2 F (*Eco*RI@AUG) + tag and reverse primer Jak2 R (*Sal*I@TCA). Products of 3429 bp corresponding to the *JAK2* coding sequence including the c-*myc* tag were obtained. The results show that the optimum annealing temperature for the enzymes is 56.0°C. **B:** Gel electrophoresis showing results for a gradient PCR using forward primer Jak2 F (*Nhe*I@AUG) and reverse primer Jak2 R (*Sal*I@TCA) + tag (lane 2-6). No products were obtained with this primer set. Using forward primer Jak2 F (*Eco*RI@AUG) and reverse primer Jak2 R (*Sal*I@TCA) (lane 7-11), bands at 3399 bp corresponding to the *JAK2* coding sequence were obtained at all annealing temperatures. [500 bp ladder: O'RangeRuler, Thermo Scientific USA; 1 kb ladder: Solis BioDyne, Medibena Austria; NTC: no template control; Ctl: positive control.]

3.2.2 Restriction digest of the pIRES2 vector

To clone the *JAK2* gene using In-Fusion® HD Cloning kit (Clontech, USA), the coding sequence was inserted into a linearised pIRES2 vector. The vector was linearised using *Eco*RI and *Sal*I enzymes, and simultaneously pDBLeu vector was similarly digested in another reaction as a control to verify the digest process. After the process, a small volume of the digestion mixture was electrophoresed and viewed under the UV transilluminator. Due to the position of the restriction sites of the enzymes, the pIRES2 vector produced a band at 5300 bp since *Eco*R1 cuts at position 629 and *Sal*1 cuts at position 639, while pDBLeu vector produced a fragment of 4580 bp and a lower band representing 2747 bp and 2576 bp given that *Eco*R1 cuts at positions 3460 and 8783 and *Sal*1 cuts at position 6207 (Figure 3.2). Once the digestion was successful, purified *JAK2* gene was ligated with linearised purified pIRES2 vector to obtain the *JAK2*-pIRES2 construct.

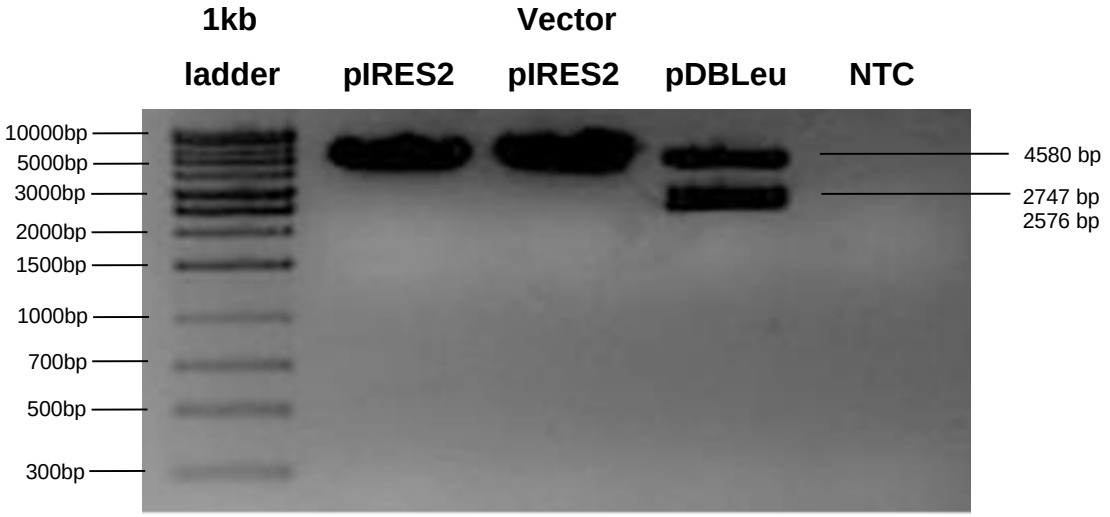


Figure 3.2: Agarose gel electrophoresis after double digest of vectors. pIRES2 produced a band at 5.3kb; pDBLeu produced bands at 4.6kb and 2.7kb/2.6kb. [1 kb ladder: Solis BioDyne, Medibena Austria; NTC: no template control.]

3.2.3 Cloning of the JAK2 construct

The *JAK2*-pIRES2 construct was transformed into competent cells. A number of colonies were picked from each plate and grown in LB broth overnight and then plasmid DNA was purified. Subsequently, a PCR using Platinum® Taq DNA polymerase was performed to establish which plasmids contain the *JAK2* ORF. Since forward primer Jak2 F (Frag 2 1200-1230) and reverse primer Jak2 R (Frag 2 2415-2385) were used, a band at 1200 bp confirmed the presence of the *JAK2* ORF (Figure 3.3).

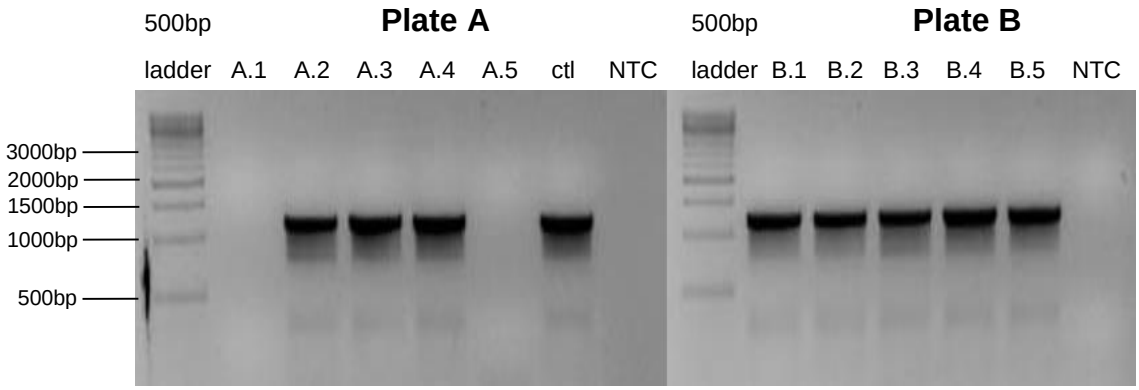


Figure 3.3: Agarose gel electrophoresis of PCR of Minipreps. Plate A: these colonies were transformed with *JAK2* amplified by forward primer Jak2 F (*Eco*RI@AUG) + tag & reverse primer Jak2 R (*Sal*I@TCA). **Plate B:** these colonies were transformed with *JAK2* amplified by forward primer Jak2 F (*Eco*RI@AUG) & reverse primer Jak2 R (*Sal*I@TCA) + tag. Colonies A.2, A.3, A.4, B.1, B.2, B.3, B.4, B.5 produced a band at 1200bp signifying the presence of *JAK2*. [500 bp ladder: O'RangeRuler, Thermo Scientific USA; NTC: no template control; ctl: positive control.]

3.2.4 Assessment of DNA quantity and purity

DNA quantity was measured using NanoDrop 2000 spectrophotometer. Table 3.2 shows an example of DNA quantification of purified DNA from various plasmids. The DNA concentration was measured twice for each plasmid and an average of the readings was then worked out to get the DNA concentration in ng/µl. Apart from the DNA quantity, the ratio of absorbance at 260nm and 280nm ($A_{260/280}$) and the ratio of absorbance at 260nm and 230nm ($A_{260/230}$) gave an indication on the purity of the DNA. The $A_{260/280}$ ranged from 1.89 to 1.95 and the $A_{260/230}$ was in the range of 1.90 to 2.23, indicating the absence of contamination, and hence suggesting that the DNA was pure and could be used for other applications.

Table 3.2: DNA concentration and purity of the plasmids. The DNA concentration and the purity of each plasmid were measured twice. DNA concentration is given in ng/µl; $A_{260/280}$ and $A_{260/230}$ indicate the purity of the DNA.

Sample ID	DNA conc	Unit	$A_{260/280}$	$A_{260/230}$
H_2O	0.6	ng/µl	0.78	0.20
A.1	210.8	ng/µl	1.90	2.23
A.1	208.5	ng/µl	1.90	2.23
A.2	197.5	ng/µl	1.91	2.22
A.2	194.3	ng/µl	1.90	2.21
A.3	135.8	ng/µl	1.91	2.16
A.3	135.7	ng/µl	1.89	2.12
A.4	147.5	ng/µl	1.90	2.19
A.4	148.4	ng/µl	1.90	2.16
A.5	166.2	ng/µl	1.90	2.14
A.5	167.2	ng/µl	1.89	2.13
B.1	145.9	ng/µl	1.90	2.21
B.1	145.9	ng/µl	1.90	2.21
B.2	135.6	ng/µl	1.89	2.16
B.2	135.9	ng/µl	1.89	2.15
B.3	93.0	ng/µl	1.94	2.08
B.3	92.7	ng/µl	1.95	2.09
B.4	54.6	ng/µl	1.93	2.03
B.4	54.8	ng/µl	1.93	2.01
B.5	43.6	ng/µl	1.93	1.92
B.5	44.6	ng/µl	1.92	1.90

3.2.5 Analysis of sequencing results

The DNA (Minipreps) derived from colonies that included the target insert, produced an amplicon of 1200 bp (i.e. containing *JAK2* sequence). The DNA samples with an optimum concentration were prepared for sequencing. Each Miniprep was sequenced using a forward primer and a reverse primer. Then each sequence was aligned with the *JAK2* coding sequence and analysed using the Geneious software. The Minipreps that showed the correct orientation of the insert and had no mutations in the 5' and 3' part of the inserts (the sequenced sections), were then sequenced using nine primers to cover all the coding sequence.

Upon analysing the results, some nucleotide changes were noticed (Figure 3.4). At position 213 of the *JAK2* coding sequence a nucleotide change from uracil to cytosine and at position 882 a nucleotide change from uracil to guanine give rise to the same amino acid, i.e. alanine (residue 71) and glycine (residue 294) respectively. However, another two mutations give rise to different amino acids. At positions 577 a nucleotide change from adenine to guanine changes the resulting amino acid from asparagine to aspartate (residue 193) and at position 1436 a nucleotide change from adenine to guanine results in the production of the amino acid serine instead of asparagine (residue 479) (Table 3.3).

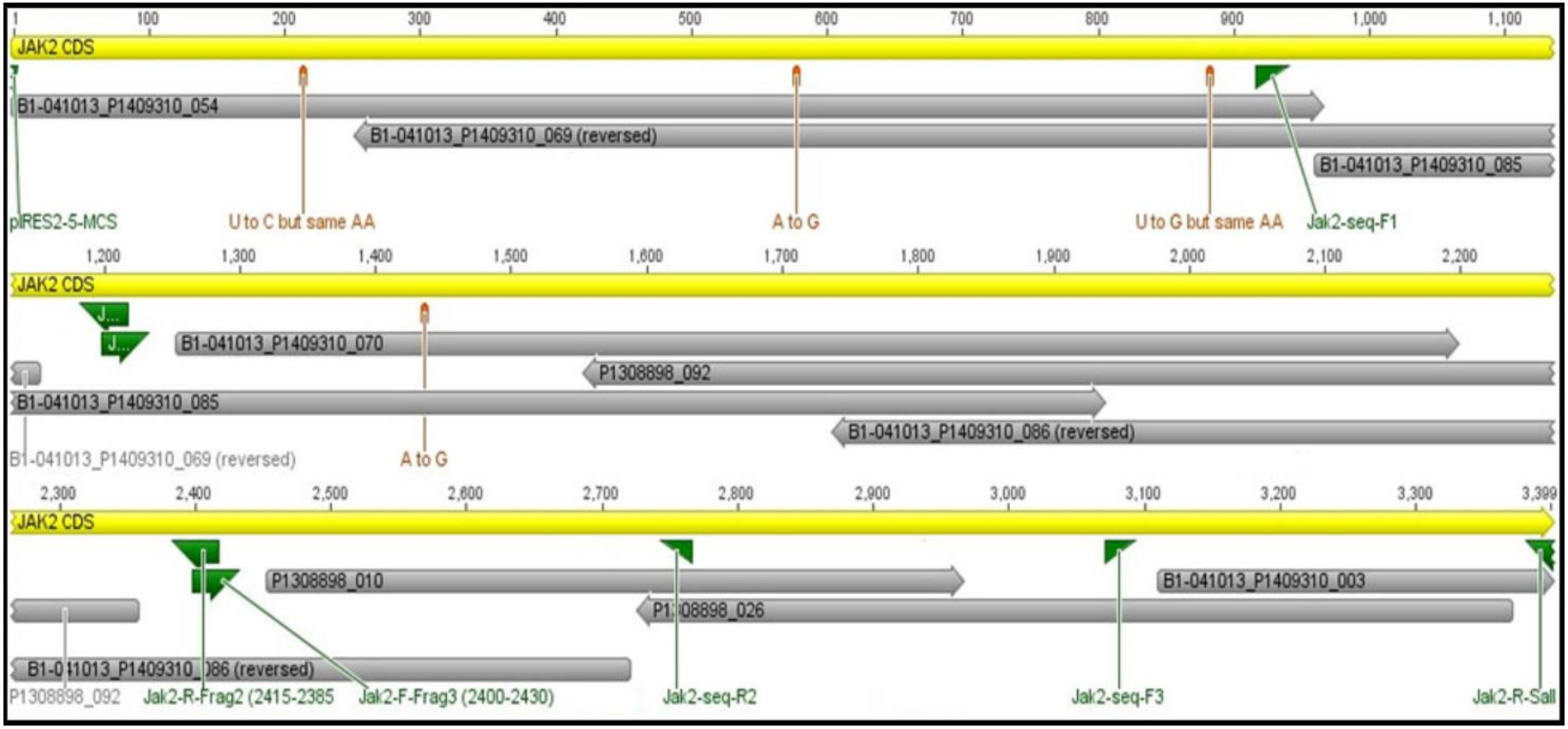


Figure 3.4: Analysis of sequencing results using Geneious software. *JAK2* coding sequence is shown in yellow; Sequencing primers are depicted in green; Sequenced area with the various primers highlighted in grey. Some nucleotide changes were observed: two of the nucleotide changes give rise to the same amino acid (at position 213 a nucleotide change from U to C and at position 882 a nucleotide change from U to G), while another two mutations give rise to different amino acids (at positions 577 and 1436 a nucleotide change from A to G). [U: uracil; C: cytosine; A: adenine; G: guanine; AA: amino acid].

Table 3.3: Sequencing results show some nucleotide changes in the *JAK2* coding sequence.

Position	Residue	Nucleotide change	Amino acid change
213	71	U to C: Uracil to Cytosine	A to A: Alanine to Alanine (same)
577	193	A to G: Adenine to Guanine	N to D: Asparagine to Aspartate
882	294	U to G: Uracil to Guanine	G to G: Glycine to Glycine (same)
1436	479	A to G: Adenine to Guanine	N to S: Asparagine to Serine

The mutation in residue 193 lies in JH6 in the FERM domain, while the mutation in residue 479 is in JH3 in the SH2 domain (UniProt, 2017). The FERM and the SH2 domains lie in the N-terminal segment of Jak2 that mediates association with the membrane-proximal region of cytokine receptors and regulate their expression (Zhao *et al*., 2010). A literature search about mutations in Jak2 did not produce published studies about these specific mutations, however studies report that other mutations in the FERM domain interfere with receptor association and disrupt the binding of Jak2 to receptors abolishing its activity (Royer *et al.,* 2005; Wernig *et al.,* 2008). It is expected that mutations of these residues result in loss of function and receptor binding because they compromise the structural integrity of the FERM and SH2 domains.

High-fidelity PCR was performed, however, no PCR products were obtained using different primer sets and different annealing temperatures. Due to these mutations and unsuccessful high-fidelity PCR, the constructs wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3 were obtained (see section *2.3.6*).

3.3 Analysis of the *JAK2* expression constructs

3.3.1 Transfection experiments

Several transfection experiments were performed to assess the expression of the Jak2 protein. Different protocols were utilised and the *JAK2* constructs were transfected in different cell lines with high transfection efficiency. A summary of these experiments is depicted in Table 3.4.

All the transfection experiments were successful as shown by the strong fluorescent results given by the pmaxGFP that was used as a positive control in every run (Figure 3.5-A,B,C). Since the vector pIRES2 has an AcGFP1 coding region which produces a green fluorescent protein, cells transfected with *JAK2*-pIRES2 exhibit fluorescence (Figure 3.5-D,E). These results show that the construct *JAK2*-pIRES2 is producing the *JAK2*-IRES-GFP transcript and the GFP protein is being expressed by the transfected cells, which indirectly shows that Jak2 is also being expressed. GFP-positive cells were visualised when *JAK2*-pIRES2 was transfected in MCF7, PC3 and GH3 cells in a 24-well plate using the FuGene transfection protocol, in MCF7 cells in a 96-well plate using the FuGene transfection protocol, and in GH3 and A549 cells in a 96-well plate using the Magnefect-nano transfection protocol (Table 3.4). Negative results (i.e. no fluorescence) could be due to lower transfection efficiency rates. Cells were also transfected with wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3, but since the vector pcDNA3 does not have a GFP, they do not display fluorescence when viewed under the microscope. Transfected cells were also prepared for Western Blot analysis in order to check if the protein Jak2 is being expressed by the cells.

Table 3.4: Optimisation of the transfection protocols. A summary of the different methods, cell lines and conditions that were used, together with the result as shown by fluorescence (where applicable).

Transfect-ion method	Plate	Construct	Cell line	DNA concentr-ation (µg)	Number of cells ($x10^6$)	Fluoresc-ence
Amaxa	6-well	*JAK2*-pIRES	K562	2.0	2.6	×
Amaxa	6-well	*JAK2*-pIRES	K562	5.0	2.2	×
Amaxa	6-well	*JAK2*-pIRES	K562	1.5	1.6	×
FuGene	96-well	*JAK2*-pIRES	MCF7	0.10	0.02	✓
FuGene	24-well	*JAK2*-pIRES	PC3	0.50	0.2	✓
FuGene	24-well	*JAK2*-pIRES	GH3	0.50	0.2	✓
FuGene	24-well	*JAK2*-pIRES	MCF7	0.50	0.2	✓
FuGene	24-well	wt*JAK2*-pcDNA3	MCF7	0.50	0.2	NA
FuGene	24-well	mut*JAK2*-pcDNA3	MCF7	0.50	0.2	NA
Magnefect	96-well	*JAK2*-pIRES	GH3	0.10	0.01	✓
Magnefect	96-well	*JAK2*-pIRES	A549	0.10	0.01	✓
Magnefect	6-well	*JAK2*-pIRES	A549	2.82	0.28	×
Magnefect	6-well	wt*JAK2*-pcDNA3	A549	2.82	0.28	NA
Magnefect	6-well	mut*JAK2*-pcDNA3	A549	2.82	0.28	NA

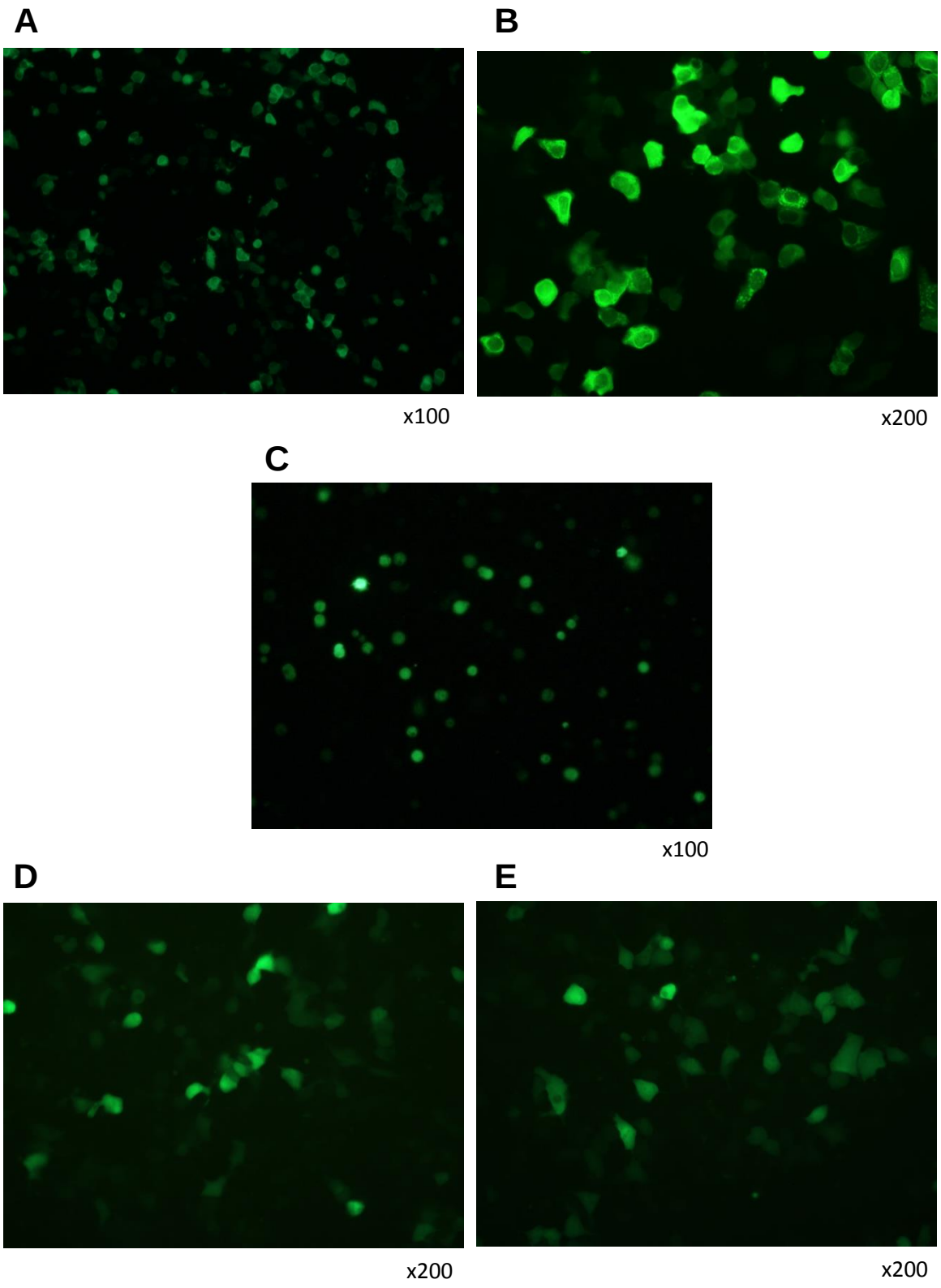


Figure 3.5: Fluorescent microscopy. Transfected cells fluoresce due to the expression of the GFP protein and are seen as green by fluorescent microscopy. **A** & **B** show pmaxGFP positive control transfected into MCF7 cells using FuGENE® HD transfection protocol. **C** shows pmaxGFP positive control transfected into $CD34^{pos}$ cells using Amaxa® Nucleofection protocol. **D & E** show *JAK2*-pIRES2 construct transfected into MCF7 cells using FuGENE® HD transfection protocol.

3.3.2 Detection of Jak2 by Western Blot

Western Blot experiments were performed to separate and identify the protein of interest, i.e. Jak2, from a mixture of proteins extracted from the cells. The optimisation of this technique is summarised in Table 3.5. Different conditions were tested, including the protein concentration loaded on the gel, the running gel concentration, and the dilutions of the antibodies. The concentration of the antibody depends on the instructions by the manufacturer, hence different dilutions of the antibodies were tested, both for Jak2 and β-actin, ranging from 1:15000 to 1:500. Different dilutions of the secondary antibodies were also tested ranging from 1:15000 to 1:5000.

Although the expression of Jak2 was visualised following transfection with *JAK2*-pIRES2, wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3 constructs in some experiments (faint bands at 125kDa were obtained), other experiments did not show such an expression. In fact, an increase in the concentration of protein loaded on the gel, did not result in optimal visualisation of the expressed protein. The use of images to show the faint Jak2 protein using Western Blots was not ideal for presentation and hence eventually another technique to enhance this result and also to show the proportion of cells overexpressing Jak2 was used (see section *5.3*).

Table 3.5: Optimisation of the Western Blot protocol. Different conditions were tested including the protein concentration loaded on the gel, the running gel concentration, and the dilutions of the antibodies.

Construct used to transfect cells	Protein concentration loaded (µg)	Running gel concentration (%)	Primary antibody dilution	Secondary antibody dilution	Bands at 125kDa
JAK2-pIRES2	15.0	12.5	1:1000	1:5000	×
JAK2-pIRES2	15.1	7.5	1:500	1:15000	✓
JAK2-pIRES2	15.6	7.5	1:500	1:15000	✓
JAK2-pIRES2	17.8	7.5	1:500	1:15000	×
JAK2-pIRES2	25.0	7.5	1:500	1:15000	×
JAK2-pIRES2	35.0	7.5	1:500	1:15000	×
wt*JAK2*-pcDNA3	11.2	7.5	1:500	1:15000	✓
wt*JAK2*-pcDNA3	27.1	7.5	1:500	1:15000	×
wt*JAK2*-pcDNA3	35.0	7.5	1:500	1:15000	×
wt*JAK2*-pcDNA3	70.0	7.5	1:500	1:15000	×
mut*JAK2*-pcDNA3	18.4	7.5	1:500	1:15000	✓
mut*JAK2*-pcDNA3	22.1	7.5	1:500	1:15000	×
mut*JAK2*-pcDNA3	35.0	7.5	1:500	1:15000	×
mut*JAK2*-pcDNA3	70.0	7.5	1:500	1:15000	×

CHAPTER 4

RESULTS:

CHARACTERISATION OF HUMAN ERYTHROID PROGENITORS CULTURED FROM PERIPHERAL BLOOD

4.1 Overview

$CD34^{pos}$ cells were harvested from human buffy coats obtained from the incomplete blood donations at the National Blood Transfusion Service (Malta) after obtaining the donors' informed consent. Harvesting was performed for 13 times: the first four harvests served for training purposes whilst also optimising the procedure; two cultures were lost due to contamination; four cultures did not expand satisfactorily and were discarded after an average of 17 days; three cultures were allowed to expand and were also used for transfection experiments, for the *in situ* hybridisation assay, and for immunophenotypic analysis. One of the latter cultures was also used for the expansion of human erythroid progenitor cells into erythroblasts and were also driven for terminal differentiation.

$CD34^{pos}$ cells harvested from Buffy coat 6, Buffy coat 8 and Buffy coat 9 were cultured in SFEM supplemented with cytokines for 17 days, 10 days, and 16 days respectively. The growth of the cells was analysed and immunophenotyping for CD34 and CD133 was performed to check the percentage of $CD34^{pos}$ cells and $CD133^{pos}$ cells obtained after harvesting. CD34 is a cell surface glycoprotein that is present on early haematopoietic stem cells. CD133 is a transmembrane glycoprotein which is also expressed in haematopoietic stem cells and is found on a subpopulation of $CD34^{pos}$ cells (Handgretinger and Kuci, 2013).

$CD34^{pos}$ cells were also cultured in ESD medium (serum-free expansion medium supplemented with Epo, SCF and Dex) in order to promote their proliferation into erythroblasts. After 16 days in SFEM medium, cells that were initially harvested from Buffy coat 9, were cultured in ESD medium. The growth of the cells was

analysed and immunophenotyping for CD71 and CD235a was performed. Expression of CD71, also known as the transferrin receptor, precedes that of CD235a, which is also known as Glycophorin A. CD71 mediates the uptake of transferrin-iron complexes and is expressed at high levels on the surface of cells of the erythroid lineage (Dong, Wilkes and Yang, 2011; Marsee, Pinkus and Yu, 2010). Its expression is highest in early erythroid precursors through the intermediate normoblast phase, after which expression decreases through the reticulocyte phase (Marsee, Pinkus and Yu, 2010; Nakahata and Okumura, 1994; Sieff *et al.,* 1982). On the other hand, CD235a expression occurs at the mid to late erythroblast stage of maturation, increases as the erythroblasts mature and is maintained through terminal red cell differentiation (Xi *et al*., 2013; Migliaccio *et al.,* 2011).

After 41 days of growing the erythroid cells in ESD medium allowing them to proliferate into erythroblasts, a differentiation experiment was performed. A culture of erythroblasts were grown in fresh ESD medium to promote proliferation and the same concentration of erythroblasts were cultured in parallel in a medium consisting of SFEM supplemented with erythropoietin and holo-Transferrin. The latter medium is intended to promote differentiation of erythroblasts into reticulocytes and erythrocytes. The growth and mean diameter of the cells were analysed. Immunophenotyping for CD71 and CD235a was performed twice, on day 2 and day 7. As the cells mature to erythrocytes, transferrin receptor expression is lost (Marsee, Pinkus and Yu, 2010; Aoun and Pirruccello, 2007). CD235a is expressed on mature erythrocytes and erythroid precursor cells and hence increases as the cells differentiate into the erythroid lineage (Xi *et al.,* 2013).

4.2 Culturing of $CD34^{pos}$ cells

4.2.1 Growth analysis

All $CD34^{pos}$ cells that were harvested from every buffy coat using the "Human whole blood / buffy coat CD34 selection kit" by EasySep (StemCell Technologies, USA) were seeded at an average concentration of 3.2 $x10^6$ /ml in SFEM supplemented with cc100 cytokines and incubated at 37°C in a 5% CO_2 humidified atmosphere. Initially, the cells were cultured in 6-well plates, but as they increased in number, they were transferred to sterile Corning® cell culture flasks having different surface areas, according to the working volume. The cultured cells were followed-up for a number of days and every two to three days they were analysed microscopically and their growth was monitored using CASY cell counter and analyser (Roche, Germany). The cells were maintained by adding fresh medium, keeping them at a concentration of about 1.5 to 2.0 $x10^6$ /ml.

Recorded parameters for the three successful harvests are shown in Tables 4.1, 4.2 and 4.3. The mean diameter data shows the mean diameter of all the viable cells (i.e. cells in the evaluation cursor size range), while the peak diameter shows the size of the viable cells that are in the majority. The cells had an average mean diameter of 11.7µm with a standard deviation of 0.6µm, while their average peak diameter was 10.2µm with a standard deviation of 0.8µm. $CD34^{pos}$ cells were cultured in supplemented SFEM medium so as to keep the cells proliferating. Hence, the size of the cells remained quite constant as the cells were not differentiating into erythroid progenitors.

Viability of the cells is expressed as a percentage of the viable cell population over the total number of cells. The percentage viability was ideally kept at more than 60%; in fact, to increase the percentage viability of cells in the culture prepared from Buffy coat 6, the culture was centrifuged at a slow speed on day 9.

The dilution factor was also calculated to obtain the cumulative number of viable cells through which the cumulative fold expansion was calculated. The cumulative number of viable cells is obtained by first determining the dilution factor by dividing the new cell culture volume by the previous volume. This is multiplied with the previous dilution factor and by the total number of viable cells. Using the cumulative number of viable cells, the fold expansion can be determined by dividing the cumulative number of viable cells between successive dates. The cumulative fold expansion on the other hand compares the cumulative number of viable cells on a particular day with day 0. Fold expansion data suggests that cells have the greatest proliferative potential between day 6 and day 9 of culture (Tables 4.1, 4.2 and 4.3).

Results show that $CD34^{pos}$ cells grew successfully in SFEM supplemented with cc100 cytokines. Figure 4.1 shows the three different buffy coat isolations. The first graph (Figure 4.1-A) represents Buffy coat 6 and shows that starting from $5.0x10^6$ cells on day 0, the cells proliferated to $9.4x10^8$ by day 17 (on day 9 the culture was centrifuged at a slow spin to increase percentage viability of cells in culture); the second graph (Figure 4.1-B) representing Buffy coat 8 shows that starting from $10.0x10^6$ cells on day 0, the cells proliferated to $1.2x10^8$ by day 10; and the third graph (Figure 4.1-C) representing Buffy coat 9 shows that starting

from $7.9x10^6$ cells on day 0, the cells proliferated to $32.3x10^8$ by day 16 (Figure 4.1; Table 4.4). Cumulative fold expansion is visually represented in Figure 4.2 and shows that while $CD34^{pos}$ cells isolated from Buffy coat 6 and Buffy coat 9 grew successfully, cells from Buffy coat 8 did not expand as efficiently and hence the culture was stopped at day 10 and the cells were used for optimisation and setting up of the immunophenotyping protocol.

Table 4.1: Parameters recorded for CD34^{pos} cells isolated from Buffy coat 6 cultured in supplemented SFEM over 17 days.

Buffy coat 6	**Day**									
Parameter	0	2	4	6	9	9*	11	13	16	17
Viable cells x10^6 (/ml)	5.0	7.2	2.1	4.5	3.8	5.2	4.7	4.3	4.4	2.9
% viable	56.4	66.2	74.7	71.5	61.2	75.2	69.6	64.0	64.1	64.7
Mean diameter (µm)	10.8	11.7	12.4	11.0	11.1	11.6	11.3	11.3	10.9	11.5
Peak diameter (µm)	9.0	7.7	11.1	9.8	10.1	10.6	10.4	10.4	10.0	10.8
Volume (ml)	1.0	1.4	4.0	4.0	9.5	4.0	8.0	11.0	12.0	18.0
Total no. of viable cells x10^6	5.0	10.1	8.4	18.0	18.1	10.4	18.8	23.8	26.6	26.4
Cumulative no. of viable cells x10^6	5.0	14.1	33.6	72.0	343.0	83.2	300.8	520.3	633.6	939.6
Fold expansion	-	2.8	2.4	2.1	4.8	-	3.6	1.7	1.2	1.5
Cumulative fold expansion	-	2.8	6.7	14.4	68.6	-	60.2	104.1	126.7	187.9

*Count and distribution of size following centrifugation.

Table 4.2: Parameters recorded for $CD34^{pos}$ cells isolated from Buffy coat 8 cultured in supplemented SFEM over 10 days.

Buffy coat 8	**Day**				
Parameter	0	2	5	8	10
Viable cells $x10^6$ (/ml)	10	2.6	3.2	2.8	1.7
% viable	77.6	54.0	80.8	88.6	86.7
Mean diameter (µm)	11.6	12.1	12.9	12.5	12.1
Peak diameter (µm)	9.5	11.3	10.8	10.2	10.4
Volume (ml)	1.0	2.0	3.0	5.5	8.5
Total no. of viable cells $x10^6$	10.0	5.2	9.6	15.4	14.4
Cumulative no. of viable cells $x10^6$	10.0	10.4	28.8	84.7	122.8
Fold expansion	-	1.0	2.8	2.9	1.4
Cumulative fold expansion	-	1.0	2.9	8.5	12.3

Table 4.3: Parameters recorded for CD34^{pos} cells isolated from Buffy coat 9 cultured in supplemented SFEM over 16 days.

Buffy coat 9	**Day**						
Parameter	0	3	6	9	11	14	16
Viable cells x10^6 (/ml)	7.9	7.3	4.5	2.3	2.6	3.6	6.1
% viable	63.5	77.9	78.3	74.7	77.2	82.4	84.8
Mean diameter (µm)	11.0	13.2	11.0	11.3	11.8	12.2	11.7
Peak diameter (µm)	9.1	11.3	9.6	10.4	10.6	10.5	10.1
Volume (ml)	1.0	1.0	4.5	14.0	18.0	23.0	9.5
Total no. of viable cells x10^6	7.9	7.3	20.3	32.2	46.8	82.8	58.0
Cumulative no. of viable cells x10^6	7.9	7.3	91.1	450.8	842.4	1904.4	3226.9
Fold expansion	-	0.9	12.5	4.9	1.9	2.3	1.7
Cumulative fold expansion	-	0.9	24.2	57.1	106.6	114.5	408.5

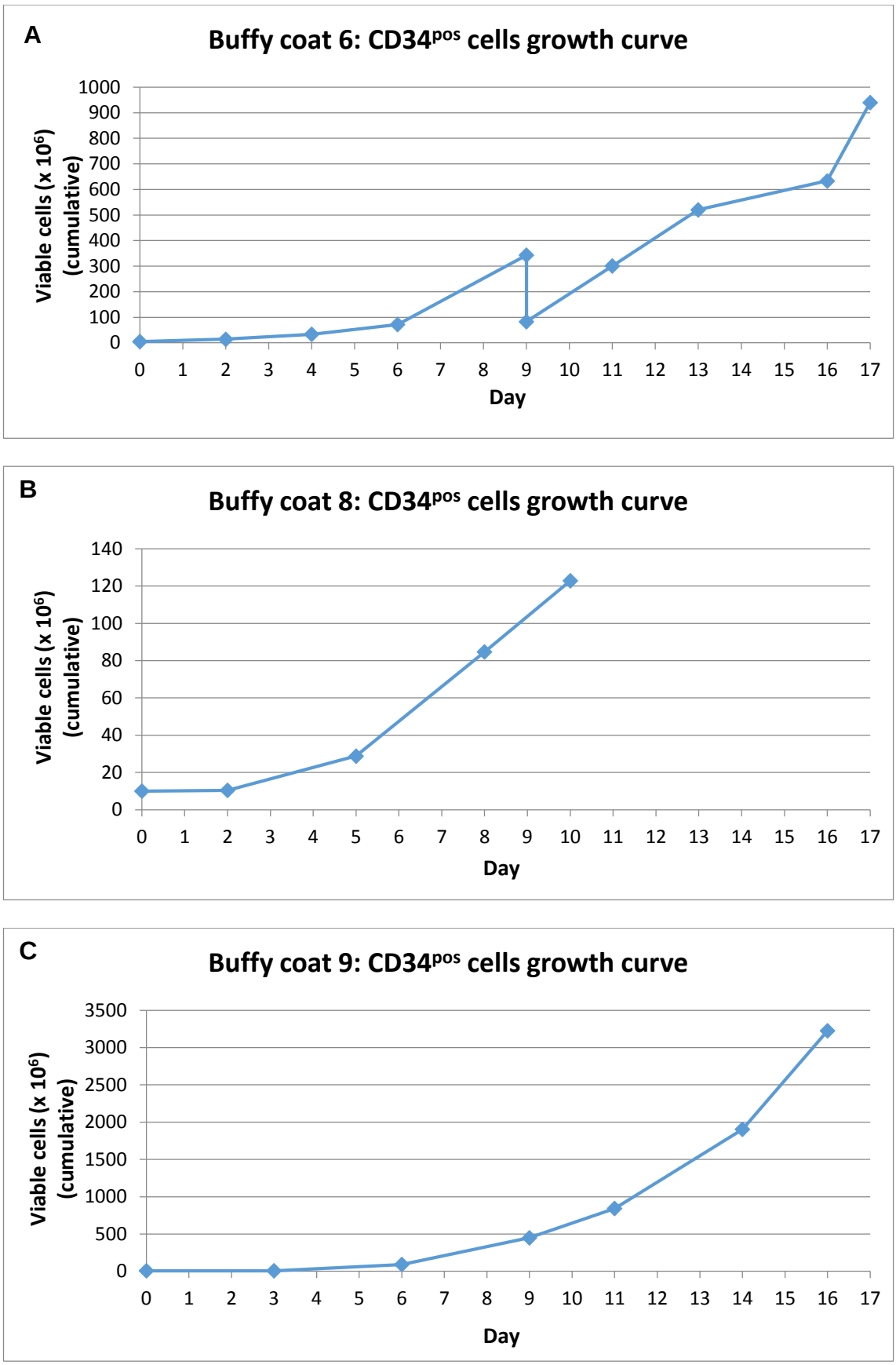


Figure 4.1: CD34^{pos} cell culture growth curves. Cumulative number of viable cells determined by CASY plotted against the day of analysis.

Table 4.4: Expansion of CD34^{pos} cells harvested from different buffy coats.

Buffy coat	Cumulative number of viable cells (x10^6)		
	Day 0	Day 9 / 10	Day 16 / 17
BC6	5.0	343.0	939.6
BC8	10.0	122.8	-
BC9	7.9	450.8	3226.9

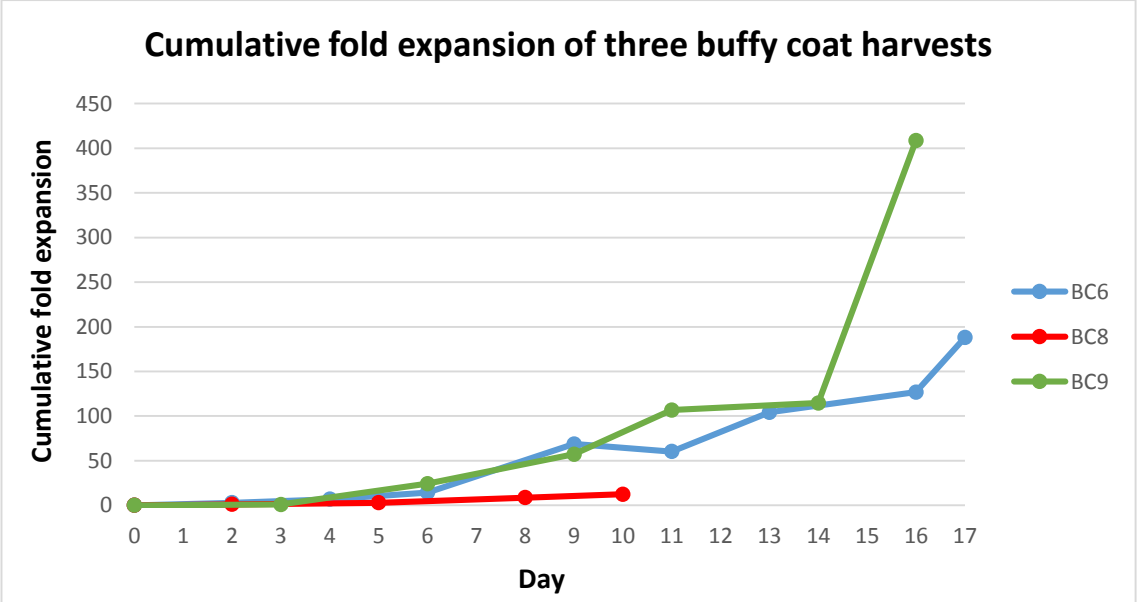


Figure 4.2: Cumulative fold expansion of CD34^{pos} cells harvested from three buffy coats against the day of analysis.

4.2.2 Immunophenotyping

The CD34^{pos} cell cultures were analysed by flow cytometry for CD34 and CD133 positivity. Flow cytometry was performed on the FACS Calibur using Cell Quest software (Becton Dickinson Biosciences, USA). Once CD34 harvesting was optimised, flow cytometry statistics demonstrate that 93% of cells were CD34^{pos}, while 68% of cells were both CD34^{pos} and CD133^{pos} (Figure 4.3).

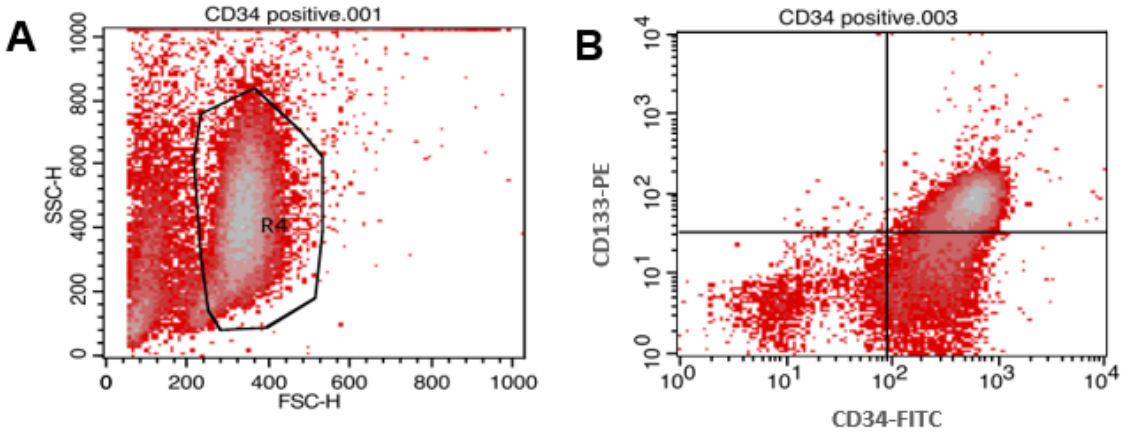


Figure 4.3: Flow cytometry analysis of CD34^{pos} cell culture. Flow cytometry analysis for the expression of antigens CD34 and CD133. Gating was set up using the unstained sample (**A**), while the quadrant was established from the isotype sample. **B** shows the gated population for the stained sample. [SSC: side scatter; FSC: forward scatter.]

4.3 Expansion of human erythroid progenitor cells

4.3.1 Growth analysis

After expanding $CD34^{pos}$ cells in SFEM supplemented with cc100, they were also cultured in ESD medium in order to promote their proliferation into erythroblasts. After 16 days in supplemented SFEM medium, 24 $x10^6$ cells that were initially harvested from Buffy coat 9, were cultured in ESD medium. The cells were incubated at 37^oC in a 5% CO_2 humidified atmosphere and they were followed-up for 41 days. The cells were maintained by adding fresh medium, keeping them at a concentration of about 1.0 to 2.0 $x10^6$ /ml. The cells were grown in different culture flasks, depending on the working volume. Every two to three days they were analysed microscopically and their growth was monitored using CASY cell counter and analyser. The CASY growth curves (Figure 4.4) provide a graphical representation of the cells by displaying the distribution of size on the x-axis versus cell count on the y-axis. The CASY program used was the one set up for $CD34^{pos}$ cells, described in section *2.5.4.1*. Recorded parameters are shown in Table 4.5. While the mean diameter of the cells remained constant (10.9 ± 0.6µm), on day 1 the peak diameter of cells was 10.0µm decreasing to 8.5µm on day 13 and 7.7µm on day 16 to day 41. The peak diameter of the cells decreased over time, indicating that when placed in ESD medium $CD34^{pos}$ cells were differentiating into erythroid progenitors (Table 4.5). This change can also be observed from the CASY graphs (Figure 4.4) where the peak of the graph shifted to the left over time indicating a decrease in the cells' diameters.

The dilution factor was calculated to obtain the cumulative cell count (Table 4.5; Figure 4.5). As expected, the $CD34^{pos}$ cells under selection of an erythroid expansion medium show a decrease in number. Since the cells were being

cultured in ESD to promote their proliferation into erythroblasts, there was a selection of cells during the first 11 days of culture. On day 11 the culture was centrifuged at a slow spin to increase percentage viability of cells in culture and to select for a pure cell culture of erythroid progenitors. At day 16, cells responsive to SCF and Epo start expansion. Between day 16 and day 31 the cells maintained a constant concentration, while from day 31 the cells grew steadily (Figure 4.5).

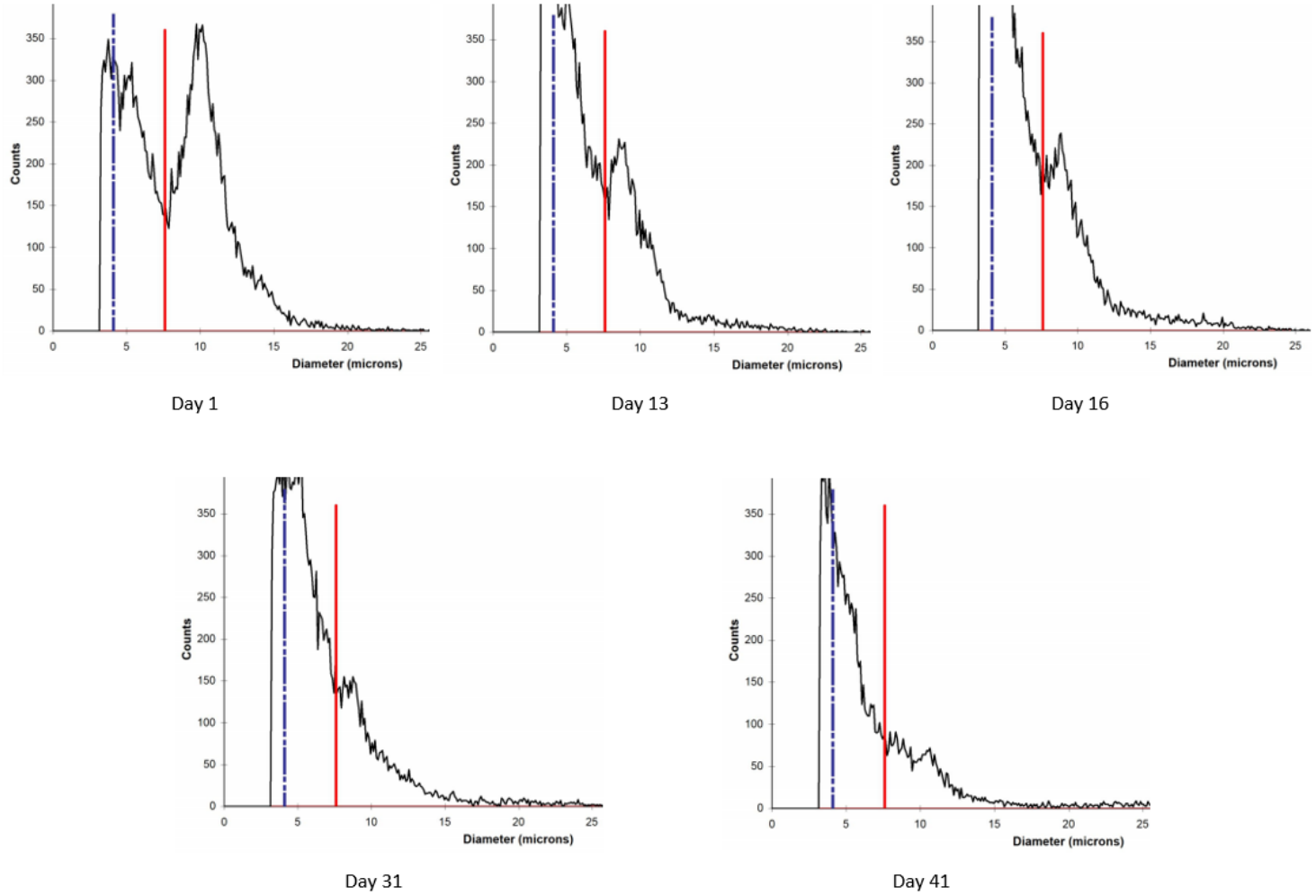


Figure 4.4: CASY growth curves of cells under ESD selection. Growth curves (obtained by CASY showing the cell count versus cell diameter in microns) of cells cultured in ESD selective medium at different time points. [Blue dotted line: left-hand normalisation cursor; Red solid line: left-hand evaluation cursor].

Table 4.5: Parameters recorded for viable cells cultured in ESD selective medium over 41 days.

	Day															
Parameter	0	1	4	7	11	11*	13	16	19	22	25	28	31	34	38	41
Viable cells $x10^6$ (/ml)	2.4	1.7	1.8	1.5	1.4	7.3	1.4	2.4	1.1	1.3	1.4	0.9	1.9	1.8	2.0	2.2
% viable	84.8	70.0	64.1	60.3	55.2	75.3	60.8	68.5	58.5	66.3	64.8	58.2	68.4	73.2	79.2	79.2
Mean diameter (µm)	11.7	10.8	10.5	10.1	10.0	10.8	10.3	10.6	10.5	10.6	11.0	10.6	10.9	11.2	12.1	12.0
Peak diameter (µm)	10.1	10.0	9.8	9.1	8.8	8.9	8.5	7.7	8.4	7.7	7.7	8.3	7.7	7.7	7.7	7.7
Volume (ml)	10.0	9.5	9.0	8.5	9.0	1.0	6.0	4.5	8.0	8.0	7.5	8.0	4.5	5.5	7.5	8.0
Total no. of viable cells $x10^6$	24.0	16.2	16.2	12.8	12.6	7.3	8.4	10.8	8.8	10.4	10.5	7.2	8.6	9.9	15.0	17.6
Cumulative no. of viable cells $x10^6$	24.0	15.3	14.6	10.8	11.3	0.7	5.0	4.9	7.0	8.3	7.9	5.8	3.8	5.4	11.3	14.1

*Count and distribution of size following centrifugation.

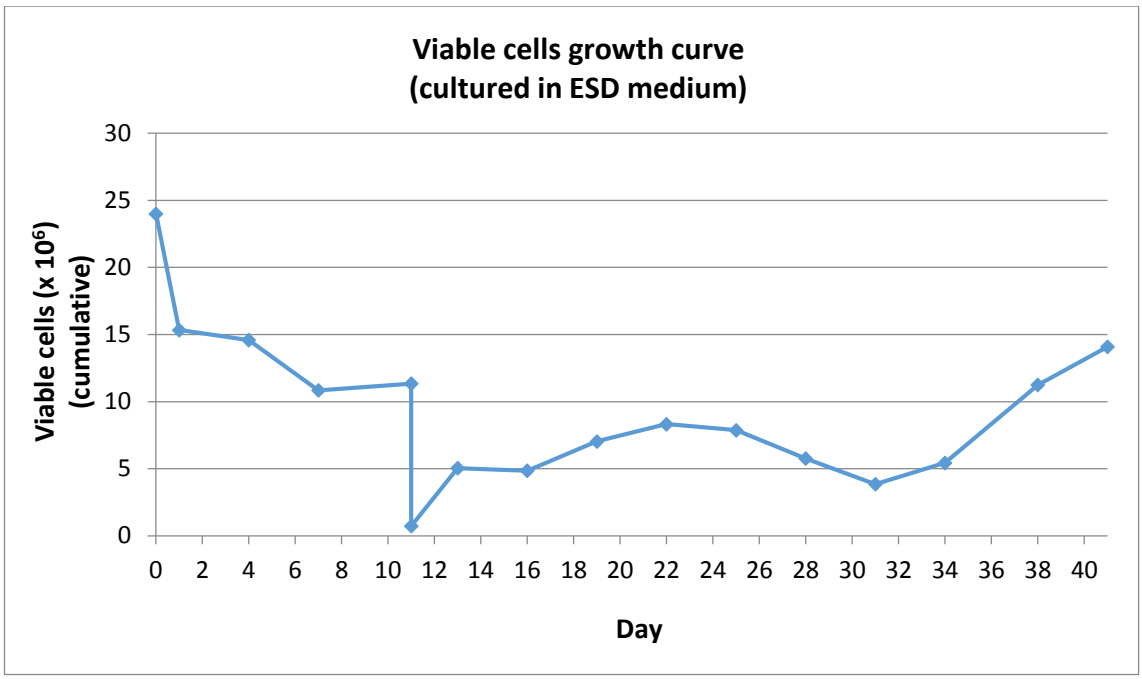


Figure 4.5: Viable cell culture growth curve. Cumulative number of viable cells, cultured in a selective ESD medium, determined by CASY plotted against the day of analysis.

4.3.2 Immunophenotyping

On day 13 the cells were analysed by flow cytometry for CD71 and CD235a positivity. Flow cytometry was performed on the FACS Canto II using FACS Diva Software (Becton Dickinson Biosciences, USA) (Figure 4.6). Flow cytometry results show that on day 13, 32.8% of cultured cells were $CD71^{pos}$ and only 6.3% were $CD235a^{pos}$ (Table 4.6). The $CD71^{pos}/CD235a^{neg}$ cells (28.1%) represent the erythroid progenitors at day 13.

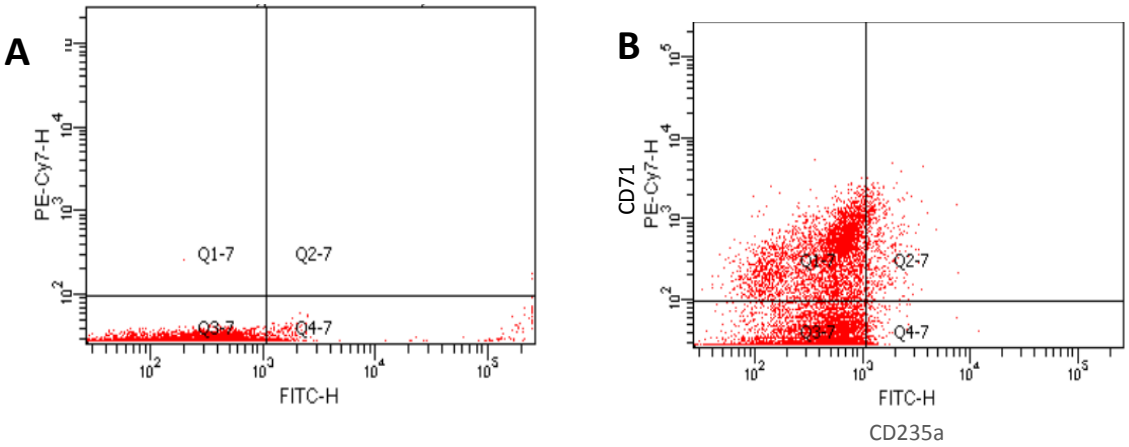


Figure 4.6: Flow cytometry analysis of cell culture growing in ESD medium. Flow cytometry analysis on day 13 for the expression of antigens CD71 and CD235a. The total cell population was gated on forward and side scatter using the unstained sample, while the quadrant was established from the isotype sample (**A**). The stained sample is shown in **B**.

Table 4.6: CD71 and CD235a percentage positivity of cells cultured in ESD selective medium on day 13.

Antigen	**Percentage Positivity (%)**
$CD71^{pos}$ / $CD235a^{neg}$	28.1
$CD71^{pos}$ / $CD235a^{pos}$	4.7
$CD71^{neg}$ / $CD235a^{pos}$	1.6

4.4 Terminal differentiation of erythroblasts

4.4.1 Growth analysis

After 41 days of growing the erythroid cells in ESD medium allowing them to proliferate into erythroblasts, a differentiation experiment was performed. A culture of 2.1 $x10^6$ erythroblasts were grown in fresh ESD medium to promote proliferation and another 2.1 $x10^6$ erythroblasts were cultured in SFEM medium supplemented with erythropoietin and holo-Transferrin in order to promote differentiation of erythroblasts into reticulocytes and erythrocytes. The two cultures were analysed microscopically every two to three days and their growth was measured using the CASY cell counter and analyser over seven days. Recorded data is shown in Table 4.7 and graphically represented in Figures 4.7 and 4.8.

Figure 4.7 illustrates the growth curve for both the proliferating cell culture and differentiating cell culture. Proliferating cells grew in the selective medium, while differentiating cells grew during the first two days, but then decreased in number. This is supported by van den Akker *et al.* (2010) who state that differentiation of erythroblasts is normally accompanied by a short burst in proliferation, followed by cell cycle arrest, a reduction in cell volume, haemoglobinisation and enucleation.

Figure 4.8 depicts the mean diameter of the cells against the day of analysis. While proliferating cells maintained a relatively constant mean diameter, the mean diameter of differentiating cells decreased. This was expected because as cells differentiate into reticulocytes, they decrease in size. While proerythroblasts

are large immature cells ranging from 12 to 20 µm in size, reticulocytes are 7 to 9 µm in size, 20% larger than mature erythrocytes (Deo, 2011).

Table 4.7: Parameters recorded for erythroid cells cultured in differentiating medium and in ESD medium over 7 days.

Differentiating cells	**Day**			
Parameter	0	2	4	7
Viable cells x10^6 (/ml)	0.7	0.9	0.5	0.6
% viable	79.2	67.9	59.9	61.3
Mean diameter (µm)	12.0	10.5	10.7	10.8
Peak diameter (µm)	7.7	7.7	8.6	8.3
Volume (ml)	3.0	3.0	3.0	3.0
Total no. of viable cells x10^6	2.1	2.6	1.6	1.7

Proliferating cells	**Day**			
Parameter	0	2	4	7
Viable cells x10^6 (/ml)	0.7	0.8	0.9	0.9
% viable	79.2	68.1	70.1	70.2
Mean diameter (µm)	12.0	11.5	11.5	11.5
Peak diameter (µm)	7.7	7.7	7.7	7.8
Volume (ml)	3.0	3.0	3.0	3.0
Total no. of viable cells x10^6	2.1	2.3	2.6	2.6

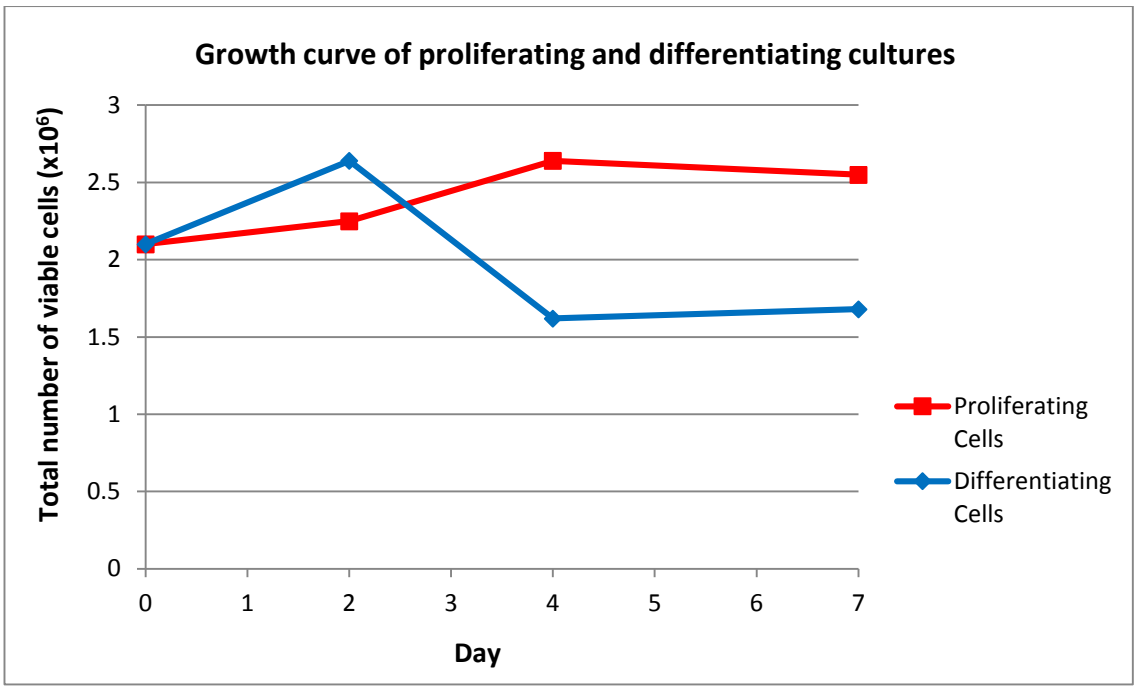


Figure 4.7: Growth curve of proliferating and differentiating cell cultures. Total number of viable cells determined by CASY plotted against the day of analysis for the different groups of cells.

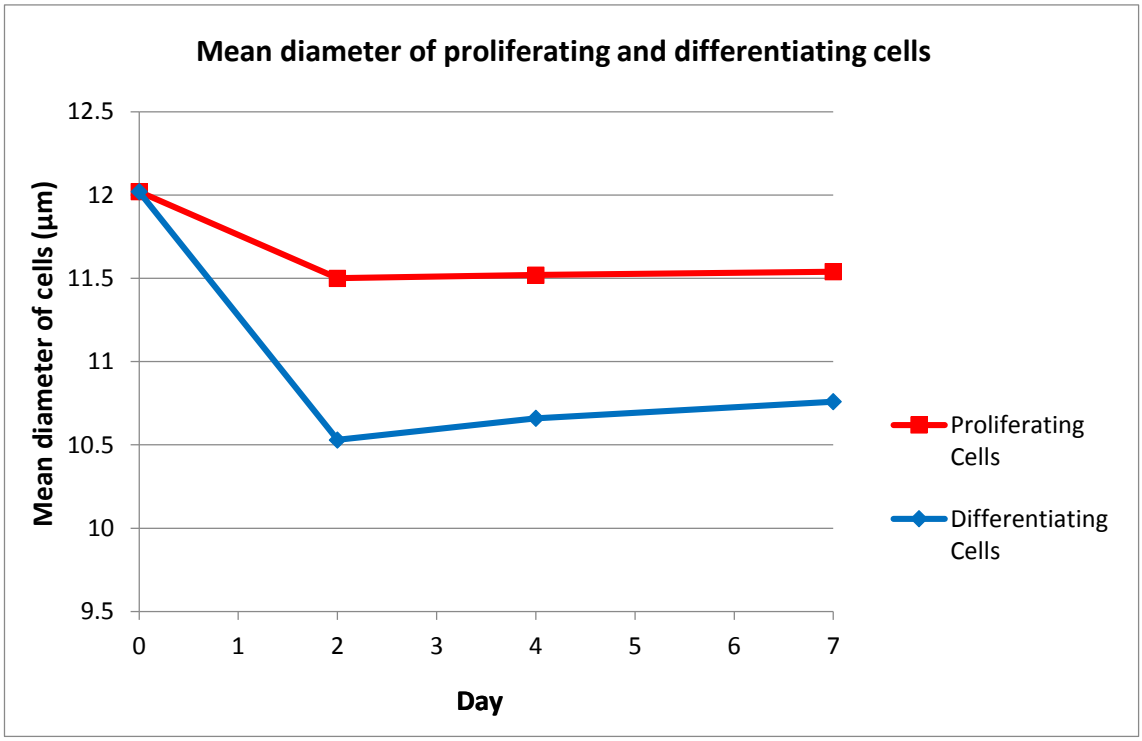


Figure 4.8: Graph of mean diameter of proliferating and differentiating cells against day of analysis.

4.4.2 Immunophenotyping

On day 2 and day 7, proliferating and differentiating cells were stained with anti-human CD71 (Transferrin Receptor) PE-Cyanine7 and anti-human CD235a (Glycophorin A) FITC (eBioscience, Affymetrix, USA). Flow cytometry was performed on the FACS Canto II using FACS Diva software (Becton Dickinson Biosciences, USA) (Figure 4.9).

Flow cytometry results demonstrate that on day 2, in the proliferating cell culture 4.7% of $CD71^{pos}$ cells were also $CD235a^{pos}$, while in the differentiating cell culture 3.7% of $CD71^{pos}$ cells were also $CD235a^{pos}$. On day 7, in the proliferating cell culture 4.8% of $CD71^{pos}$ cells were also $CD235a^{pos}$, while in the differentiating cell culture 10.9% of $CD71^{pos}$ cells were also $CD235a^{pos}$.

The results demonstrate that while the proliferating cells have a similar constant CD71 / CD235a positivity over time, differentiating cells show an increase in CD235a positivity. This is in-line with various studies and observations that declare that mature non-nucleated red blood cells are typically $CD235a^{pos}$ but $CD71^{neg}$ (Dong, Wilkes and Yang, 2011; Marsee, Pinkus and Yu, 2010; Nakahata and Okumura, 1994).

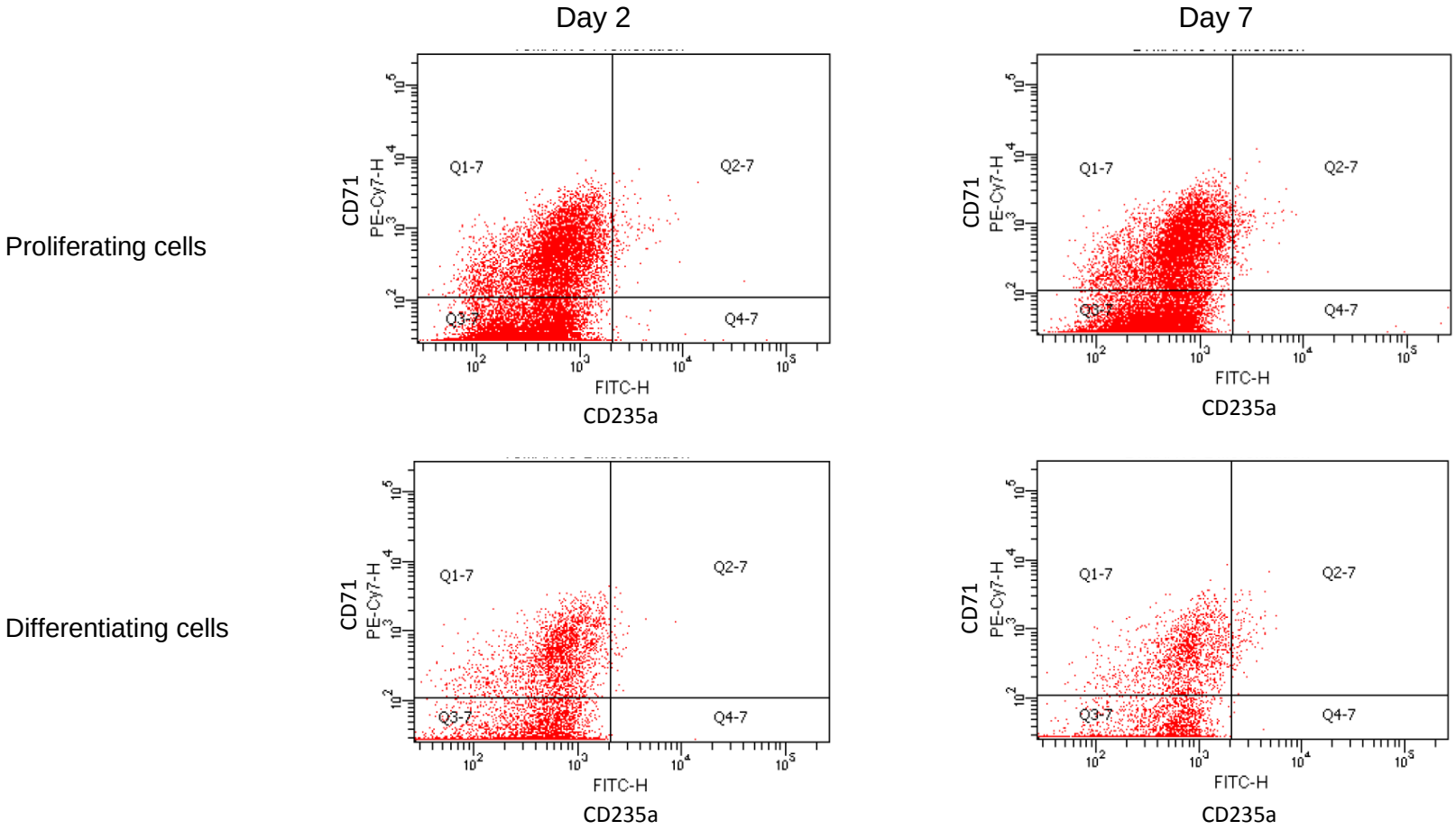


Figure 4.9: Flow cytometry analysis of proliferating and differentiating cell cultures. Flow cytometry analysis for the expression of antigens CD71 and CD235a. Gating was set up using the unstained sample, while the quadrant was established from the isotype sample.

CHAPTER 5

RESULTS:

OVEREXPRESSION OF JAK2 IN HAEMATOPOIETIC CELLS

5.1 Overview

Transient transfections were performed in which $CD34^{pos}$ cells were transfected with the wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3 constructs, using the Amaxa® Nucleofection protocol. Transfections were performed to overexpress Jak2 (wild-type and mutant) in $CD34^{pos}$ cells and to study the differential gene expression following transfection with the two constructs.

In the first experiment, $CD34^{pos}$ cells harvested from Buffy coat 6 and cultured in SFEM supplemented with cytokines for 17 days were transfected with the wild-type and mutant *JAK2* constructs and cultured in SFEM supplemented with cytokines. On day 2 the medium was changed to ESD and the growth of the cells was observed until day 31.

In the second transfection experiment, $CD34^{pos}$ cells harvested from Buffy coat 9 and cultured in SFEM supplemented with cytokines for 16 days were transfected with the constructs wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3 in three replicates and cultured in SFEM supplemented with cytokines. After two days, one set of transfected cells had their medium changed to ESD and the growth of the cells was observed until day 31. The second set of transfected cells were analysed using the PrimeFlow™ RNA assay (an *in situ* hybridisation assay), where the cells were surface stained with CD34-FITC and CD133-PE and then labelled with Type 1 (APC channel) human JAK2 Alexa Fluor® 647 target probe for the detection of *JAK2* mRNA. Western Blot was not performed due to the limited number of cells in culture and also due to the unsatisfactory results obtained during optimisation of Western Blot experiments. The expression of Jak2 was confirmed by flow

cytometry through the detection of *JAK2* mRNA with the target probe. Transfection efficiency was also assessed through flow cytometry by analysing the number of $CD34^{pos}$ cells which were also positive for *JAK2* mRNA. After confirming the presence of the *JAK2* transcript by the *in situ* hybridisation assay, the third set of transfected cells had their RNA extracted and RNA sequencing analysis was performed.

5.2 Growth analysis of transfected cells

CD34^{pos} cells that were isolated from human buffy coats were left to grow and proliferate in SFEM supplemented with 1% cc100 cytokines, 100 U/ml penicillin and 0.1mg/ml streptomycin. Transfection experiments were performed in which the CD34^{pos} cells were transfected with the constructs wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3, and also with pmaxGFP as a positive control. At first the cells were cultured in SFEM supplemented with 1% cc100 cytokines, but after two days, the medium was changed to ESD in order to allow them to proliferate into the erythroid lineage. The cells were cultured in a 6-well plate and incubated at 37ºC in a 5% CO_2 humidified atmosphere and they were followed-up for 31 days.

Cells that were transfected with pmaxGFP construct served as a control to check whether or not transfection was successful. On day 2, the cells were viewed under the microscope and strong fluorescence was observed (Figure 5.1), indicating that transient transfection was obtained efficiently.

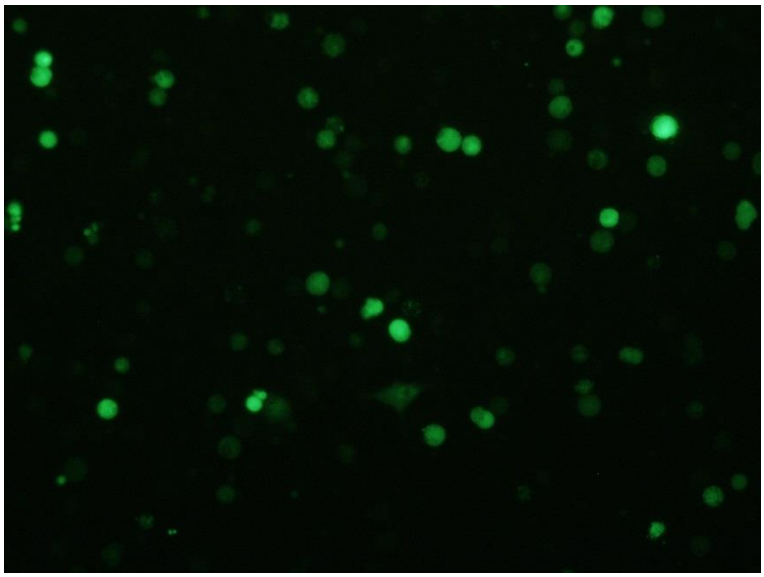


Figure 5.1: Fluorescent microscopy on day 2 of CD34^{pos} cells transfected with pmaxGFP using Amaxa® Nucleofection protocol. [Magnification x100].

In the first experiment, 2.0 x 10^6 $CD34^{pos}$ cells were transfected per sample and cultured in 2ml of medium in a 6-well plate. Fresh medium was added frequently to provide the cells with fresh supplements. Every two to four days the cells were analysed microscopically and their growth was monitored using CASY cell counter and analyser (Figure 5.2). The CASY program used was the one set up for $CD34^{pos}$ cells, described in section *2.5.4.1*. Recorded parameters are shown in Table 5.1. There was no observable difference in the cells' characteristics between the three groups, i.e. between cells transfected with wt*JAK2*-pcDNA3, cells transfected with mut*JAK2*-pcDNA3 and cells transfected with pmaxGFP (control cells) over time. The cells had an average mean diameter of 10.8 ± 0.6µm over 31 days. On the day of transfection the peak diameter of cells was on average 10.4µm decreasing to 9.2µm on day 2, 8.0µm on day 10 and 7.8µm on day 31. This change can also be observed from the CASY graphs (Figure 5.2) where the peak of the graph shifted to the left over time indicating a decrease in the cells' diameters. This indicates that when placed in ESD medium $CD34^{pos}$ cells were differentiating into erythroid progenitors. Of interest, erythroblasts were visible on day 18 in the culture of cells transiently transfected with mut*JAK2*-pcDNA3.

The total number of viable cells and percentage viability decreased over time for the three groups of transfected cells. Interestingly, in this experiment, cells transfected with mut*JAK2*-pcDNA3 showed an enhanced survival up to 31 days in ESD selective medium (Figure 5.3). No viable cells were observed at day 18 following culturing of $CD34^{pos}$ cells transfected with wt*JAK2*-pcDNA3 or pmaxGFP.

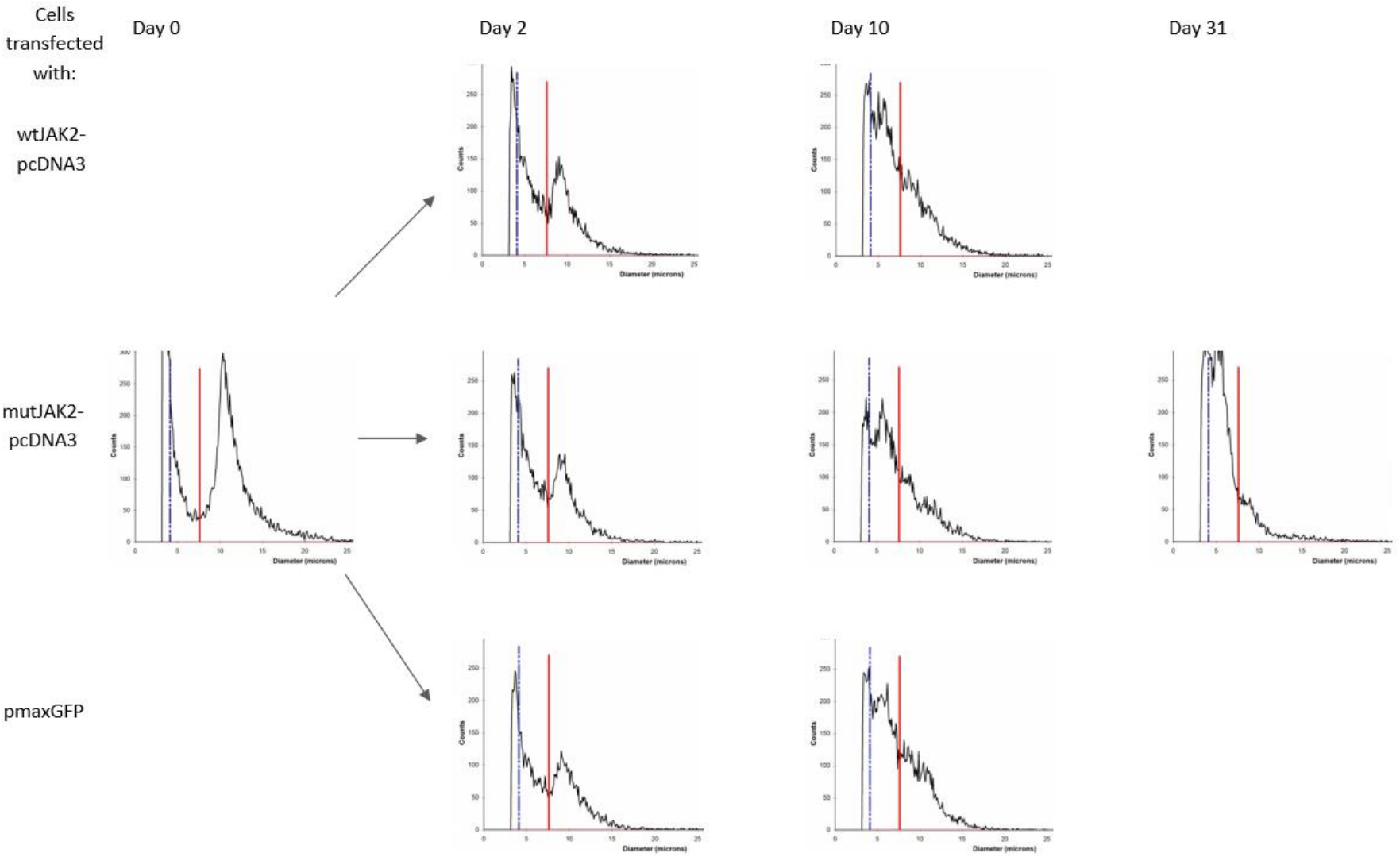


Figure 5.2: CASY growth curves of transfected cells in Experiment 1. Growth curves (obtained by CASY showing the cell count versus cell diameter in microns) of cells transfected with different constructs at different time points. [Blue dotted line: left-hand normalisation cursor; Red solid line: left-hand evaluation cursor].

Table 5.1: Parameters recorded for CD34^{pos} cells transfected with different constructs over 31 days (Experiment 1).

Day	Cells transfected with	Viable cells (x10^6) /ml	Volume (ml)	Total no. of viable cells (x10^6)	Viable cells (%)	Mean diameter (µm)	Peak diameter (µm)
0	wt*JAK2*-pcDNA3	1.0	2.0	2.0	86.7	12.1	10.4
	mut*JAK2*-pcDNA3	1.0	2.0	2.0	86.7	12.1	10.4
	pmaxGFP	1.0	2.0	2.0	86.7	12.1	10.4
2	wt*JAK2*-pcDNA3	0.8	1.7	1.3	69.0	10.5	9.2
	mut*JAK2*-pcDNA3	0.7	1.7	1.2	66.6	10.5	9.2
	pmaxGFP	0.7	1.7	1.1	70.7	10.6	9.3
6	wt*JAK2*-pcDNA3	1.1	1.1	1.2	69.0	10.7	8.6
	mut*JAK2*-pcDNA3	0.9	1.1	1.0	66.9	10.6	8.6
	pmaxGFP	1.4	1.1	1.5	67.7	10.9	8.7
10	wt*JAK2*-pcDNA3	1.5	1.0	1.5	71.9	10.6	7.7
	mut*JAK2*-pcDNA3	1.2	1.0	1.2	71.0	10.7	7.7
	pmaxGFP	0.9	1.0	0.9	62.8	10.4	8.6
13	wt*JAK2*-pcDNA3	0.7	1.0	0.7	60.5	10.3	8.3
	mut*JAK2*-pcDNA3	0.5	1.0	0.5	55.4	10.3	8.5
	pmaxGFP	0.8	1.0	0.8	58.5	10.2	8.6
31	wt*JAK2*-pcDNA3	-	-	-	-	-	-
	mut*JAK2*-pcDNA3	0.8	1.2	1.0	55.7	11.0	7.8
	pmaxGFP	-	-	-	-	-	-

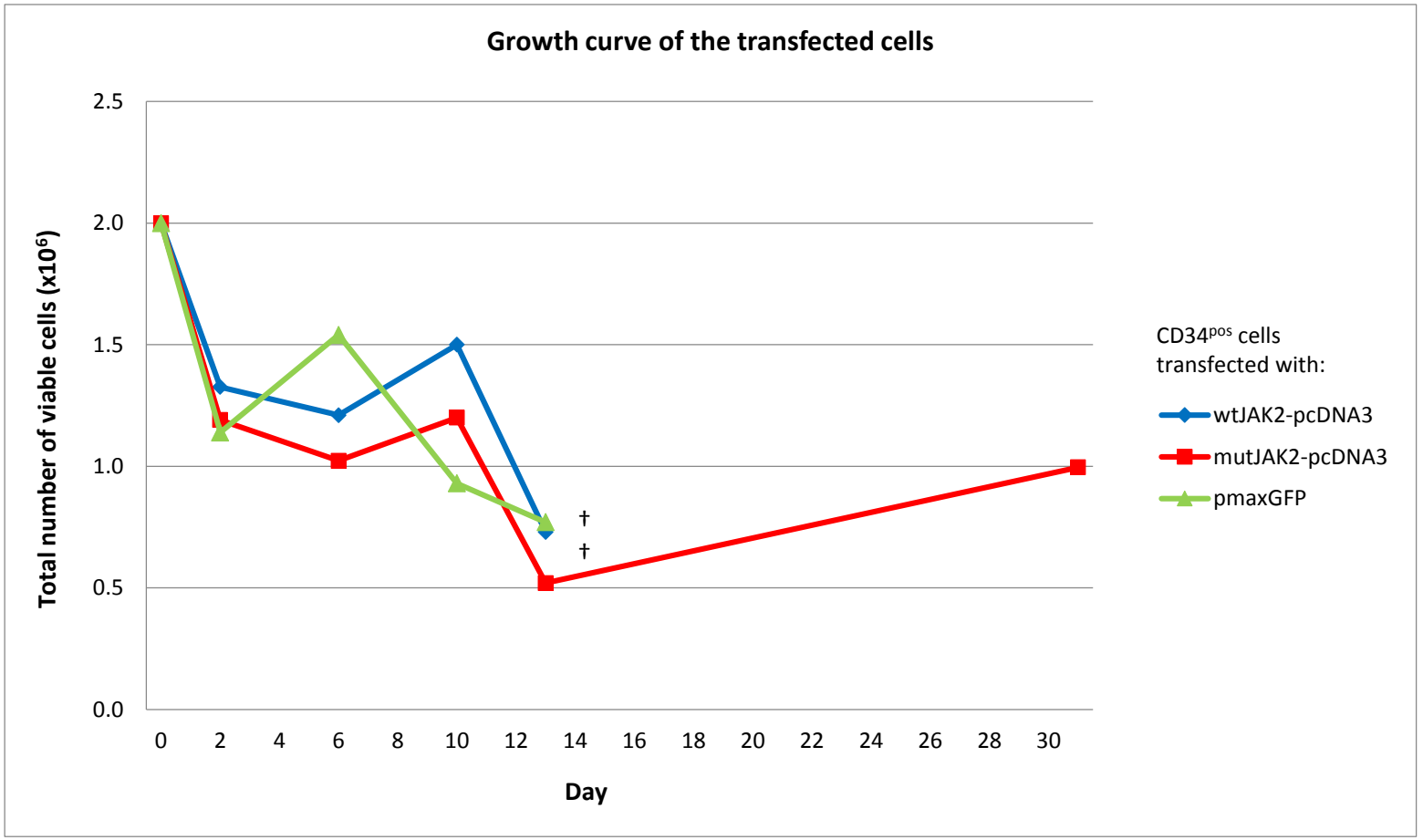


Figure 5.3: Growth curve of the transfected cells in Experiment 1. Total number of viable cells determined by CASY plotted against the day of analysis for the different groups of transfected cells. [Day 0: cells transfected with the constructs; Day 2: medium change to ESD]. [† on day 18 no viable cells were visible in cultures of cells transfected with wt*JAK2*-pcDNA3 and pmaxGFP].

The experiment was repeated, this time starting from double the number of cells to be transfected per well. In fact, 4.2 x 10^6 $CD34^{pos}$ cells were transfected per sample and cultured in 2ml of medium in 6-well plates. The same procedure and monitoring was followed as for the first experiment. Recorded parameters are shown in Table 5.2. Similar to Experiment 1, there was no observable difference in the cells' characteristics between the three groups of transfected cells over time. The cells had an average mean diameter of 10.4 ± 0.7µm over 31 days. On the day of transfection the peak diameter of cells was on average 10.5µm decreasing to 9.5µm on day 2, 8.7µm on day 10 and 8.0µm on day 31. This change can also be observed from the CASY graphs (Figure 5.4) where the peak of the graph shifted to the left over time indicating a decrease in the cells' diameters. This indicates that when placed in ESD medium $CD34^{pos}$ cells were differentiating into erythroid progenitors.

As expected, after transfection the number of viable cells decreased due to the electroporation process. Starting from 4.2 x 10^6 cells, after 48 hours post-nucleofection the total number of viable cells was on average 2.9 x 10^6. The "Amaxa® human $CD34^{pos}$ cell Nucleofector kit" claims a transfection efficiency up to 83% and a viability up to 70% (Lonza, 2009). In this experiment the viability was determined at 69%, 48 hours post-electroporation, while transfection efficiency was calculated at 57.5% for transfection with wt*JAK2*-pcDNA3 construct and 67.7% for transfection with mut*JAK2*-pcDNA3 construct.

On day 2, the medium was changed to ESD to promote proliferation into erythroblasts. As expected, the transfected $CD34^{pos}$ cells under selection of the erythroid expansion medium show a decrease in number until about day 16.

Between day 16 and day 31 the cells that were transfected by the wild-type and mutant *JAK2* constructs and pmaxGFP control maintained a constant and similar growth rate (Figure 5.5).

Although a loss of survival in the cells that were transfected by the wild-type *JAK2* construct and pmaxGFP control was expected, as observed in Experiment 1, the repeat experiment (Experiment 2) shows that there is no difference between cells that were transfected by the wild-type *JAK2* construct, cells transfected by the mutant *JAK2* construct and cells transfected with the pmaxGFP control. This result prompted to study the differential gene expression between $CD34^{pos}$ cells transfected with mutant *JAK2* construct and $CD34^{pos}$ cells transfected with wild-type *JAK2* construct.

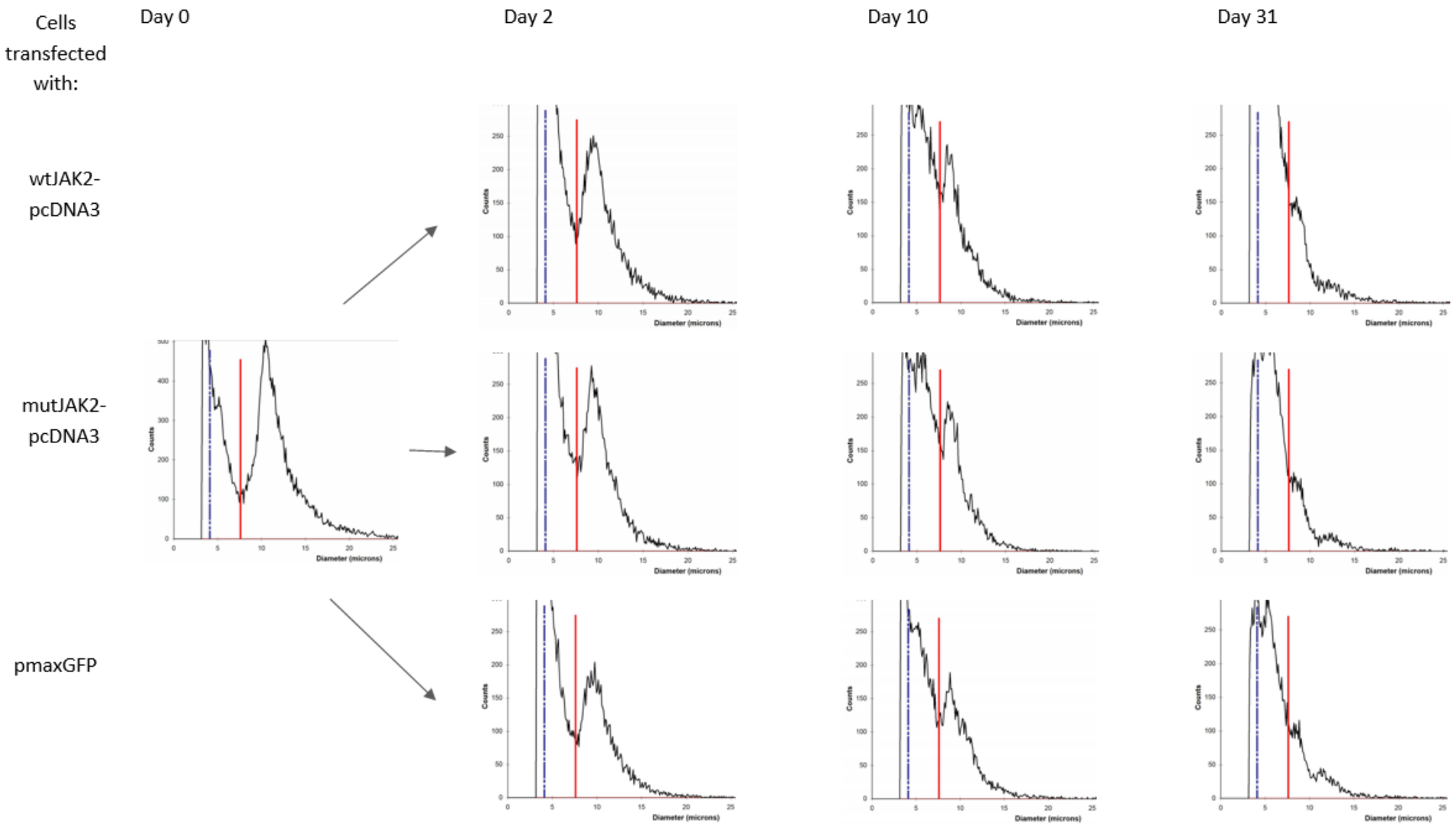


Figure 5.4: CASY growth curves of transfected cells in Experiment 2. Growth curves (obtained by CASY showing the cell count versus cell diameter in microns) of cells transfected with different constructs at different time points. [Blue dotted line: left-hand normalisation cursor; Red solid line: left-hand evaluation cursor].

Table 5.2: Parameters recorded for CD34^{pos} cells transfected with different constructs over 31 days (Experiment 2).

Day	Name	Viable cells (x10^6) /ml	Volume (ml)	Total no. of viable cells (x10^6)	Viable cells (%)	Mean diameter (µm)	Peak diameter (µm)
0	wt*JAK2*-pcDNA3	2.1	2.0	4.2	82.4	12.2	10.5
	mut*JAK2*-pcDNA3	2.1	2.0	4.2	82.4	12.2	10.5
	pmaxGFP	2.1	2.0	4.2	82.4	12.2	10.5
2	wt*JAK2*-pcDNA3	1.6	2.0	3.2	67.8	10.9	9.5
	mut*JAK2*-pcDNA3	1.5	2.0	3.0	65.4	10.7	9.5
	pmaxGFP	1.2	2.0	2.4	64.6	10.8	9.6
10	wt*JAK2*-pcDNA3	1.2	1.6	1.9	60.8	10.1	8.6
	mut*JAK2*-pcDNA3	1.0	1.6	1.6	57.9	10.0	8.6
	pmaxGFP	1.0	1.6	1.6	62.2	10.4	8.8
16	wt*JAK2*-pcDNA3	0.9	1.6	1.4	52.5	9.9	8.7
	mut*JAK2*-pcDNA3	0.8	1.6	1.2	50.8	9.8	8.5
	pmaxGFP	0.8	1.6	1.2	58.0	10.1	8.7
19	wt*JAK2*-pcDNA3	0.9	1.6	1.4	50.6	9.8	8.6
	mut*JAK2*-pcDNA3	0.7	1.6	1.1	46.7	9.7	8.7
	pmaxGFP	0.8	1.6	1.3	55.8	10.1	8.6
22	wt*JAK2*-pcDNA3	0.8	1.5	1.2	50.0	9.9	8.5
	mut*JAK2*-pcDNA3	0.8	1.5	1.2	48.4	9.9	8.5
	pmaxGFP	0.9	1.5	1.4	57.9	10.3	8.6
25	wt*JAK2*-pcDNA3	1.0	1.6	1.6	52.0	9.7	7.7
	mut*JAK2*-pcDNA3	0.7	1.6	1.2	48.4	9.8	8.4
	pmaxGFP	1.1	1.6	1.8	69.6	10.8	7.7
28	wt*JAK2*-pcDNA3	0.9	1.2	1.1	45.4	10.2	7.9
	mut*JAK2*-pcDNA3	0.8	1.2	0.9	48.8	9.8	7.7
	pmaxGFP	1.1	1.2	1.3	60.3	10.4	7.7
31	wt*JAK2*-pcDNA3	0.9	1.2	1.1	46.3	10.2	8.5
	mut*JAK2*-pcDNA3	0.8	1.2	0.9	48.9	10.0	7.7
	pmaxGFP	0.9	1.2	1.1	57.8	10.4	7.7

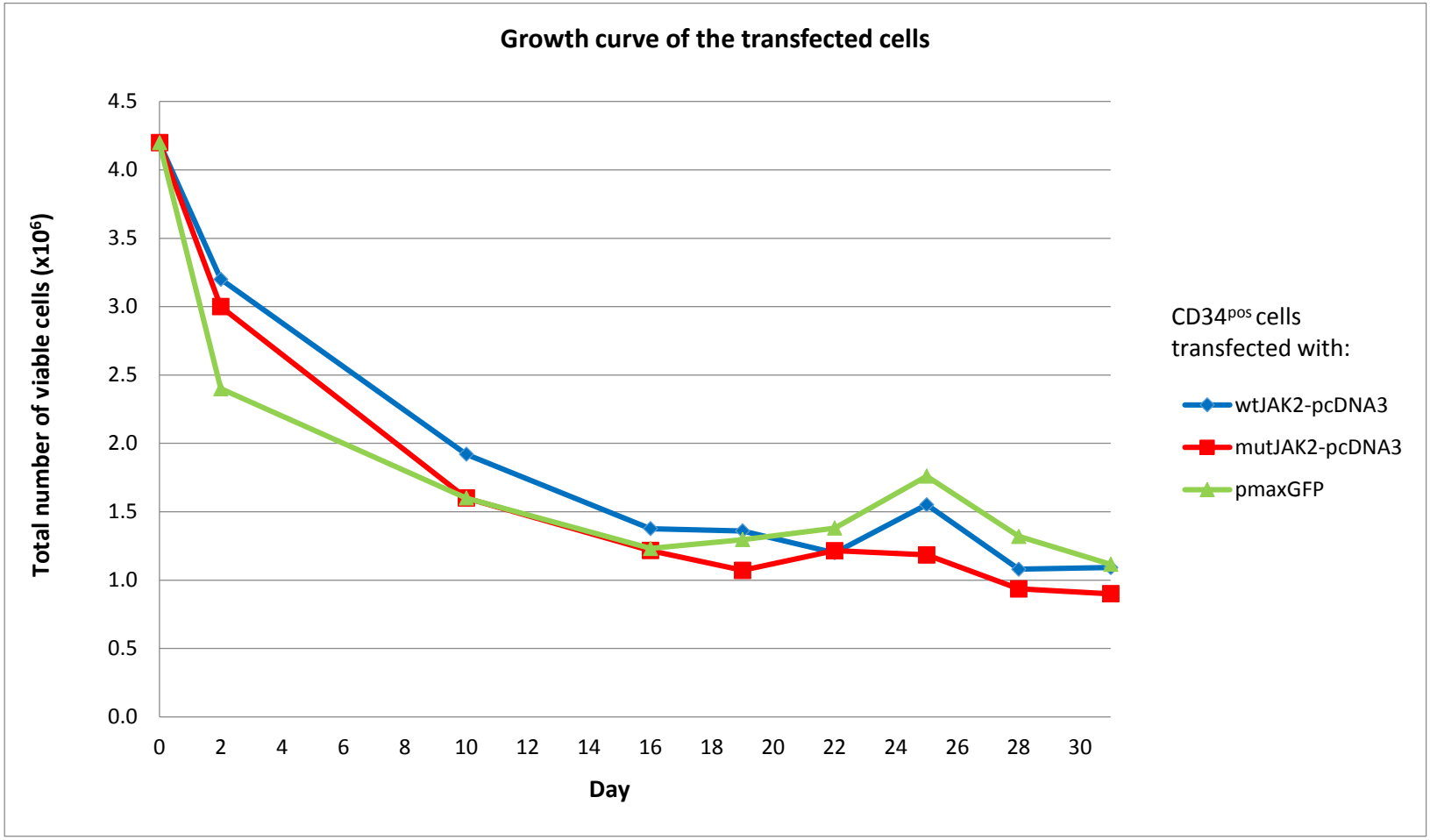


Figure 5.5: Growth curve of the transfected cells in Experiment 2. Total number of viable cells determined by CASY plotted against the day of analysis for the different groups of transfected cells. [Day 0: cells transfected with the constructs; Day 2: medium change to ESD].

5.3 *In situ* hybridisation assay analysis

The transfected cells of Experiment 2 were also analysed using an *in situ* hybridisation assay (PrimeFlow™ RNA assay) on day 2, but not with Western Blotting due to the low concentration of cells. Cells transfected with pmaxGFP construct served as a control for transfection and hence were not analysed further as the green fluorescence could interfere with the detection channel of the flow cytometer. The other transfected cells as well as untransfected $CD34^{pos}$ cells (to serve as controls), were surface stained with CD34-FITC and CD133-PE and then labelled with Type 1 (APC channel) human JAK2 Alexa Fluor® 647 target probe for the detection of *JAK2* mRNA. Flow cytometry was performed on the FACS Canto II using FACS Diva Software (Becton Dickinson Biosciences, USA).

A group of untransfected $CD34^{pos}$ cells were probed with Type 1 positive control probe. This positive control includes a probe for human ribosomal protein RPL13A which is highly expressed in various cell types (Affymetrix, 2000). When analysed by flow cytometry, a positive population for APC was observed and 79.5% of $CD34^{pos}$ cells were positive for the Type 1 positive control probe indicating that the assay was performed successfully (Figure 5.6). Cells transfected with wild-type and mutant *JAK2* constructs and another group of untransfected cells were probed with the target probe, i.e. human JAK2 probe. Cells transfected with the wt*JAK2*-pcDNA3 construct and cells transfected with the mut*JAK2*-pcDNA3 construct showed a positive population in the APC channel, while untransfected cells were negative for APC. Flow cytometry statistics demonstrate that 57.5% of $CD34^{pos}$ cells that were transfected with wt*JAK2*-pcDNA3 were positive for *JAK2* and 67.7% of $CD34^{pos}$ cells that were

transfected with mut*JAK2*-pcDNA3 were positive for *JAK2* (Figure 5.6). This demonstrates that both wild-type and mutant *JAK2* was present in the cultured cells and that *JAK2* mRNA was being expressed.

Histograms of APC versus Count were created to show the APC-positive cells, i.e. cells expressing the *JAK2* transcript. A region of interest was selected on the negative control and the same region was applied to all the analysed samples (Figure 5.7). The intensity of APC positivity for cells transfected with wt*JAK2*-pcDNA3 (Figure 5.7-C) and for cells transfected with mut*JAK2*-pcDNA3 (Figure 5.7-D) is equivalent to the positive control (Figure 5.7-A). No Jak2 is expressed in the negative control and this lack of APC positivity (Figure 5.7-B) indicates that there is no endogenous Jak2 being expressed by untransfected $CD34^{pos}$ cells.

$CD34^{pos}$ cells that were transfected with the wild-type and with the mutant *JAK2* constructs and were confirmed to be expressing the *JAK2* transcript, were selected for RNA sequencing analysis in order to analyse the differential expression of genes of mutant Jak2 against wild-type Jak2 expressing cells.

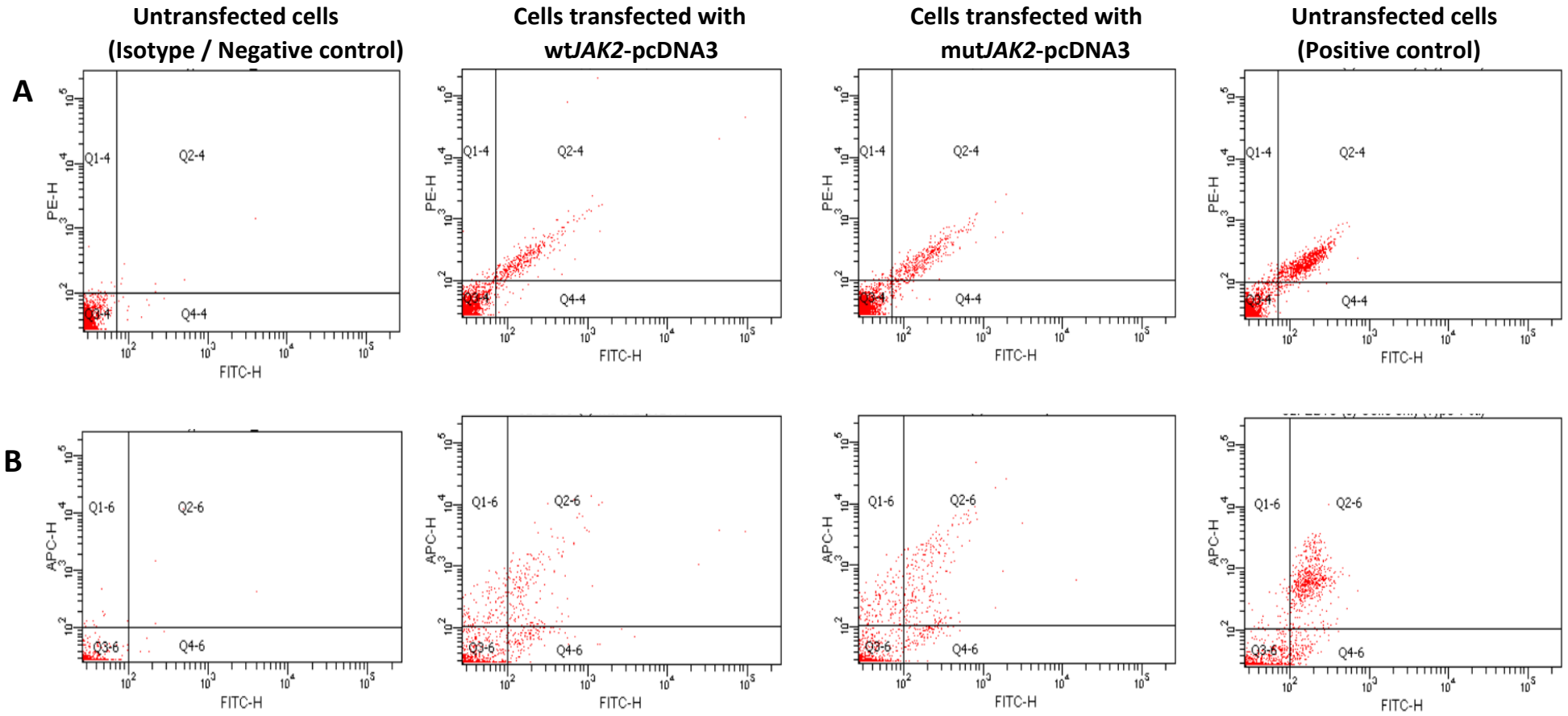


Figure 5.6: CD34^{pos} cells transfected with *JAK2* constructs and then analysed using the PrimeFlow™ RNA assay (Experiment 2). Cells were surface stained with CD34-FITC and CD133-PE (**A**) and then labelled with Type 1 (APC channel) human JAK2 Alexa Fluor® 647 target probe for the detection of *JAK2* mRNA (**B**).

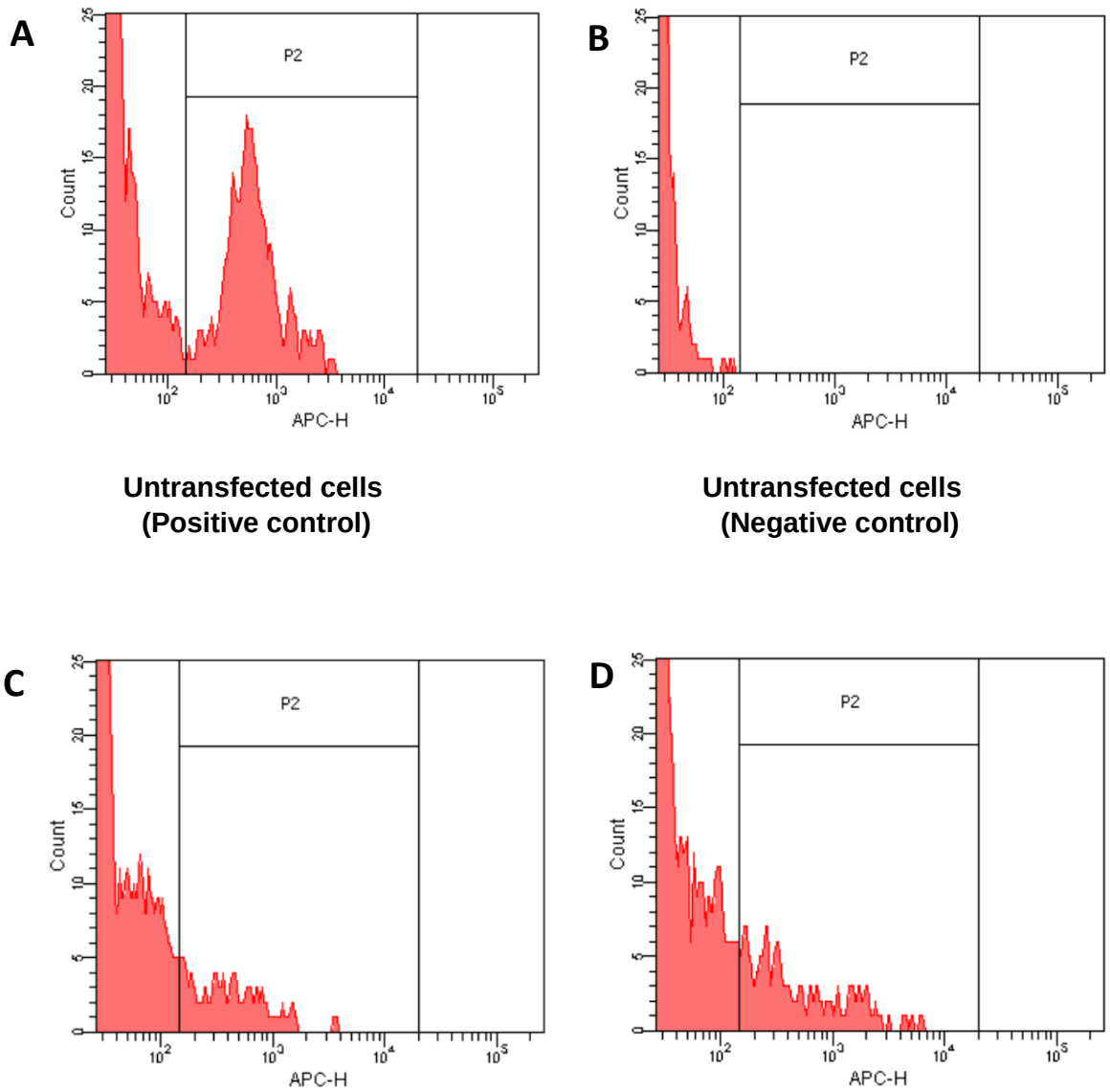


Figure 5.7: Histograms of APC (presence of *JAK2* transcript) versus count. A region of interest was selected on the Negative control and applied to all the analysed samples in Experiment 2.

5.4 RNA sequencing results

On day 2 after transient transfection, RNA was extracted from the cells that were transfected with the constructs wt*JAK2*-pcDNA3 and mut*JAK2*-pcDNA3. The presence of *JAK2* transcript was confirmed by the *in situ* hybridisation assay. Due to the unstable nature of RNA, cDNA synthesis was performed. The concentration of the cDNA together with the absorbance ratios were measured using the NanoDrop spectrophotometer (Table 5.3).

Table 5.3: Measurements of cDNA concentration and purity. RNA extracted from the transfected cells was transcribed to cDNA. Concentration is given in ng/µl; $A_{260/280}$ and $A_{260/230}$ indicate the purity of the cDNA.

cDNA	Concentration (ng/µl)	Absorbance 260/280	Absorbance 260/230
Cells transfected with wt*JAK2*-pcDNA3	1660.6	1.79	2.20
Cells transfected with mut*JAK2*-pcDNA3	1557.7	1.79	2.20

The samples were sent to BGI Genomics (Hong Kong) for RNA sequencing (described in section *2.9.4*). Two samples were sent for RNA sequencing, i.e. RNA extracted from $CD34^{pos}$ cells transfected with wt*JAK2*-pcDNA3 construct and from $CD34^{pos}$ cells transfected with mut*JAK2*-pcDNA3 construct. RNA sequencing provided a list of expressed genes for the two samples. These results were analysed with the programme AltAnalyze (described in section *2.9.5*) to obtain the differential expression of mut*JAK2*- versus wt*JAK2*- transfected cells. A gene list was produced and the resulting networks of genes were then analysed

using another programme called Ingenuity Pathway Analysis (IPA) described in section *2.9.6*).

RNA sequencing analysis provided a numerous list of overexpressed genes for both wt*JAK2*- and mut*JAK2*- transfected cells. To compare the effect of transfected mut*JAK2* with wt*JAK2* on $CD34^{pos}$ cells, the top 100 differentially expressed genes (Appendix E) were selected and IPA was used to analyse the network between various genes. These differentially expressed genes are interconnected with sets of genes that are associated with a particular biological function or pathway.

The top pathways affected when comparing mut*JAK2*- and wt*JAK2*- transfected cells are eukaryotic initiation factor 2 (eIF2) signalling (p-value 6.42^{-42}), mammalian target of rapamycin (mTOR) signalling (p-value 1.02^{-10}), regulation of eukaryotic initiation factor 4 (eIF4) and p70S6 kinase (p70S6K) signalling (p-value 1.29^{-10}).

When comparing mut*JAK2*-transfected $CD34^{pos}$ cells over wt*JAK2*-transfected $CD34^{pos}$ cells, IPA presented five networks, two of which were selected, being the most interesting and relevant (Figure 5.8 and 5.9). Network 1 involves ribosomal proteins and translation initiation and elongation factors; while Network 2 involves signalling pathways. The other three networks were not selected for discussion because Network 3 is composed of a ribosomal network that is also represented in Network 1, Network 4 involves NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and p38 MAPK (a regulator of skeletal muscle development), while Network 5 involves keratin associated proteins.

Both of the selected networks have a key gene of interest, namely nucleophosmin 1 (*NPM1*), that encodes a phosphoprotein which moves between the nucleus and the cytoplasm and is involved in several cellular processes such as ribosome biogenesis, centrosome duplication, protein chaperoning, histone assembly, cell proliferation, and regulation of tumour suppressors p53/TP53 and ARF. Interestingly, both networks show a connection between *NPM1* and eukaryotic elongation factor-1 (eEF1) subunits *eEF1A1* (eukaryotic translation elongation factor 1, alpha-1) and *eEF1G* (eukaryotic translation elongation factor 1, gamma).

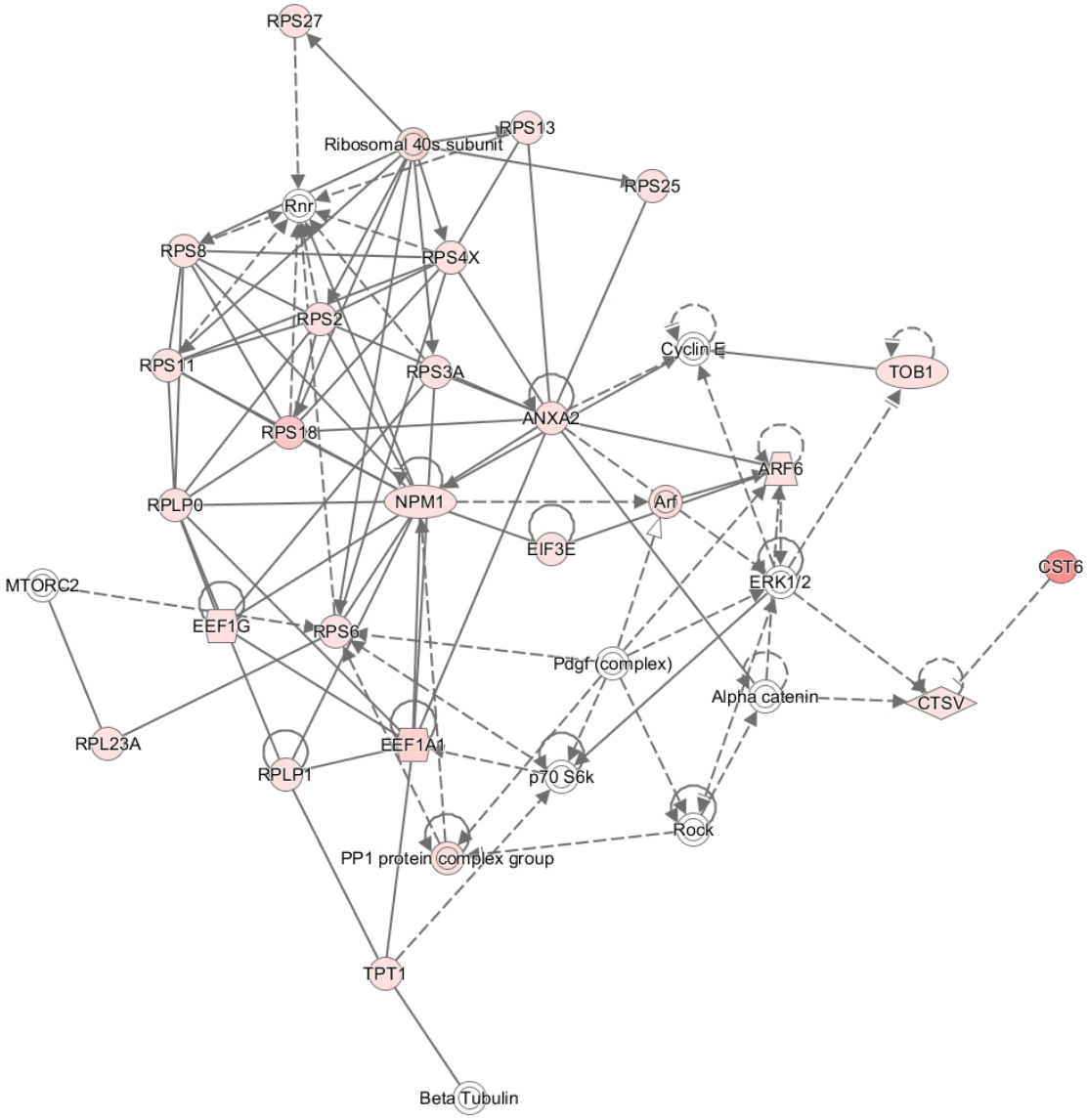


Figure 5.8: Network 1. Ingenuity Pathway Analysis (IPA) of top 100 differentially expressed genes when comparing *JAK2* mutant and wild-type expressing CD34^{pos} cells. Network showing interactions between NPM1 (nucleophosmin 1) and RPS (ribosomal proteins) and control of translation initiation and elongation through EEF (eukaryotic elongation factors) and EIF (eukaryotic initiation factors) proteins. [Red highlights: overexpressed genes when comparing mut*JAK2*- with wt*JAK2*- transfected cells; Solid arrows: direct regulation between the genes; Dashed arrows: indirect regulation between the genes; Straight line: binding to each other].

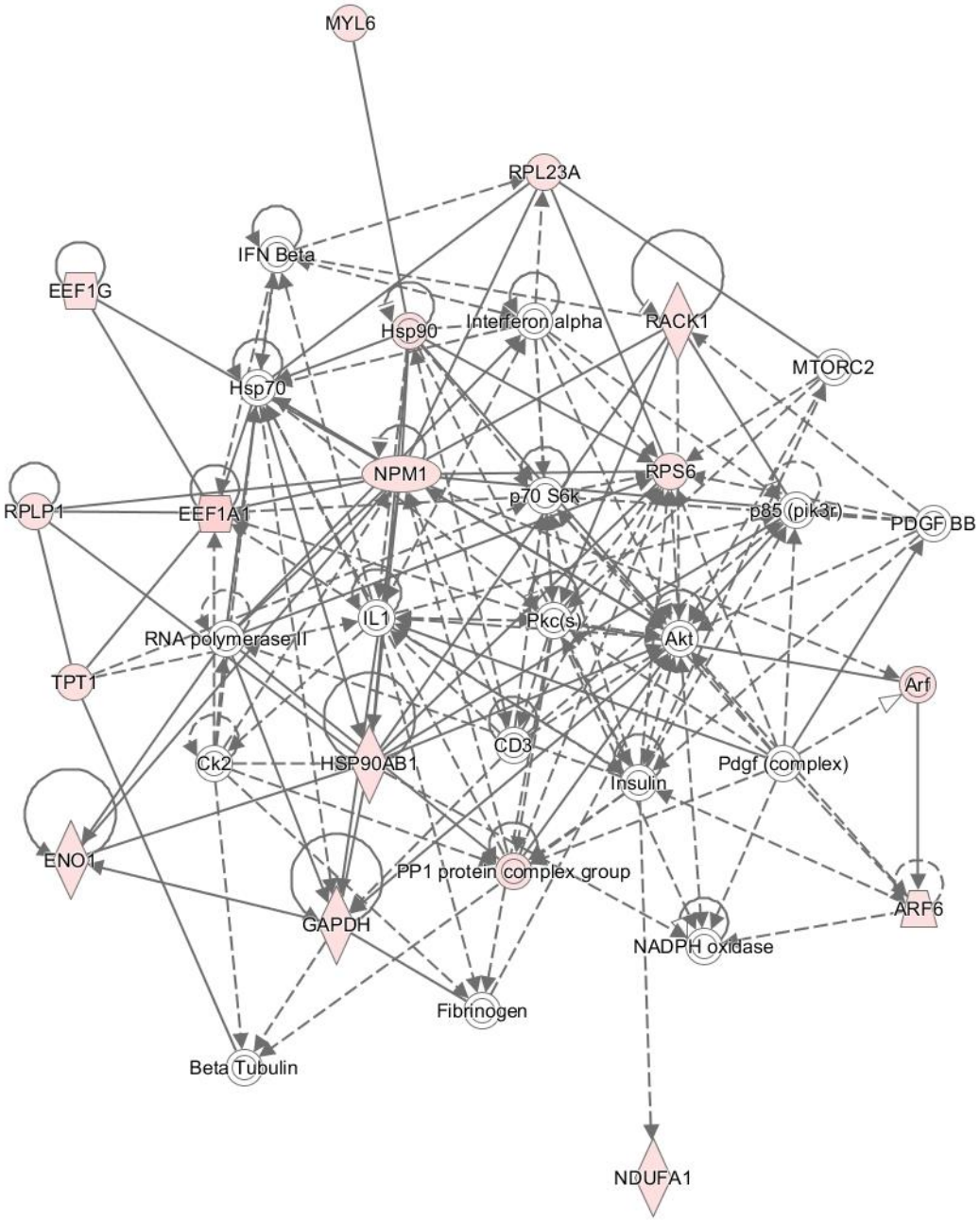


Figure 5.9: Network 2. Ingenuity Pathway Analysis (IPA) of top 100 differentially expressed genes when comparing *JAK2* mutant and wild-type expressing CD34^{pos} cells. Network showing interactions between NPM1 (nucleophosmin 1) and signaling pathways including mTOR (shown as AKT/ p70 S6K), Pkc (protein kinase C), Insulin, and Pdgf (platelet derived growth factor). Although IL1 (interleukin-1) is also included in the network, this interaction is not significant in the context of erythroblasts. [Red highlights: overexpressed genes when comparing mut*JAK2*- with wt*JAK2*- transfected cells; Solid arrows: direct regulation between the genes; Dashed arrows: indirect regulation between the genes; Straight line: binding to each other].

From the observed gene expression and gene interactions, a number of upstream regulators that are connected to dataset genes through a set of direct or indirect relationships were identified. These are molecules upstream of the genes in the dataset that potentially explain the observed expression changes. Those with a p-value greater than 0.001 were not taken into account, resulting in 96 upstream regulators. These were grouped according to their biological function; 19% are cytokines and involved in growth factor signalling, 16% are transcription regulators, 11% are enzymes, while 54% have other functions such as biological drugs, chemical drugs, chemical reagents, chemical toxicants, complexes and transporters (Figure 5.10).

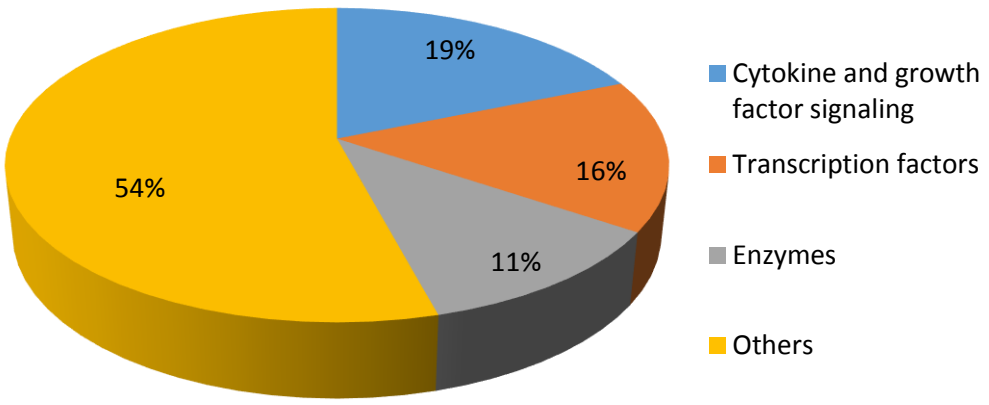


Figure 5.10: Distribution of biological function. Proportion of upstream regulators of differentially expressed genes when comparing mut*JAK2*- and wt*JAK2*- transfected cells.

Looking at the differentially expressed *NPM1* gene and interacting genes, some key upstream regulators that activate *NPM1* are observed (Table 5.4). The strongest relationships (i.e. having the lowest p-values) are with the transcription regulators MYCN (V-Myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog) and MYC (V-Myc avian myelocytomatosis viral oncogene homolog).

Interestingly Epo, KITLG (kit ligand) and Dex are also identified as regulators that are involved in the over expression of the differentially expressed genes including *NPM1*. The supplements Epo, SCF and Dex are components of ESD medium that promote proliferation of cells into the erythroid lineage. In cells transfected with mutant *JAK2,* the regulators Epo, KITLG and Dex activate a number of genes including *NPM1.* This observation prompts a question whether mut*JAK2* regulates $CD34^{pos}$ cells in a way to prepare them for erythroid expansion. Another cytokine, Endothelin 1 (EDN1) and the transcription factor Tumour protein p53 (TP53) were also identified as regulators of the differentially expressed genes, including *NPM1.*

Table 5.4: Upstream regulators of the differentially expressed *NPM1* gene.

Upstream regulator	Biological function	p-value
MYCN	transcription regulator	1.48^{-42}
MYC	transcription regulator	7.15^{-22}
EPO	cytokine	3.37^{-07}
KITLG	growth factor	5.16^{-07}
DEX	glucocorticoid	6.31^{-06}
EDN1	cytokine	1.86^{-05}
TP53	transcription regulator	2.34^{-04}

CHAPTER 6

DISCUSSION

6.1 Long-term *in vitro* culturing of red blood cells

In this project, the aim was to study the effect of exogenous *JAK2* (mutant and wild-type) on cultured haematopoietic cells and to understand the expression signatures that are different when comparing $CD34^{pos}$ cells that overexpress wild-type Jak2 and $CD34^{pos}$ cells overexpressing mutant Jak2. In addition, erythroid cells were cultured *in vitro* and immunophenotyping was performed in order to characterise the cells produced along this expansion process.

Peripheral blood was used as a source of HSCs and $CD34^{pos}$ cells were positively selected from the MNCs derived from human buffy coats extracted from the incomplete blood donations obtained from the Malta National Blood Transfusion Service. The disadvantage of using stem cells from peripheral blood is that they have limited expansion capacity compared to those from cord blood. However, they are easier to obtain and they display mature forms of haemoglobin, whereas those from cord blood only express foetal haemoglobin (Lu *et al.*, 2009). Nevertheless, studies demonstrated that while erythroblasts generated *in vitro* from cord blood express mainly foetal haemoglobin, when injected *in vivo* using mice these cells generate RBCs that express adult haemoglobin (Fujimi *et al.*, 2008; Neildez-Nguyen *et al.*, 2002).

The culture protocol for the *in vitro* culturing of RBCs used during this research is similar to that described by Heideveld *et al.* (2015), van den Akker *et al.* (2010) and Leberbauer *et al.* (2005) where culturing was performed in phases and serum-free medium enhanced with similar supplements was used. Culturing of RBCs was performed in three phases. Phase 1 involved the proliferation of $CD34^{pos}$ cells for 16 days. The cells were cultured in serum-free expansion

medium (SFEM) (containing BSA, insulin, transferrin and 2-mercaptoethanol) supplemented with a combination of recombinant human cytokines formulated to support the proliferation of human haematopoietic progenitors. The fold increase (FI) for the $C34^{pos}$ proliferation experiment was $4.1x10^2$.

In phase 2a $CD34^{pos}$ cells were transferred to ESD selective medium, consisting of SFEM supplemented with 100 ng/ml SCF, 1 µM Dex, 2 U/ml Epo, and a lipid-mixture supplement containing 0.22 mg/ml cholesterol. Culturing in ESD medium promotes the proliferation of HSCs into erythroblasts and a decrease in the number of cultured cells shows the selection of cells that differentiate towards this lineage. This selection phase took 11 days.

In phase 2b, cells were still cultured in ESD, but during the subsequent 30 days, a rise in the number of cells was observed. At this stage, cells responsive to SCF and Epo start expansion and proliferate into erythroblasts resulting in a FI of 20.1. Immunophenotyping on day 13 of culturing in ESD selective medium confirms that 28.1% of cells displayed characteristics consistent with erythroid progenitors.

The third phase involved the terminal differentiation of the erythroblasts into erythrocytes. On day 57 proliferating erythroblasts were transferred to a culture medium consisting of SFEM supplemented with 5 U/ml Epo and 0.5 mg/ml holo-Transferrin. This differentiation phase was followed for 7 days. Flow cytometry analysis of these cells demonstrated an increase in CD235a positivity over time. This observation correlates with other studies that established that mature non-nucleated red blood cells are typically $CD235a^{pos}$ and $CD71^{neg}$ (Dong, Wilkes and Yang, 2011; Marsee, Pinkus and Yu, 2010; Nakahata and Okumura, 1994). Table

6.1 provides a summary of the culture conditions used during this research for culturing RBCs *in vitro*.

A further experiment involved the culturing of $CD34^{neg}$ cells. The $CD34^{neg}$ cell culture consisted of residual MNCs after $CD34^{pos}$ selection. The culture was selected for erythroblasts by growing the cells in ESD. This experiment has showed that a stromal cell layer derived from cord blood enhanced the proliferation of the $CD34^{neg}$ cells (data not shown as the aim of the research did not involve $CD34^{neg}$ cells). This cell layer has not been studied yet and it would be interesting to follow-up this observation. This is supported by Giarratana *et al*. (2005) who achieved substantial amplification of $CD34^{pos}$ cells with 100% terminal maturation into RBCs by co-culturing $CD34^{pos}$ cells with a murine stromal cell line. The drawback of this protocol is the use of a xenogeneic cell line. Xi *et al*. (2013) made the culture system safer by co-culturing erythroid progenitors with human foetal liver stromal cells. They also observed that large numbers of erythroid cells could be produced from a small amount of peripheral blood and concluded that unidentified populations of human stromal cells mimicked the microenvironment of the bone marrow and stimulated the maturation of erythroid progenitor cells. Other studies have showed that mouse and human stromal cells also promote the *in vitro* generation of erythroid cells derived from human embryonic stem cells (Lee *et al*., 2011; Ma *et al*., 2008; Ma *et al*., 2007).

Table 6.1: Long-term *in vitro* culturing of red blood cells.

Phase	**Phase 1**	**Phase 2**	**Phase 3**
Aim	Proliferation of $CD34^{pos}$ cells	Proliferation of HSCs to EBs	Terminal differentiation into RBCs
Days	0 – 16	17 – 57	58 – 64
Culture conditions	SFEM (containing BSA, insulin, transferrin and 2-mercaptoethanol) supplemented with: cc100 cytokines	SFEM (containing BSA, insulin, transferrin and 2-mercaptoethanol) supplemented with: Epo (2U/ml) SCF (100ng/ml) Dex (1µM) Lipids (0.22mg/ml)	SFEM (containing BSA, insulin, transferrin and 2-mercaptoethanol) supplemented with: Epo (5U/ml) Holo-Transferrin (0.5mg/ml)

[BSA: bovine serum albumin; Dex: dexamethasone; EB: erythroblast; Epo: erythropoietin; HSC: haematopoietic stem cell; RBC: red blood cell; SCF: stem cell factor; SFEM: serum-free expansion medium].

6.2 Expansion of haematopoietic cells transfected with human *JAK2* constructs

One of the objectives of this research was to overexpress both wild-type and mutant *JAK2* in $CD34^{pos}$ cells. It was hypothesised that constitutive erythropoietin receptor signalling induced by exogenous *JAK2* expression results in enhanced proliferation resulting in mass-production of erythroid cells and that transfection of *JAK2* mutant in $CD34^{pos}$ cells provides a transcriptional program that enhances selection and proliferation of erythroid progenitors, also resulting in increased numbers of cells.

During this research, the human *JAK2* ORF was successfully amplified with PCRBIO Ultra Polymerase using forward primer 5'- CTCAAGCTTCGAATTC**ATG** GGAATGGCCTGCCTTACG-3' and reverse primer 5'-CCGCGGTACCGTCGA CTCATCCAGCCATGTTATCCC-3'. This PCR generated a coding sequence that was then inserted into a linearised pIRES2 vector to create the *JAK2*-pIRES2-AcGFP1 construct. This construct was cloned using the In-Fusion® HD Cloning Kit and sequenced in full. Sequencing results showed the presence of a couple of non-synonymous variants at position 577bp and position 1436bp. Nonetheless, transfection experiments show that the *JAK2*-pIRES2 construct produced the *JAK2*-IRES-GFP transcript successfully as the GFP protein was expressed by the transfected cells. However, due to the mutations and unsuccessful high-fidelity PCR, another two published human *JAK2* constructs were obtained, namely the wild-type *JAK2* construct (wt*JAK2*-pcDNA3) and the mutant *JAK2* construct that contains the V617F mutation (mut*JAK2*-pcDNA3).

During this research, transfection was performed using a Nucleofection method utilising electroporation technology whose transfection efficiency was assessed at 57.5% when $CD34^{pos}$ cells were transfected with wt*JAK2*-pcDNA3 construct and 67.7% when $CD34^{pos}$ cells were transfected with mut*JAK2*-pcDNA3 construct. This method provides transient transfection whereby the gene is not integrated into the cells' genome. As the gene is not replicated, it is expressed for a finite period of time and then it is lost through cell divisions. Hence, the transfected cells could not be followed for a long time. In fact, cells transfected with pmaxGFP were analysed by fluorescence microscopy after 19 days and very few fluorescent cells were noted (data not shown). This observation revealed that over time the transfected plasmid was lost from the cells. Stable transfection would be more ideal, as the foreign gene is integrated into the cells' genome and hence would be replicated, leading to daughter cells that also express the gene. However, stable transfection was not an option because the research facilities in Malta are not equipped for viral transduction methods.

Nonetheless, the application of stable gene transfer for clinical applications is restricted because most methods require the use of viruses such as lentiviruses, gamma-retroviruses, and spumaviruses that can cause upregulation of proto-oncogenes and/or inactivation of tumour suppressor genes resulting from insertional mutagenesis (Xue *et al.,* 2009). Hacein-Bey-Abina *et al.* (2008) report that four out of ten patients suffering from severe combined immune deficiency, that were treated by gene therapy using gamma-retroviral vectors to transduce $CD34^{pos}$ cells carrying the therapeutic gene, developed T-cell acute lymphoblastic leukaemia. Non-viral gene transfer would be more ideal for clinical use as they offer more advantages including simplicity of the method, low cost,

ease of handling, potential for large-scale production, and most importantly biosafety. The main disadvantage of the use of non-viral vectors is that genes do not integrate with the cells' genome and thus does not result in a stable transgene expression.

One could argue that using gene transfer to enhance the proliferation of erythroid progenitor cells is quite safe because the end product to be used for clinical use, i.e. red blood cells, do not have a nucleus and thus cultured RBCs should not pose an oncogenic risk. However, there is still potential danger as it is challenging to achieve synchronised terminal differentiation into erythrocytes and to ascertain that the transfusable product consists of enucleated RBCs only.

A study by Lee *et al.* that was published in April 2016, also studied the possibility of producing massive quantities of mature RBCs by overexpressing *JAK2* in HSCs. They transduced wild-type and mutant *JAK2* constructs into cord blood-derived $CD34^{pos}$ cells using a lentivirus system. After transduction, $CD34^{pos}$ cells were seeded in 24-well plates at a density of 1×10^5 cells/ml and followed-up for 21 days. Similar to this research, culturing was performed in phases, in order to enhance proliferation of $CD34^{pos}$ cells initially, then to induce proliferation of the haematopoietic stem cells into erythroblasts and finally to drive terminal differentiation into RBCs. During Phase 1 (day 0 to day 7) Lee *et al.* (2016) cultured the transduced cells in IMDM supplemented with FBS, SCF, IL-3 and Flt-3 to stimulate proliferation. In the second phase, from day 8 to day 14, cells were cultured in StemPro-34 SFM complete medium supplemented with SCF, IL-3 and Epo in order to drive differentiation into erythroblasts. In the third phase, from day 15 to day 18, the medium was replaced with serum-free medium

supplemented with SCF, Epo and poloxamer 188 (a membrane resealing agent that repairs damaged cell membranes). During the last phase, no cytokines were added and cells were cultured in serum-free medium with poloxamer 188 from day 19 to day 21.

During the first 10 days of culture, all transduced cells expanded similarly. Then, from day 10 to day 17, cells transduced with wild-type and mutant *JAK2* proliferated more than control untransfected cells. In particular, on day 14 wild-type and mutant *JAK2*-transduced cells exhibited a higher fold expansion. This shows that wild-type and mutant *JAK2* introduced in the cells, affect the cells resulting in an expansion advantage during the later period of culture. In the current research, the expansion of cells transfected by the wild-type and mutant *JAK2* constructs was shown to be similar to control cells transfected with pmaxGFP. Being a transient transfection, the constructs were eventually lost through cell divisions. These observations, reinforced by conclusions from Lee *et al.* (2016), suggest that overexpression of Jak2 in $CD34^{pos}$ cells is not enough to drive over-expansion of cells and perhaps *JAK2* does not impact haematopoietic stem cells but has an effect on cells at a different maturity stage.

It would be interesting to study at what maturity stage of the cells would exogenous wild-type and mutant *JAK2* have an effect. Initial studies were undertaken during this project. Primers for different promoters, i.e. erythropoietin receptor (EpoR), c-kit, and CD34, were synthesised and PCR was performed to obtain the promoters. Different promoters were selected as they are present during the different maturity stages of erythroid cells; CD34 promoter is present in early erythroid cells, EpoR is present in later erythroid cells, while c-kit is

present all through the erythroid maturity lineage. Up to now, the synthesised promoters were inserted into linearised pGL4.10 luciferase vector and cloned. The next stage is to analyse the activity of the promoters with a luciferase assay. Then the CMV promoter will be removed from pIRES2 vector and different promoters will be inserted upstream to the inserted *JAK2* coding sequence. The different constructs will be transfected into $CD34^{pos}$ cells and the expansion activity of the cells will be analysed and compared to control untransfected cells.

Lee and colleagues (2016) also reported that during the first 14 days there was no difference in the proportion of erythroblasts among the three types of transduced cells. Mature erythroid cells appeared at day 17 and more mature RBCs were observed in the control culture, suggesting delayed differentiation of cells transduced with wild-type and mutant *JAK2*. However on day 21, more mature RBCs were present in mutant *JAK2*-transduced cells than in wild-type *JAK2*-transduced cell cultures and control cell cultures. In addition the morphology of the cells was healthier for the mutant *JAK2*-transduced cell culture.

Interestingly, during one of the experiments of this research, it was observed that transient transfection of mutant *JAK2* into $CD34^{pos}$ cells enhanced survival of cells during erythroid selection. Does mutant *JAK2* induce a survival characteristic to erythroid cells? This observation correlates with the findings of Lee *et al.* (2016) and with Fernandez-Luna's group that observed an increased survival of erythroid-lineage cells derived from PV patients (Silva *et al.,* 1998).

6.3 Promoting Epo-independency of human erythroid progenitor cells

This study aspired to generate cells with a greater proliferative potential that could possibly lead to achieving Epo-independency, thus making the culture system more cost-effective. Culture medium is heavily supplemented with growth factors and other reagents, which make it quite expensive (Table 6.2). Epo is the most expensive supplement in a standard culture medium and by removing Epo from the medium, the cost would diminish considerably.

During this study, it was hypothesised that by introducing exogenous *JAK2* into HEPs, erythropoietin receptor will be constitutively active, possibly promoting Epo-independency. However, the cells did not expand even in the presence of Epo in the culture medium. Lee *et al*. (2016) cultured the transduced cells in the absence of Epo and the wild type *JAK2*- and mutant *JAK2*-transduced cells expanded similarly to control untransduced cells. On the other hand, when the cells were cultured in the presence of Epo, wild type *JAK2*- and mutant *JAK2*-transduced cells had greater proliferative capacity than control cells. This is supported by a study performed by Laubach *et al.* (2009) which concluded that erythroblasts derived from PV patients did not survive and proliferate when cultured in the absence of either Epo or SCF. Conversely, other studies show that PV erythroid cells grow in the absence of exogenous Epo (Bogeska and Pahl, 2013; Dupont *et al.*, 2007; James *et al.,* 2005; Levine *et al.,* 2005; de Wolf *et al.,* 1994), while other studies discuss that PV erythroid cells can grow dependent or independent of Epo (Fisher *et al.,* 1994; Cashman *et al.,* 1983).

Table 6.2: Cost of a standard complete medium required to produce one unit of red blood cells (RBC). (Timmins and Nielsen, 2009; Rousseau, Giarratana and Douay, 2014).

Reagent / Supplement	Cost per RBC unit	Percentage
Basic medium	€260	3.5%
Erythropoietin	€2450	33%
Stem cell factor	€1140	15.5%
Insulin	€240	3%
Interleukin-3	€640	9%
Transferrin	€1260	17%
Human serum albumin	€1420	19%
Total	**€7410**	**100%**

6.4 Mut*JAK2* expression prepares the cells for proliferation, differentiation and survival

Another objective of this research was to study differential gene expression following transfection with wild-type *JAK2* or mutant *JAK2* construct. RNA sequencing analysis was performed once on two samples and it provided some interesting observations when comparing mut*JAK2*-transfected $CD34^{pos}$ cells to wt*JAK2*-transfected $CD34^{pos}$ cells. Preferably $CD34^{pos}$ cells are sorted before RNA sequencing analysis. The decision to express the *JAK2* coding sequence in a GFP-encoding vector was to be able to sort transfected $CD34^{pos}$ cells through fluorescence. However, due to the low concentration of cells, no sorting was performed and $CD34^{pos}$ cells that were transfected with the wild-type and with the mutant *JAK2* constructs and were confirmed to be expressing the *JAK2* transcript (by the *in situ* hybridisation assay) were selected for RNA sequencing analysis.

Mut*JAK2*-transfected $CD34^{pos}$ cells were compared to wt*JAK2*-transfected $CD34^{pos}$ cells and a list of differentially expressed genes was obtained. No comparison to untransfected $CD34^{pos}$ cells was performed due to background noise. The top differentially expressed genes encode ribosomal proteins and translation factors, such as *RPL41* (ribosomal protein L41) which encodes a protein component of the large 60S ribosomal subunit (Sim *et al*., 2010), *RPS18* (ribosomal protein S18) which encodes a protein component of the 40S ribosomal subunit (NCBI, 2016b), *MTRNR2L2* which is transcribed into the large 16S mitochondrial ribosomal RNA (rRNA) (Kearsey and Craig, 1981), and *eEF1A1* (eukaryotic translation elongation factor 1, alpha-1) which encodes a protein that promotes binding of transfer RNA (tRNA) to the ribosomes during protein synthesis (Sasikumar, Perez and Kinzy, 2012). These ribosome and translation

factors are upregulated during proliferation of cells and overexpression is consistent with this activity. This observation indicates that $CD34^{pos}$ cells that were transfected with mut*JAK2* were being prepared for proliferation.

Using Ingenuity Pathway Analysis, the top 100 differentially expressed genes (Appendix E) were analysed and networks of the genes were obtained. Interestingly, the gene *NPM1* was observed in two selected networks. This gene encodes a phosphoprotein which shuttles between the nucleus and the cytoplasm (Grisendi *et al.,* 2006). NPM1 is a ubiquitous and multifunctional protein (Zhang *et al.,* 2012) involved in numerous cellular processes including control of cell growth and proliferation, cell differentiation and programmed cell death (You et al., 1999; Hsu and Yung, 1998; Liu and Yung, 1998). NPM1 is more abundant in tumour and proliferating cells than in normal resting cells. In fact, overexpression of NPM1 promotes cell growth and proliferation by enhancing ribosome biogenesis as a result of aiding the interactions between ribosomal proteins and rRNA (Lindström, 2011; Grisendi *et al.,* 2006). Overexpression of NPM1 also promotes cell survival through the inhibition of apoptosis (Grisendi *et al.,* 2006; Ye 2005). Using a murine model, Maggi *et al.* (2008) showed that when NPM1 expression is increased, export of newly synthesised RNAs is enhanced and the rate of protein synthesis is increased.

NPM1 is also overexpressed in many tumours of different origins (gastric, colon, liver, breast, ovarian, prostate, bladder, thyroid, brain and multiple myeloma) suggesting its role as a proto-oncogene (Yip *et al.,* 2011; Grisendi *et al.,* 2006). Genetic alterations in *NPM1* gene are found frequently in haematopoietic malignancies, especially in acute myeloid leukemia (AML) (Grisendi *et al*., 2006).

In addition, both networks show a connection between *NPM1* and eukaryotic elongation factor-1 (eEF-1) subunits *eEF1A1* and *eEF1G*. Both genes encode proteins of the eEF-1 complex and are responsible for the recruitment of aminoacyl-tRNAs to the ribosome during protein synthesis (Sasikumar, Perez and Kinzy, 2012).

The top pathways affected when comparing mut*JAK2*- and wt*JAK2*- transfected cells were eIF2 signalling, mTOR signalling and regulation of eIF4 and p70S6K signalling. These signals constitute part of the PI3K/PKB/mTOR pathway and lead to cell proliferation and enhanced survival of the developing erythroid progenitors (Somervaille, Linch and Khwaja, 2001). It has been established that in addition to activating the JAK/STAT pathway, the *JAK2*-V617F mutation also constitutively activates the PI3K/PKB/mTOR pathway, resulting in an increased expansion of affected cells. This aberrant activation of the PI3K/PKB/mTOR pathway has been documented in *JAK2*-V617F mutated cells (Bartalucci *et al.,* 2013; James *et al.,* 2005). Apart from the effect of the *JAK2*-V617F mutation on the PI3K/PKB/mTOR pathway, *NPM1* is also associated with this pathway and it is believed that it may play a critical role in the molecular mechanisms related to cell growth, proliferation and survival (Chan *et al.,* 2015).

When evaluating the over-expressed genes and their different interactions, a number of upstream regulators were observed, most of which are cytokines and involved in growth factor signalling, transcription factors, or enzymes. The top upstream regulators that are connected to the dataset genes were MYCN and MYC. The *MYC* family consists of three closely related transcription factors

encoding the proto-oncogenes *MYC*, *MYCN*, and *MYCL* (also known as c-*myc*, N-*myc*, and L-*myc*, respectively) (Edsjö *et al.,* 2004) which regulate transcription of specific target genes. MYC proteins are multifunctional and are involved in regulating cell growth and proliferation, apoptosis and differentiation (Dang *et al.,* 1999). Overexpression of *MYC* causes tumourigenesis by inducing hyperproliferation and transformation (Li, Boone and Hann, 2008). The MYCN protein has a role in the formation of tissues and organs during embryonic development and is necessary for normal development of the limbs, heart, kidneys, nervous system, digestive system and lungs. Overexpression of this gene is associated with a variety of tumours, most notably neuroblastomas (Kuzyk *et al.,* 2015; Edsjö *et al.,* 2004).

One of the transcriptional targets of MYC is *NPM1* which explains the association of NPM1 overexpression with increased proliferation (Zeller *et al*., 2001). In fact, several studies have shown that the expression of MYC correlates with the expression of NPM1 and that an increase in MYC expression results in elevated NPM1 expression (Neiman *et al.,* 2001; Guo *et al.,* 2000; Kim *et al.,* 2000). This positive regulation is achieved by the binding of MYC to *NPM1* promoter.

Interestingly, Epo, Kit ligand and Dex were also identified as upstream regulators that activate a number of the over-expressed genes including *NPM1.* Co-operation between Epo, SCF and Dex leads to an expansion of immature erythroid progenitors resulting in an increase in erythropoiesis (Kolbus *et al.,* 2003). The observation of these upstream regulators that activate *NPM1* and other connected differentially expressed genes in $CD34^{pos}$ cells transfected with mut*JAK2*, indicates that the cells were being prepared for erythroid expansion

and shows that mut*JAK2*-transfected cells have a more proliferative potential than wt*JAK2*-transfected cells. A summary of the RNA sequencing analysis is depicted in Figure 6.1.

The enhanced survival of mut*JAK2*-transfected cells observed in this study, can be attributed to the overexpression of *NPM1* and the activation of the PI3K/PKB/mTOR pathway. In addition, the enhanced activity of the regulators Epo, SCF and Dex, activation of the PI3K/PKB/mTOR pathway, overexpression of the transcription factor *MYC*, together with their association with *NPM1,* and *NPM1*'s connection with eEF-1 subunits *eEF1A1* and *eEF1G,* indicate that mut*JAK2*-transfected cells were being prepared for proliferation as all the mentioned activities are involved in promoting cell growth and proliferation, and an increase in erythropoiesis. RNA sequencing analysis was performed 48 hours post-nucleofection and the results show that mut*JAK2*-transfected cells were being programmed for proliferation. However, since transfection was transient, the mut*JAK2* ORF was lost through cell divisions. This could explain why the transfected cells did not show enhanced proliferation when cultured in ESD selective medium over the successive 29 days.

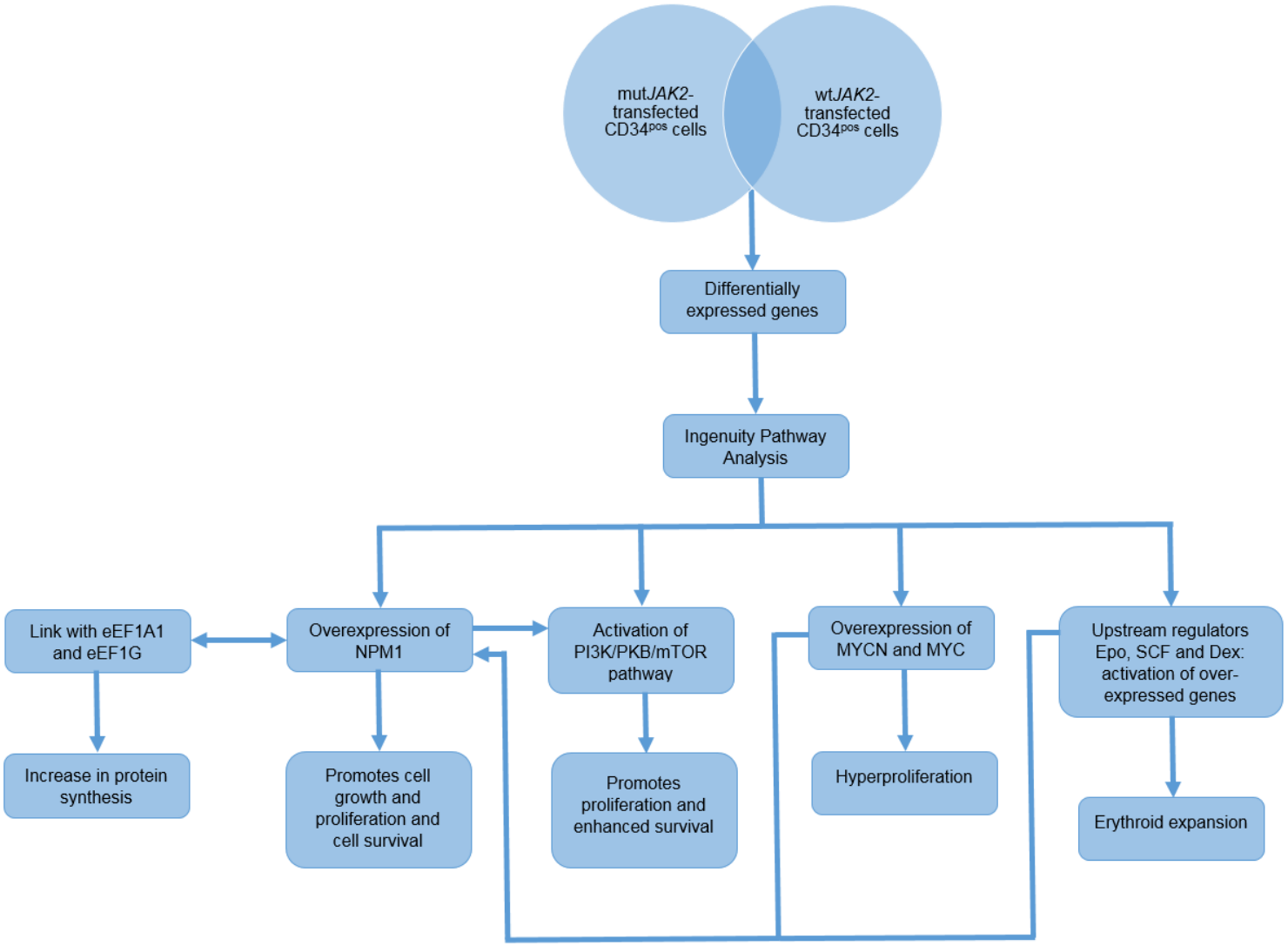


Figure 6.1: Summary of RNA sequencing analysis.

6.5 Transfusion of *in vitro* cultured cells

More than a decade of research about the *in vitro* culturing of RBCs led to considerable progress, suggesting that the *in vitro* generation of RBCs for transfusion may become a reality in the future. The first transfusion of cultured erythroblasts was accomplished in 2008 using an animal model, where Nakamura and colleagues successfully transfused mice suffering from haemolytic anaemia with erythroblasts generated *in vitro* from embryonic stem cells (Hiroyama *et al.*, 2008). The first-in-man injection of RBCs generated *in vitro* was reported in 2011 by Luc Douay and colleagues. A total of 10^6 $CD34^{pos}$ cells were isolated from the peripheral blood of a consenting donor. These cells were subsequently expanded and differentiated to 10^{10} RBCs and transfused into the same donor. The cultured RBCs survived *in vivo* as long as RBCs obtained by classical blood collection and behaved as normal RBCs when injected into the autologous recipient (Giarratana *et al.*, 2011). This was a major breakthrough and enhanced the possibility of cultured cells as an alternative transfusion product, however even though the cultured RBCs had similar morphological and functional characteristics to *in vivo* RBCs, based on the protocol of this study, therapeutic transfusion is still unrealistic. The safety of transfusing large number of cultured cells rather than an injection of small volume has not been assessed and this can create immunological problems and can also lead to neoplastic transformation (Tolar *et al.*, 2007). The long-term effects of administration of RBCs expanded *in vitro* remain unknown (Migliaccio *et al.*, 2012). Nevertheless, this study leads the way for industrial application due to the utilisation of a combination of nutrients and growth factors in the culture medium that are compatible with the automated, large-scale manufacturing process.

Another undergoing project led by Professor Forrester at the Centre for Regenerative Medicine at the University of Edinburgh follows previous research that proved that RBCs can be generated from HSCs and the promising results obtained by Luc Douay's team. Their aim is to improve the yield and reduce the cost of RBC production through the scaling-up of the RBC production process on a commercial scale (CRM, 2014). The first in-man trial was projected to take place by late 2016. Early trials would involve injecting patients with small amounts of the blood and then moving on to larger transfusions in a bigger group of people. Obviously, before starting the clinical studies, a lot of regulatory and quality requirements should be in place and the cultured RBCs have to be manufactured according to Good Manufacturing Practice (GMP) standards.

6.6 Large-scale production of transfusable red blood cells

Culture systems developed by Giarratana *et al.* (2005) and Miharada *et al.* (2006) produced efficient enucleation rates and reasonable yields of 1.95 x 10^6 FI and 0.72 x 10^6 FI producing 4.9 and 1.8 transfusable RBC units respectively. The culture conditions which generated the most erythroblasts (3.63 x 10^6 FI) with nearly a 100% enucleation rate, was developed in 2008 by Fujimi *et al.*, producing 1.76 x 10^{13} RBCs from one unit of cord blood (i.e. 8.8 transfusable units). However, such yield is insufficient to replace all the blood donations to meet the demand and the existing culture methods are unsuitable for large-scale manufacture.

Since large numbers of RBCs are required for transfusion, generation of RBCs in large numbers to viable industrial production and under GMP conditions still remains a major challenge. The number of RBCs necessary for transfusion (one

therapeutic unit contains on average 2 x 10^{12} RBCs (Migliaccio *et al.*, 2012; Whitsett, Vaglio and Grazzini, 2012; Giarratana *et al.*, 2005; Neildez-Nguyen *et al.*, 2002; Goodell, 1999)) cannot be easily produced and current technologies have only led to successful transfusion of cultured RBCs in mice.

Timmins and Nielsen (2009 and 2011) calculated that with current techniques that use static tissue culture flasks to produce artificial blood, 660 to 1000 litres of culture media would be needed to generate one RBC unit with a total cost of at least €7000 per unit (Mercier Ythier, 2015; Timmins and Nielsen, 2009). This is far more expensive than the price of a standard donated RBC concentrate unit, where in Malta it is estimated that it costs about €170, while in the UK it costs about €150 (NHSBT, 2015), in France it costs €180 (Mercier Ythier, 2015) and in the USA it costs about €200 (Whitaker and Hinkins, 2011). Hence, the current culture techniques are not feasible for the large-scale production of RBCs, though it would be practical to produce some units for the treatment of alloimmunised patients, patients with haemoglobinopathies and patients with rare blood phenotypes, as the price for a phenotypically-matched blood unit is estimated to be around €600 to €1000 (Zeuner *et al.,* 2012).

The solution to decrease the consumption of culture medium would be to develop suitable automated large-scale bioreactors that can control and monitor parameters such as temperature, pH, oxygen tension and waste products, which is essential to attain GMP standards (Timmins and Nielsen, 2009). In 2011 Timmins *et al.* developed the first large-scale bioreactor for HSC expansion and differentiation into RBCs, but these provided similar yields as in static cultures. After 21 days of culture, the FI was of 1.73 x 10^6 with a 90% enucleation rate,

which is similar to the study by Giarratana *et al.* (2005). While Giarratana *et al.* suggest that feeder cells are required for terminal maturation, Leberbauer *et al.* (2005) and Miharada *et al.* (2006) demonstrated that they are not necessary. Timmins *et al.* (2011) did not use feeder cells as it would be complicated to use them in a large-scale process and demonstrated that HSCs proliferated and matured efficiently in the absence of feeder-cells. The cultures were extended to day 33, achieving a FI of 2.25 x 10^8 (i.e. over 500 units of RBCs), however terminal enucleation was not efficient. The use of bioreactors for RBC culture is relatively a new area of study and to date, the large-scale production of transfusable RBCs in a cost-effective manner still remains a major challenge.

6.7 The importance of this study and future work

Active investigations aim to develop a cost effective automated industrial cell culture system that can mass-produce safe clinical-grade RBCs for transfusion purposes. Manufacturing blood on an industrial scale will help end shortages and also limit the transmission of infections, cause fewer allergic reactions, and reduce alloimmunisation. However, the highest possible standards of cell culture and manufacture must be achieved and issues relating to the possibility of neoantigen formation must be addressed.

When this study was initiated, it was the first trial to test the effect of wild-type and mutant *JAK2* transfected into peripheral blood-derived $CD34^{pos}$ cells. In April 2016, Lee *et al.* published a paper presenting a study similar to this, where they transduced cord blood-derived $CD34^{pos}$ cells with wild-type and mutant *JAK2.* However, this research is the first one to study the differential gene expression of mutant Jak2 against wild-type Jak2 expressing cells.

NPM1 mutations account for about 50 to 60% of adult AML (Pasqualucci *et al.,* 2008). Studies have also observed that the *JAK2*-V617F mutation can be found in AML patients although this is very rare (Pasqualucci *et al.,* 2008). The co-existence of *NPM1* mutations and the *JAK2*-V617F mutation has also been reported in AML (Schnittger *et al.,* 2011; Pasqualucci *et al.,* 2008; Vicente *et al.,* 2007) although this is extremely rare. The current study has showed an interesting association between the *JAK2*-V617F mutation and *NPM1* in the context of driving over-expansion of haematopoietic cells. In fact, it is the first study to show an association between the *JAK2*-V617F mutation and wild-type *NPM1* in this area.

The encouraging results obtained from this study and from the study by Lee *et al.* (2016), show a promising way to enhance proliferation of haematopoietic progenitor cells and achieve an efficient *in vitro* culture system. The production of a large volume of RBCs is still a major challenge and further research should be aimed to exploit to the maximum the proliferative and differentiation capacity of HSCs in order to obtain large amounts of RBCs in the shortest time possible. As already discussed, it would be very interesting to elucidate at what stage of cell maturity would exogenous Jak2 have an effect, as this would help to target the optimal cells in order to mass-produce RBCs efficiently. However, biosafety is of paramount importance and GMP, including quality control and quality assurance parameters, are needed to produce clinical-grade RBCs. This should encompass the choice and origin of stem cells. Ideally they should have unlimited availability, be suitable to attain full erythroid maturation and not be immunogenic.

The source of reagents, and the use of chemically-defined and non-xenogeneic media formulations is also important in order to produce products that can be used for clinical applications in a safe, efficient and reproducible manner since the final product is aimed to be used for human transfusion. Chemically-defined means that all the components and their concentrations are known and reproducible, while non-xenogeneic refers to a formulation that is free of non-human-derived components, denoting that factors are either of human origin or recombinant (made synthetically) (Baron, 2016). For therapeutic applications, the presence of non-human components is not acceptable due to the possibility of xeno-transfer of immunogens or pathogens.

During this research cells were cultured in serum-free medium that consists of IMDM with BSA, recombinant human insulin, human transferrin, and 2-mercaptoethanol. The medium was then supplemented with other additives depending on the aim of the culture. Supplements used include recombinant human cc100 cytokines, Epo, SCF, Dex, and a non-animal derived lipid mixture. In the early years when culture conditions to grow RBCs *in vitro* were being developed, Epo was obtained from the plasma of polycythaemic sheep. Eventually the genes encoding Epo, as well as SCF, were cloned and the proteins were produced through recombinant DNA technology (Migliaccio *et al*., 2011). Dex is also chemically synthesised making it appropriate to be used for the production of cultured RBCs. The culture used in this research contains BSA, which is widely used in routine cell culture practices. However, since BSA is a serum albumin protein derived from cows' blood, the medium is inadequate for the production of a clinical product due to the possibility of contamination by animal-derived infectious pathogens. In order to produce a non-xenogeneic

medium, BSA can be replaced by Human Serum Albumin as a protein source (Baron, 2016). However, this will make the medium too expensive and impractical for routine use. The complete reliance on recombinant supplements results in high costs which needs to be taken in consideration, particularly in a research context where funding may be limited.

Although cultured RBCs have shown clinical application potential, their use in transfusion requires further development. The first clinical use of *in vitro* generated RBCs is expected to be for alloimmunised patients and patients with rare blood phenotypes (Whitsett, Vaglio and Grazzini, 2012; Giarratana *et al.*, 2011; Migliaccio *et al.*, 2011). The results of several studies show that there is potential that cultured RBCs will replace RBC donations and eventually, transfusion products will become available for the general population in the future.

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APPENDICES

APPENDIX A:

EXPRESSION VECTORS

pIRES2-AcGFP1 vector

The pIRES2-AcGFP1 vector is a mammalian expression bicistronic vector which is 5307bp long. It contains an IRES of the encephalomyocarditis virus (ECMV) for expressing a gene, together with the *Aequorea coerulescens* green fluorescent protein (AcGFP1).

The IRES is situated between the MCS and the AcGFP1 coding region. The MCS in pIRES2-AcGFP1 is between the immediate early promoter of CMV and the IRES sequence.

pIRES2-AcGFP1 can be used to quickly identify cells expressing a gene of interest by screening for AcGFP1 fluorescence by flow cytometry or fluorescence microscopy. A gene of interest is cloned into the MCS of the vector and then the plasmid is transfected transiently into mammalian cells. The gene, together with the *AcGFP1* gene, is translated from a single bicistronic mRNA allowing the cells expressing AcGFP1 and the protein of interest to be selected efficiently.

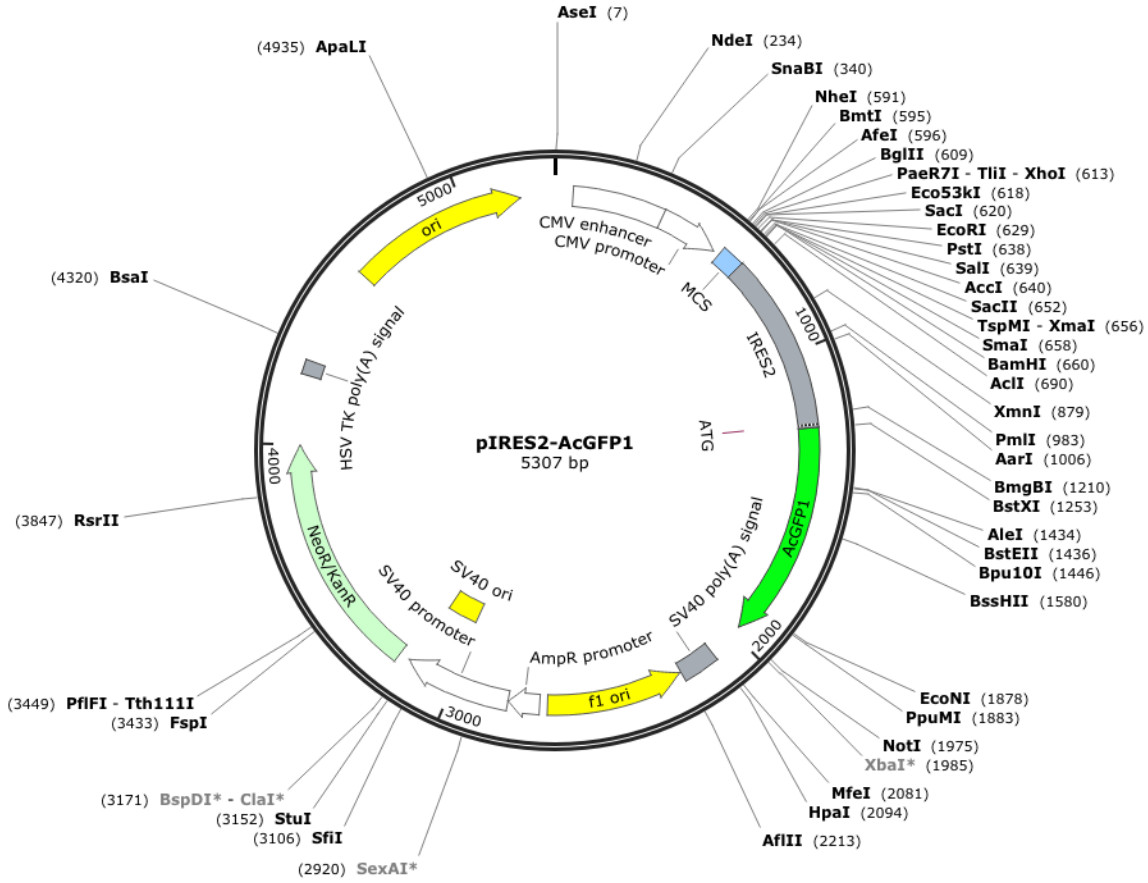

Figure A.1: pIRES2-AcGFP1 vector information. CMV enhancer/promoter: human cytomegalovirus immediate early promoter; MCS: multiple cloning site; IRES2: internal ribosomal entry site; AcGFP1: *Aequorea coerulescens* green fluorescent protein; SV40 poly(A) signal: early mRNA polyadenylation signal; f1 ori: single-strand DNA origin; AmpR promoter: bacterial promoter for expression of KanR gene; SV40 ori: SV40 origin of replication; SV40 promoter: early promoter/enhancer; NeoR/KanR: neomycin/kanamycin resistance gene; HSV TK poly(A) signal: herpes simplex virus thymidine kinase polyadenylation signal; ori: plasmid replication origin[1]. (Used with permission from SnapGene).

[1] Image obtained from http://www.snapgene.com/resources/plasmid_files/mammalian_expression_vectors/pIRES2-AcGFP1/

pcDNA3 vector

The pcDNA3 vector is a mammalian expression vector with the CMV promoter and a neomycin-resistance marker. This vector is 5446 bp long.

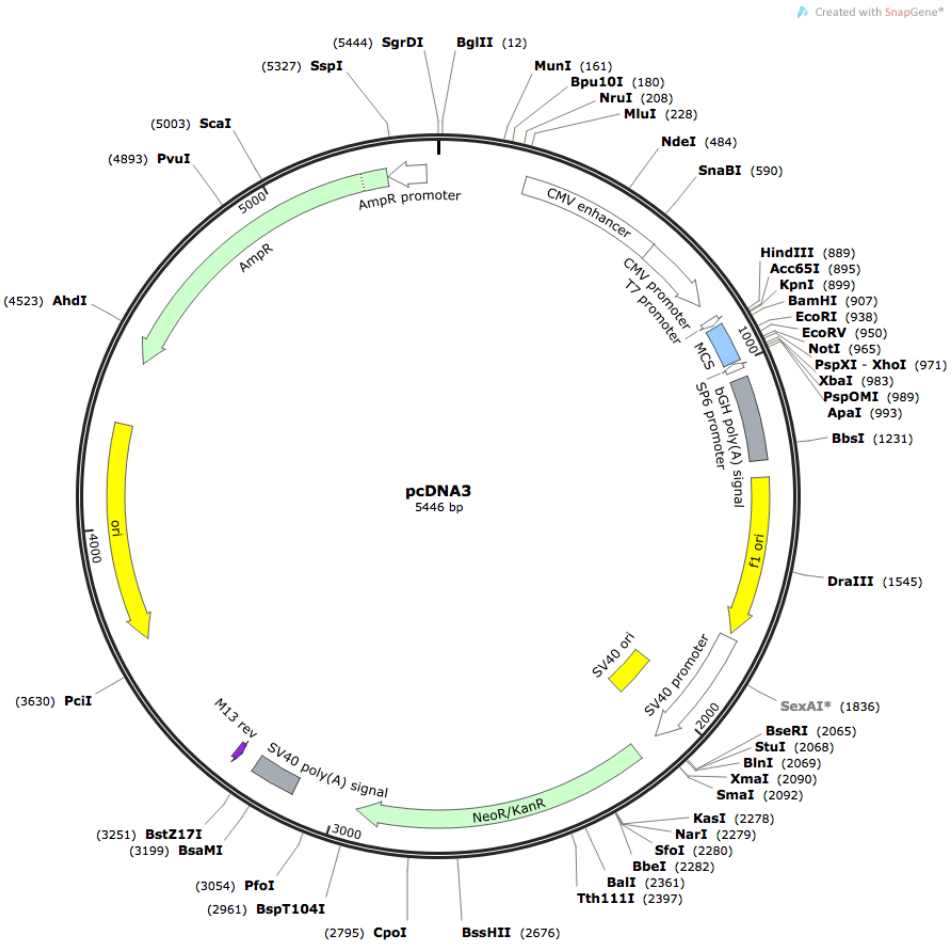

Figure A.2: pcDNA3 vector information. CMV enhancer/promoter: human cytomegalovirus immediate early enhancer and promoter; T7 promoter: promoter for bacteriophage T7 RNA polymerase; MCS: multiple cloning site; SP6 promoter: promoter for bacteriophage SP6 RNA polymerase; bGH poly(A) signal: bovine growth hormone polyadenylation signal; f1 ori: single-strand DNA origin of replication; SV40 promoter: Simian virus 40 early promoter; SV40 ori: Simian virus 40 origin of replication; NeoR/KanR: neomycin/kanamycin resistance gene; SV40 poly(A) signal: early mRNA polyadenylation signal; ori: plasmid replication origin; AmpR: ampicillin resistance gene; AmpR promoter: promoter for expression of AmpR gene [2]. (Used with permission from SnapGene).

[2] Image obtained from http://www.snapgene.com/resources/plasmid_files/basic_cloning_vectors/pcDNA3/

APPENDIX B:

DNA MOLECULAR MARKERS

DNA ladders data

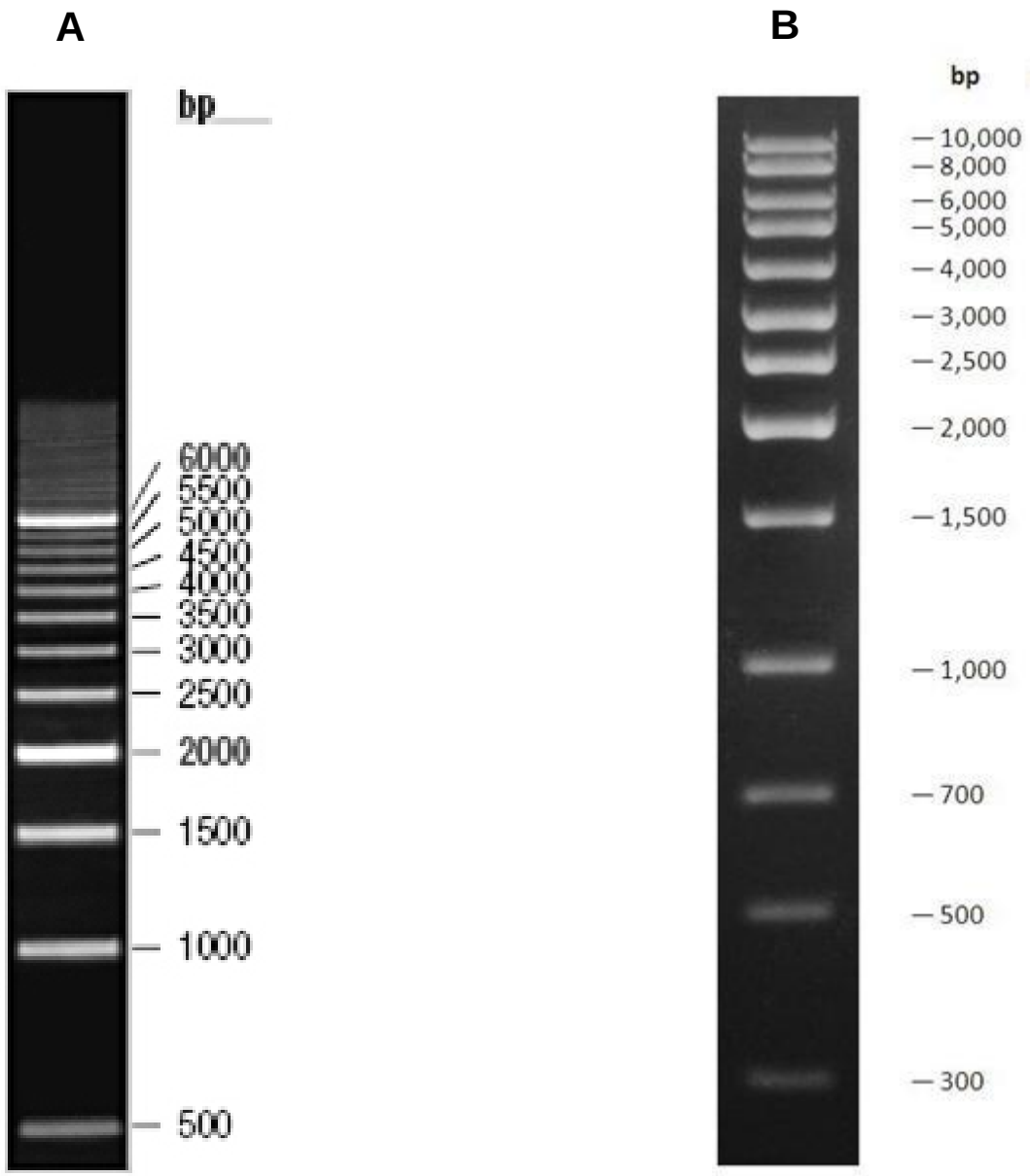

Figure B.1: DNA ladders. (A) O'RangeRuler 500bp DNA ladder (Thermo Fisher Scientific, USA). (Reproduced courtesy of Thermo Fisher Scientific; all rights on the image are owned by Thermo Fisher Scientific). **(B)** Solis BioDyne 1kb DNA ladder (Medibena, Austria). (Used with permission from Solis BioDyne).

APPENDIX C:

PREPARATION OF REAGENTS

Preparation of Luria-Bertani (LB) Broth

LB is a nutrient-rich microbial broth that contains peptides, amino acids, water-soluble vitamins, and carbohydrates in a low-salt formulation. LB is used for maintenance and propagation of *Escherichia coli*. The *E. coli* grow faster in LB because the tryptone and yeast supply essential growth factors that the *E. coli* would otherwise have to synthesise. Luria Broth also contains essential electrolytes for transport and osmotic balance, due to the NaCl component.

Components:

10 g/L Tryptone

5 g/L Yeast Extract

5 g/L NaCl

LB broth was prepared at a concentration of 20g / L by weighing 10g LB broth powder (Sigma-Aldrich, USA) and adding 500ml deionised water. The broth was sterilised by autoclaving at 121^{o}C for 20 minutes.

The autoclaved broth was left to cool and then the desired antibiotic was added at the required concentration (see below).

Preparation of Luria-Bertani (LB) Agar

LB agar is basically LB broth with the addition of agar to provide a solid medium for microbial growth.

Components:

15 g/L Agar

10 g/L Tryptone

5 g/L Yeast Extract

5 g/L NaCl

LB agar was prepared at a concentration of 35g / L. For about 20 petri dishes, 17.5g LB agar powder (Sigma-Aldrich, USA) was weighed and dissolved in 500ml deionised water. The liquid agar was sterilised by autoclaving at 121^{o}C for 20 minutes.

The autoclaved agar was left to cool in a water bath at 55^{o}C for about one hour. Then the desired antibiotic was added at the required concentration (see below). Finally, the agar was poured into petri dishes, left to cool down to solidify and stored at 2 to 8^{o}C.

Preparation of Ampicillin

This antibiotic was freshly prepared by weighing 0.1g Ampicillin Sodium Salt (Sigma-Aldrich, USA) and adding 1ml deionised water to get a stock concentration of 100mg/ml.

0.5µl of freshly-prepared ampicillin was added for every 1 ml of agar to obtain LB agar with 50µg/ml ampicillin.

1µl of freshly-prepared ampicillin was added for every 1 ml of broth to obtain LB broth with 100µg/ml ampicillin.

Preparation of Kanamycin

0.01g Kanamycin powder (Sigma-Aldrich, USA) was weighed and 1ml deionised water was added to get a stock concentration of 10mg/ml.

3µl of stock kanamycin was added for every 1 ml of agar to obtain LB agar with 30µg/ml kanamycin.

5µl of stock kanamycin was added for every 1 ml of broth to obtain LB broth with 50µg/ml kanamycin.

APPENDIX D:

ETHICAL APPROVAL, INCLUDING:

- FINAL LETTER OF APPROVAL,
- INFORMATION SHEET,
- CONSENT FORM,
- PERMISSION LETTER.

Letter of approval

L-UNIVERSITÀ TA' MALTA

Msida – Malta
Skola Medika
Sptar Mater Dei

UNIVERSITY OF MALTA

Msida – Malta
Medical School
Mater Dei Hospital

Ref No: 75/2011

22nd December, 2011

Ms. Sephora Aquilina
101 Shangri-la
Trincetta Street
Mosta
MST4356

Dear Ms. Aquilina,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

DEVELOPMENT OF IN VITRO LONG TERM CULTURES OF ERYTHROBLASTS FOR TRANSFUSION PURPOSES

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Dr. Mario Vassallo
Chairman
Research Ethics Committee

Email: umrec@um.edu.mt • Web: http://www.um.edu.mt/rec

Information Sheet in English

INFORMATION SHEET

I have been asked to participate in a research study entitled:

"Development of *in vitro* long term cultures of erythroblasts for transfusion purposes"

You are being asked to take part in this study and to help you decide if you want to agree to take part, we are providing you with information on how your participation will be helpful for this study.

Please take your time to read this sheet carefully.

The purpose and details of the study have been explained to me by _____ and any difficulties which I raised have been adequately clarified.

The Study

The demand for blood is always increasing and being the only National Blood Transfusion Service we are always in need of blood, to ensure a safe and sustainable supply for those who might need it. We rely on voluntary blood donors, however only a small percentage of the Maltese population donate blood. Red blood cell transfusion has become a routine and indispensable procedure for many clinical purposes; however, the supply of transfusable red blood cell units is not always sufficient.

The aim of this research is to study the effect of *JAK2* (mutant and wild-type) on cultured erythroid progenitor cells. The main goal is to understand the expression signatures that are different when comparing $CD34^{pos}$ cells that overexpress wild-type Jak2 and $CD34^{pos}$ cells overexpressing mutant Jak2 which is associated with polycythaemia vera disease.

What happens to the participant?

If you agree to participate in this study, you will be asked to sign an informed Consent Form. No further details or other blood samples will be collected; only the blood which you have given is needed for this study.

Demographics and Personal Data

No information will be required for this study. Your personal data has been registered at the Donation Area and stored in its database; however we will not have access to this database and hence cannot identify the donor. For further details, see section entitled "Data Protection and Privacy" below.

Blood Sample

We will only require the blood you have just given, which cannot be used for patient transfusion as it is an incomplete blood donation. No further samples will be required.

Proliferation & Differentiation of Cells

No genetic studies will be done during this research study. Mononuclear cells will be extracted from whole blood by gradient centrifugation. These cells will be cultured on selective media to achieve erythroid progenitor cells and they will be expanded. Certain cell types will then be targeted with wild type and mutant *JAK2* constructs, in order to try to achieve increased numbers of human erythroblasts in a short time. The cultured cells will also be allowed to differentiate and will be characterised during the process.

Benefits

This project aims to produce reticulocytes *in vitro* which will eventually lead to the production of erythrocytes that can be used to transfuse patients requiring red blood cells. A successful *in vitro* culture system will enable the production of red blood cell units from an individual to be used also for self-transfusion and it will also allow the banking of certain rare red cell phenotype combinations which would enable the production of red blood cells to be given to patients requiring them within a minimal time.

The culturing of these cells *in vitro* is also invaluable for investigating red blood cell development and diseases of the erythroid lineage, especially where only small amounts of blood may be available for study.

Risks, Inconveniences & Discomfort

There is no significant physical risk to your health for participating in this study. Only the usual risks, inconveniences and discomforts associated with a normal blood donation can be experienced.

Informed Consent

In order to participate in this study, you will be asked to provide your written consent on the attached consent form. You may ask any question and you can also contact the Chief Investigator whose details can be found towards the end of this sheet and on the consent form.

Data Protection & Privacy

This study has been approved by the University of Malta Research Ethics Committee and the Genetically Modified Microorganisms board.

The blood you have just given is labelled by a donation number. The consent form will also be labelled with this same donation number in order to identify it. However, we do not have access to the database which can link the donation number to the donor and hence you will not be personally identified in any way, either individually or collectively.

Chief Investigator:	Sephora Aquilina
Contact Address:	Donor Serology Laboratory National Blood Transfusion Service St. Luke's Hospital
Contact Number:	2595 1599 / 99880216

Information Sheet in Maltese

INFORMAZZJONI

Talbuni biex nieħu sehem fi studju ta' riċerka bl-isem ta':

"Development of *in vitro* long term cultures of erythroblasts for transfusion purposes"

Int qed tiġi mistoqsi biex tieħu sehem f'dan l-istudju. Aħna ser nispjegawlek fiex jikkonsisti dan l-istudju u ngħinuk tiddeċiedi jekk tridx tieħu sehem.

Jekk jogħġbok aqra dan li ġej.

L-għan u d-dettalji ta' l-istudju ġew spjegati lili minn _____ li wkoll iċċarali/iċċaratli xi mistoqsijiet li għamilt.

L-Istudju

Id-domanda għad-demm qed dejjem tikber u bħala l-uniku Ċentru ta' l-Għoti tad-Demm nazzjonali dejjem ikun hemm bżonn ta' demm, sabiex niżguraw li jkollna provvista ta' demm adekwata għal kull min jiġi bżonnu. Aħna dipendenti fuq nies li jiġu jagħtu d-demm volontarjament, imma sfortunatament huma persentaġġ żgħir tal-popolazzjoni Maltija li jagħtu d-demm. It-trasfużjoni ta' ċelloli ħomor kważi saret proċedura ta' rutina u hija indispensabbli għal ħafna każijiet kliniċi; sfortunatament il-provvista ta' ċelloli ħomor ma tkunx dejjem suffiċenti.

L-għan ta' dan l-istudju hija biex nistudjaw l-effett ta' *JAK2* (mutanti u normali) fuq ċelloli ħomor immaturi. L-għan huwa li nifhmu d-differenza ġenetika li tiġi espressa meta nqabblu ċellolli $CD34^{pos}$ li qed jipproduċu Jak2 normali ma' ċellolli $CD34^{pos}$ li qed jipproduċu Jak2 mutanti li huwa assoċjat mal-marda msejħa *polycythaemia vera*.

X'jiġri lill-pazjent?

Jekk inti taċċetta li tieħu sehem f'dan l-istudju, aħna ser nitolbuk biex tiffirma il-Formola tal-Kunsens. M'aħniex ser nistaqsuk għal iktar dettalji personali jew nitolbuk biex tagħti iktar demm; kulma għandna bżonn huwa dak id-demm li diġa tajt.

Dettalji Personali u Demografiċi

L-ebda informazzjoni mhi meħtieġa għal dan l-istudju. Id-dettalji personali tiegħek huma reġistrati u storjati fid-*database* ta' dan iċ-Ċentru; għalkemm aħna m'għandniex aċċess għal din id-*database* u għalhekk bl-ebda mod ma nistgħu nidentifikaw id-donatur. Għal aktar informazzjoni ara "Privatezza u Protezzjoni tad-Dettalji" iktar 'il quddiem.

Kampjuni tad-Demm

Għal dan l-istudju rridu biss dak id-demm li għadek kif tajt u li ma jistax jiġi mogħti lil xi pazjent għaliex hi “donazzjoni mhux kompluta”. Mhumiex meħtieġa iktar kampjuni ta’ demm.

Tkabbir u Żvilupp ta’ Ċelloli

F’dan l-istudju mhumiex se jsiru testijiet ġenetiċi. *Mononuclear cells* ser jiġu estratti mid-demm permezz ta’ metodu imsejjaħ *gradient centrifugation*. Dawn iċ-ċelloli ser jiġu mkabbra fuq *media* apposta sabiex ikollna ħafna ċelloli immaturi. Dawn imbagħad ser jiġu esposti għal *JAK2*, sabiex nippruvaw nipproduċu ammonti ikbar ta’ ċelloli ħomor immaturi f’temp qasir. Iċ-ċelloli li nkunu ipproduċejna mbagħad jitħallew jiżviluppaw u jiġu kkaratterizzati matul il-proċess.

Benefiċċji

L-għan ta’ l-istudju huwa li nipproduċu *reticulocytes in vitro* u li eventwalment inwasslu għal produzzjoni ta’ ċelloli ħomor li jistgħu jintużaw bħala terapija għall-pazjenti. Jekk din is-sistema tkun ta’ suċċess, inkunu nistgħu nipproduċu ċelloli ħomor minn individwu u li jkunu jistgħu jintużaw bħala terapija għalih stess. Ikun ukoll possibli li nżommu demm li jkollu profil ta’ *antigens* rari u meta jkun hemm pazjent li jkollu bżonn dan id-demm, ikun jista’ jiġi mmedikat f’qasir żmien.

Din is-sistema tkun ukoll ta’ valur kbir biex ikunu jistgħu jsiru aktar investigazzjonijiet fuq iċ-ċelloli ħomor u fuq mard assoċjat magħhom, speċjalment meta volum żgħir ta’ demm ikun disponibbli.

Riskju, Inkonvenjenza u Skumdita

Dan l-istudju ma jimponi l-ebda riskju għal saħħtek. L-uniċi riskji, inkonvenjenza u skumdita li tista’ tesperjenza huma dawk li normalment huma assoċjati ma’ donazzjoni tad-demm normali.

Formola tal-Kunsens

Biex tieħu sehem f’dan l-istudju, inti ser tiġi mitlub/a biex timla l-formola tal-kunsens li tinsab mehmuża. Tista’ tistaqsi li trid u tista’ wkoll tikkuntattja lil Investigatur Prinċipali li d-dettalji tagħha jinsabu fl-aħħar ta’ din l-ittra u fuq il-formola tal-kunsens.

Privatezza u Protezzjoni tad-Dettalji

Dan l-istudju ġie approvat mill-“*University of Malta Research Ethics Committee*” u l-bord inkarigat mill-użu ta’ “*Genetically Modified Microorganisms*”.

Id-demm li għadek kif tajt huwa mmarkat bin-numru tad-donazzjoni. Il-formola tal-kunsens ser tkun immarkata bl-istess numru, sabiex id-demm ikun jista’ jiġi identifikat. Madankollu, aħna mhux ser ikollna aċċess għad-*database* fejn tkun tista’ tidentifika d-donatur. Għalhekk inti b’ebda mod mhu ser tiġi identifikat/a, la individwalment u lanqas bħala parti minn grupp.

Investigatur Prinċipali: Sephora Aquilina

Indirizz: Donor Serology Laboratory
National Blood Transfusion Service
St. Luke's Hospital

Numru: 2595 1599 / 99880216

Consent Form in English

CONSENT FORM

I am a Maltese citizen and I am over eighteen (18) years of age. I have been asked to participate in a research study entitled:

"Development of *in vitro* long term cultures of erythroblasts for transfusion purposes"

I understand that the blood that I have just donated is considered as an "incomplete donation" which cannot be used for patient transfusion and thus will be discarded. I hereby give my consent to Sephora Aquilina to use this blood for her study and to derive cellular blood components.

I understand that the results of this study may be used for scientific purposes and that the results achieved from this study in which I am participating may be reported or published. However, I shall not be personally identified in any way, either individually or collectively.

I am under no obligation to participate in this study and am doing so voluntarily.

In case of any queries I may contact Ms Sephora Aquilina on telephone number 2595 1599 / 99880216.

_____ _____

Participant's Name Participant's Signature

_____ _____

Chief Investigator's Signature
SEPHORA AQUILINA
ID: 150882(M)

Supervisor's Signature
Dr GODFREY GRECH

_____ _____

Donation Number Date

Consent Form in Maltese

FORMOLA TAL-KUNSENS

Jiena ċittadin/a Malti/ja u għalaqt tmintax (18)-il sena. Talbuni biex nieħu sehem fi studju riċerka bl-isem ta':

"Development of *in vitro* long term cultures of erythroblasts for transfusion purposes"

Nifhem li d-demm li għadni kif tajt huwa kkunsidrat bħala "donazzjoni mhux kompluta" li ma tistax tingħata lill-pazjenti u għalhekk ħa tiġi mormija. Jiena qed nagħti l-kunsens tiegħi lil Sephora Aquilina biex tuża dan id-demm għar-riċerka tagħha biex tkabbar ċellolli tad-demm.

Jiena nifhem li r-riżultati ta' dan l-istudju jistgħu jintużaw għal skopijiet xjentifiċi u jista' jiġi ppublikat rapport bil-miktub. Jekk isir hekk b'ebda mod ma nista' nkun identifikat/a, individwalment jew bħala parti minn grupp.

Jiena m'għandi l-ebda dmir li nieħu sehem f'dan l-istudju u dan qed nagħmlu minn rajja.

Jekk ikolli xi diffikulta, nista' nikkuntatja lil Ms Sephora Aquilina fuq in-numri 2595 1599 / 99880216.

_____	_____
Isem tal-Parteċipant/a	Firma tal-Parteċipant/a
_____	_____
Firma tal-Investigatur Prinċipali SEPHORA AQUILINA ID: 150882(M)	Firma tas-Supervisor Dr GODFREY GRECH
_____	_____
Numru tad-Donazzjoni	Data

Permission Letter

National Blood Transfustion Centre
Guardamangia Hill, Pietà
PTA1314 Malta
Tel: 2124 8451
Fax: 2125 0163

Blood Donation Centre
St. Luke's Square, Guardamangia
PTA1010 Malta
Tel: 2123 4767
Fax: 2206 6216

Email:
nbts@gov.mt
Website:
www.health.gov.mt/nbts

11th August 2011

To whom it may concern:

I, the undersigned, as the Director of the National Blood Transfusion Centre (Malta), give my permission to Sephora Aquilina to use the incomplete blood donations for her research entitled "Development of *in vitro* long term cultures of erythroblasts for transfusion purposes", after obtaining the donors' consent.

Dr Alex Aquilina
Director NBTC (Malta)

APPENDIX E:

RNA SEQUENCING RAW DATA

Top 100 differentially expressed genes when comparing mut*JAK2*- versus wt*JAK2*- transfected CD34^{pos} cells

RPL41	TPT1	RPL7A
SPRR4	ANXA1	C6orf62
CST6	KRTAP9-3	ACTG1
SPINK5	TMEM45A	CTSL2
KRTAP4-1	CXCL14	KRT75
RPS18	B2M	SRSF6
DPYSL2	RPS11	MT1X
MTRNR2L2	RPL21	MUCL1
KRTAP3-1	UBB	S100A11
EEF1A1	RPL9	EIF3E
ANXA2	KRT6B	CAPZA1
RPL3	RPL30	NPM1
RPLP0	RPS8	HSP90AA1
RPS27	RPLP1	KRTAP9-9
RPL7	RPL4	RPL8
TIMP3	KRTAP3-2	UGDH-AS1
RPS6	MYL6	FABP5
RPL19	EEF1G	ENO1
MALAT1	SPRR1A	RPL10
KRT15	LY6G6C	RPL26
MTRNR2L8	UBC	RPS3A
KRTDAP	RPS2	FLG2
KRTAP9-4	RPL37A	NDUFA1
DSP	RPL11	RPL10A
KRT14	RPS4X	ARF6
KRT5	RPS25	RHOA
KRT6A	PERP	CALM2
TMSB4X	ASPRV1	TOB1
KRTAP3-3	LAPTM4A	RPS13
RPL23	GAPDH	RPL23A
ARPC2	PPP1CB	H3F3B
EFNA5	GNB2L1	PSME1
RPL5	HSP90AB1	ARF
ACTB		

APPENDIX F:

COLLABORATIVE INSTITUTES

Collaborations

- National Blood Transfusion Service (Malta).
- University of Malta: All laboratory work involved in this research was performed at the Laboratory of Molecular Pathology and the Allied Research Unit at the University of Malta.
- Sanquin Blood Supply (The Netherlands): Underwent a two-week internship at the Department of Haematopoiesis at the Division Research.
- Bioneer (Republic of Korea): Acquisition of *JAK2* primers and sequencing primers.
- Dr P.P. Sayeski at the University of Florida, College of Medicine (USA): Provision of the *JAK2* constructs.
- McGill University and Genome Quebec Innovation Centre (Canada): DNA sequencing.
- Affymetrix (USA): Design of the ViewRNA® Probe Human JAK2 and acquisition of the PrimeFlow™ RNA assay.
- BGI Genomics (Hong Kong): RNA sequencing.