Modelling severe paediatric aplastic anaemia using induced pluripotent stem cell technology

Dario Melguizo Sanchis BSc MSc

PhD Thesis

A thesis submitted for the degree of Doctor in Philosophy in the Institute of Genetic Medicine, Newcastle University

June 2017

Abstract

Aplastic anaemia (AA) is a disorder resulting in pancytopenia and hypocellular bone marrow. Although the immunological nature of AA pathogenesis is widely accepted, there is an increasing recognition that a significant number of AA patients might present dysfunctional haematopoietic stem or progenitor cells. In this study, induced pluripotent stem cell (iPSC) technology was used to reprogram fibroblasts from four paediatric severe AA (SAA) patients and three unaffected controls. SAA-iPSC lines were successfully differentiated into erythroid and myeloid progenitors and cells. Two key differences were observed in three of the four SAA patients: (1) SAA-iPSC generated a reduced number of erythroid and myeloid cells and (2) SAA-iPSC failed to elongate their telomeres during the reprogramming process. These deficiencies comprise two key features of AA and indicate that the iPSC model closely mimics the disease phenotype. These deficiencies also suggest that some (but not all SAA) may be characterised by an underlying genetic predisposition which impacts the proliferation and/or differentiation of erythroid and myeloid cells.

A detailed flow cytometric analysis indicated a significant reduction in the fraction of proliferative iPSC-derived-haematopoietic progenitors in three SAA patients. Likewise, significant levels of replicative stress-associated DNA damage were observed in iPSC-derived-haematopoietic progenitors from one of the SAA patients, which may suggest an impaired DNA damage response in the face of replicative stress.

Finally, thrombopoietin-receptor agonist eltrombopag was investigated in the iPSC model system and was shown to have no significant effect on the, proliferation, DNA repair and erythroid/myeloid colony-forming potential of SAA-iPSC derived haematopoietic progenitors under normal or stress conditions.

In summary, the data generated from this study highlights the utility of patient specific iPSC in providing a disease model for SAA, in identifying likely constitutional cases for further genetic studies and predicting patient specific responses to available and future drugs.

Acknowledgments

First of all I would like to thank my supervisor Professor Majlinda Lako for giving me the opportunity to undertake my doctoral studies under her supervision. I really appreciate her guidance, support and encouragement throughout my PhD without which I would have not achieved nearly as much. In addition, I would like to thank Dr. Sujith Samarasinghe and Professor Lyle Armstrong for their support and ideas. Thanks to my assessors during my PhD Dr. Colin Miles, Dr. David Steel and Dr. Caroline Relton for their advice and critique that has been a very important part of this study. I also thank Mr. Ian Dimmick, Dr. Andrew Filby, Dr. David McDonald, Mr. Lothar Marischen, Mr. Andrew Fuller and Mrs. Gill Hulme for their technical help and advice. Additionally I thank our collaborators Dr. Gabriele Saretzki, Dr. Carmen Martin-Ruiz, Dr. Jo Mountford and Dr. Scott Cowan for sharing their expertise and scientific knowledge. Special thanks to Dr. Kim Pearce for her scientific advice and patience.

I want to thank current and former members of the Stem Cell Group for their help and advice in times when it was especially needed. Special thanks to Mr. Dheraj Taheem, Dr. Jarmila Spegarova, Dr. Ellie Meader, Dr. Katarzyna Tilgner, Ms. Adriana Buskin, Dr. Min Yu and Dr. David Lee for sharing ideas and expertise. I would like to particularly thank Ms. Katja Gassner who has provided generous friendship and support and with whom I had numerous interesting discussions that will surely be missed.

I thank all my family and friends for their support and encouragement from the distance. And especially to my wife Alicia and my son Leo, because they make me persevere and thrive as we have always done. This work is as much theirs as it is mine.

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Abbreviations

γH2AX	Phosphorylated Histone Variant H2AX at Serine 139
AA	Idiopathic Aplastic Anaemia
AGM	Aorta-Gonad-Mesonephros
AMD	Acute Macular Degeneration
AML	Acute Myeloid Leukaemia
AP	Alkaline Phosphatase
APLN	Apelin Receptor
ATG	Anti-Thymocyte Globulin
bFGF	Basic Fibroblast Growth Factor
BFU-E	Burst Forming Unit-Erythroid
BMFS	Bone Marrow Failure Syndromes
BMP4	Bone Morphogenetic Protein 4
BrdU	5-Bromo-2-Deoxyuridine
Cas9	CRISPR Associated Protein 9
CAMT	Congenital Amegakaryocytic Thrombocytopenia
CD43	Leukosialin
CFU	Colony-Forming Unit
CFU-E	Colony Forming Unit-Erythroid
CFU-G	Colony Forming Unit-Granulocyte
CFU-GM	Colony Forming Unit-Granulocyte, Macrophage
CFU-GEMM	Colony Forming Unit-Granulocyte, Erythrocyte, Macrophage, Megakaryocyte
CFU-M	Colony Forming Unit-Macrophage
CGH	Comparative Genomic Hybridization
CN-LOH	Copy-Neutral Loss Of Heterozygosity
CNV	Copy Number Variation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI	4', 6-Diamino-2-Phenylindole (DAPI)
DBA	Diamond-Blackfan Anaemia

DC	Dyskeratosis Congenita
DMSO	Dimethyl Sulfoxide
DSB	DNA Double-Strand Break
EB	Embryoid Bodies
ECC	Embryonic Carcinoma Cells
EGC	Embryonic Germ Cell
EHT	Endothelial To Haematopoietic Transition
ELISA	Enzyme-Linked Immunosorbent Assay
EP	Eltrombopag
ERG	ETS Transcription *Factor*
EryP	Erythroid Progenitors
Ery/MkP	Erythroid/Megakaryocytic Progenitors
ESC	Embryonic Stem Cell
ETV2	ETS Variant 2
FA	Fanconi Anaemia
FACS	Fluorescence-Activated Cell Sorting
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
FGF2	Fibroblast Growth Factor-2
Flt3-L	Fms-Related Tyrosine Kinase 3 Ligand
FSC-A	Forward Scatter Area
FSC-H	Forward Scatter Height
G-CSF	Granulocyte Colony-Stimulating Factor
GATA2	GATA Protein 2
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HbF	Foetal Haemaglobin
HDF	Human Dermal Fibroblast
HE	Haemogenic Endothelium
HLA	Human Leukocyte Antigen
HMVP	Haematovascular Mesodermal Progenitors
HOXA9	Homeobox A9

HOXB4	Homeobox B4
HPC	Haematopoietic Progenitor Cell
HR	Homologous Recombination
HSC	Haematopoietic Stem Cell
HSCT	Haematopoietic Stem Cell Transplantation
HSPC	Haematopoietic Stem And Progenitor Cells
HU	Hydroxyurea
IBMX	3-Isobutyl-1-Methylxanthine
ICM	Inner Cell Mass
IFN-γ	Interferon Gamma
IL-3	Interleukin-3
IL-6	Interleukin-6
Inhibitor VII	GSK3β Inhibitor
iPSC	Induced Pluripotent Stem Cells
IRF-1	Interferon Regulatory Factor-1
IST	Immunosuppressive Therapy
ITP	Immune Thrombocytopenia Purpura
LCOR	Ligand Dependent Nuclear Receptor Corepressor
MDS	Myelodysplastic Syndrome
MEF	Mouse Embryonic Fibroblast
miRNA	Microrna
MkP	Megakaryocytic Progenitors
mRNA	Messenger RNA
MSC	Mesenchymal Stem Cells
MYB	MYB Proto-Oncogene
MyeP	Myeloid Progenitors
NHEJ	Non-Homologous End Joining
NE	Neutrophil Elastase
NGS	Next-Generation Sequencing
NO	Nitric Oxide
NOS	Nitric Oxide Synthase

NSAA	Non-Severe Idiopathic Aplastic Anaemia
PARP	Poly (ADP-Ribose) Polymerase-1
PCR	Polymerase Chain Reaction
PDGFR	Platelet-Derived Growth Factor Receptor
PFA	Paraformaldehyde
PGC	Primordial Germ Cell
PNH	Paroxysmal Nocturnal Haemoglobinuria
PS	Primitive Streak
PSC	Pluripotent Stem Cell
qPCR	Quantitative Polymerase Chain Reaction
RPA	Replication Protein A
RPE	Retinal Pigment Epithelium
RORA	RAR-Related Orphan Receptor A
RT	Reverse Transcription
RUNX1	Runt-Related Transcription Factor 1
SAA	Severe Idiopathic Aplastic Anaemia
SCF	Stem Cell Factor
SCID	Severe Combined Immunodeficiency Disease
SCN	Severe Congenital Neutropenia
SCNT	Somatic Cell Nuclear Transfer
SDS	Schwachman-Diamond Syndrome
SeV	Sendai Virus
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variation
SOX4	Sry-Box 4
SOX17	SRY-Box 17
SPI1	Spi-1 Proto-Oncogene
SSEA	Stage Specific Embryonic Antigen
SSEA-4	Stage Specific Embryonic Antigen-4
T	Brachyury
TAL1	T Cell Acute Lymphocytic Leukaemia 1

TIF	Telomere-Induced Foci
TNAP	Tissue Non-Specific Alkaline Phosphatase
TNF-α	Tumour Necrosis Factor Alpha
TPO	Thrombopoietin
TRAP	Telomere Repeat Amplification Protocol
VEGF	Vascular Endothelial Growth Factor
VSAA	Very Severe Idiopathic Aplastic Anaemia
WES	Whole Exome Sequencing
YS	Yolk Sac

Chapter 1. Introduction

1.1 Aplastic anaemia

1.1.1 Definition

Aplastic anaemia is a rare and heterogeneous disorder defined as a clinical syndrome that results from a marked reduction of marrow blood cell production and characterized by low blood cell counts (pancytopenia) and hypocellular bone marrow (**Figure 1**) in the absence of neoplasia and reticulin fibrosis (Killick *et al.*, 2016).

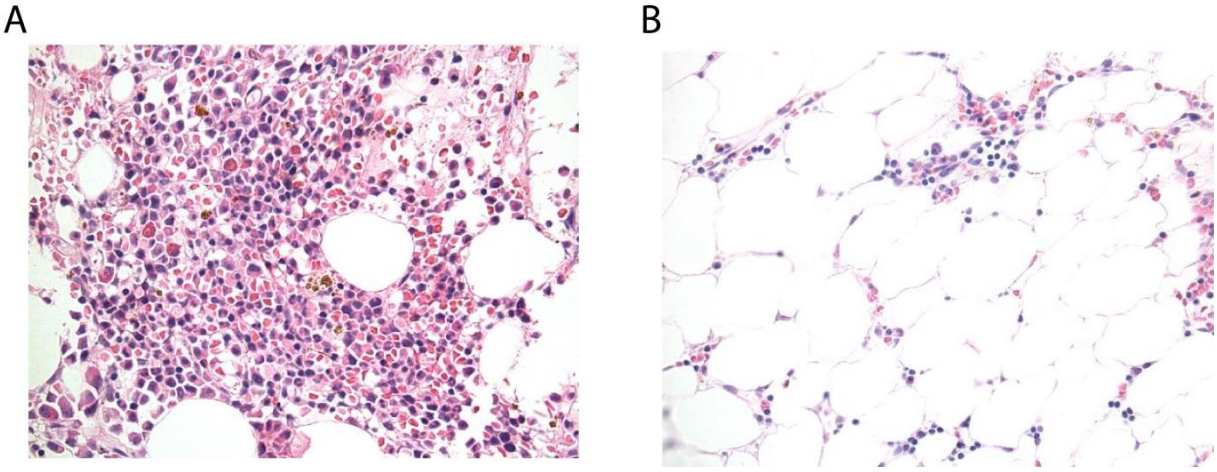


Figure 1. Normal and aplastic anaemia bone marrow biopsies.

(A) Normal bone marrow characterised by presence of haematopoietic cells and fat cells; (B) Aplastic anaemia bone marrow with extremely reduced number of haematopoietic cells and elevated presence of fat cells. Reproduced from Raghupathy *et al*. 2012 (Raghupathy and Derman, 2012)

1.1.2 Classification of aplastic anaemia

According to its aetiology, aplastic anaemia can be classified as inherited or acquired (**Table 1**). Inherited syndromes represent a 15-20% of aplastic anaemia cases the most common being Fanconi anaemia (FA) followed by telomeropathies such as dyskeratosis congenita (DC). On the other hand, aplastic anaemia might be acquired by exposure to environmental factors such as radiotherapy, drugs, toxins or viruses that would lead to the destruction of the haematopoietic stem cells (HSC) in the bone marrow. However, they account for a small fraction of the cases and no causal aetiology to the pathogenesis of acquired aplastic anaemia has been found for any of these agents (Young *et al.*, 2008). To date 70-80% of the total cases of diagnosed aplastic anaemia account for idiopathic cases where the aetiology remains unknown

or poorly understood (Marsh *et al.*, 2009). In this study we will focus on the study of the potential pathogenic mechanisms associated with idiopathic aplastic anaemia (AA).

Aetiology	Syndrome	Cause associated
Inherited	Fanconi Anaemia (FA)	Mutations in FANC complementation groups
	Dyskeratosis congenita (DC)	Mutations in DKC1, TERC, TERT, NOP10, ACD, NOLA2, NOLA3, TCAB1, TINF2, RTEL1 genes
	Schwachman-Diamond syndrome (SDS)	Mutations in SBDS gene
	Congenital amegakaryocytic thrombocytopenia (CAMT)	Mutations in MPL gene
Acquired	Acquired aplastic anaemia	Environmental factors (irradiation, drugs, chemicals, viruses)
	Idiopathic aplastic anaemia (AA)	Unknown

Table 1. Classification of aplastic anaemia according to aetiology

1.1.3 Epidemiology and clinical presentation of AA

1.1.3.1 Epidemiology

The incidence rate of AA is 2-3 per million per year in Europe and North America and 2-3 fold higher in East Asia (Montane *et al.*, 2008). Incidence of AA follows a bimodal distribution, with peaks among young adults (from 10 to 25 years) and elderly patients (over 60 years), and incidence ratio of approximately of one between male and female in all modern studies (Young and Kaufman, 2008).

1.1.3.2 Clinical presentation

Patients with AA show a clinical presentation related to anaemia due to the low numbers of red blood cells, skin/mucosal/retinal haemorrhage caused by the thrombocytopenia, and recurrent neutropenia-related infections (Killick *et al.*, 2016). Likewise, AA patients do not present abnormal lymph nodes (lymphadenopathy) or enlarged liver and spleen (hepatosplenomegaly) in the absence of infection (Gordon-Smith, 1991).

1.1.4 Diagnosis and management of AA

1.1.4.1 Diagnosis of AA

AA diagnosis is characterised by neutrophil count fewer than $1.5x10^9$/L, a haemoglobin concentration less than 100g/L and a platelet count fewer than $50x10^9$/L according to established Camitta criteria (Camitta *et al.*, 1975) and two lineages in peripheral blood cells must be affected. The number of lymphocytes does not appear to be affected in AA patients. Based on the blood counts and the degree of marrow hypocellularity, AA has been stratified into moderately severe, severe and very severe (Camitta *et al.*, 1975; Bacigalupo *et al.*, 1988) (**Table 2**).

Degree	Bone marrow cellularity	Blood cell count	
Non-severe AA (NSAA)	Patients not fulfilling the criteria for severe and very severe AA		
Severe AA (SAA)	<25% Or 25-50% with <30% residual haematopoietic cells compared to normal controls	At least one of the following	Neutrophils $<0.5x10^9/L$
			Platelets $<20x10^9/L$
			Reticulocytes $<20x10^9/L$
Very Severe AA (VSAA)	<25% Or 25-50% with <30% residual haematopoietic cells compared to normal controls	At least one of the following	Neutrophils $<0.2x10^9/L$
			Platelets $<20x10^9/L$
			Reticulocytes $<20x10^9/L$

Table 2. Degrees of severity of AA according to bone marrow cellularity and blood cell counts

However, appropriate diagnosis and management of AA can be challenging since clinical features as hypocellular bone marrow and low blood cell counts can be also found in inherited and clonal disorders associated with aplastic anaemia (**Figure 2**). This overlap observed in AA and inherited forms of aplastic anaemia, referred as bone marrow failure syndromes (BMFS) thereinafter, such as DC and Schwachman-Diamond syndrome (SDS), is likely due to shared pathogenic mechanisms (Young *et al.*, 2008). Likewise, reduced stem cell compartment, which is characteristic for AA, can favour the evolution to clonal disorders such as myelodysplastic syndrome (MDS) paroxysmal nocturnal haemoglobinuria (PNH) and acute myeloid leukaemia (AML).

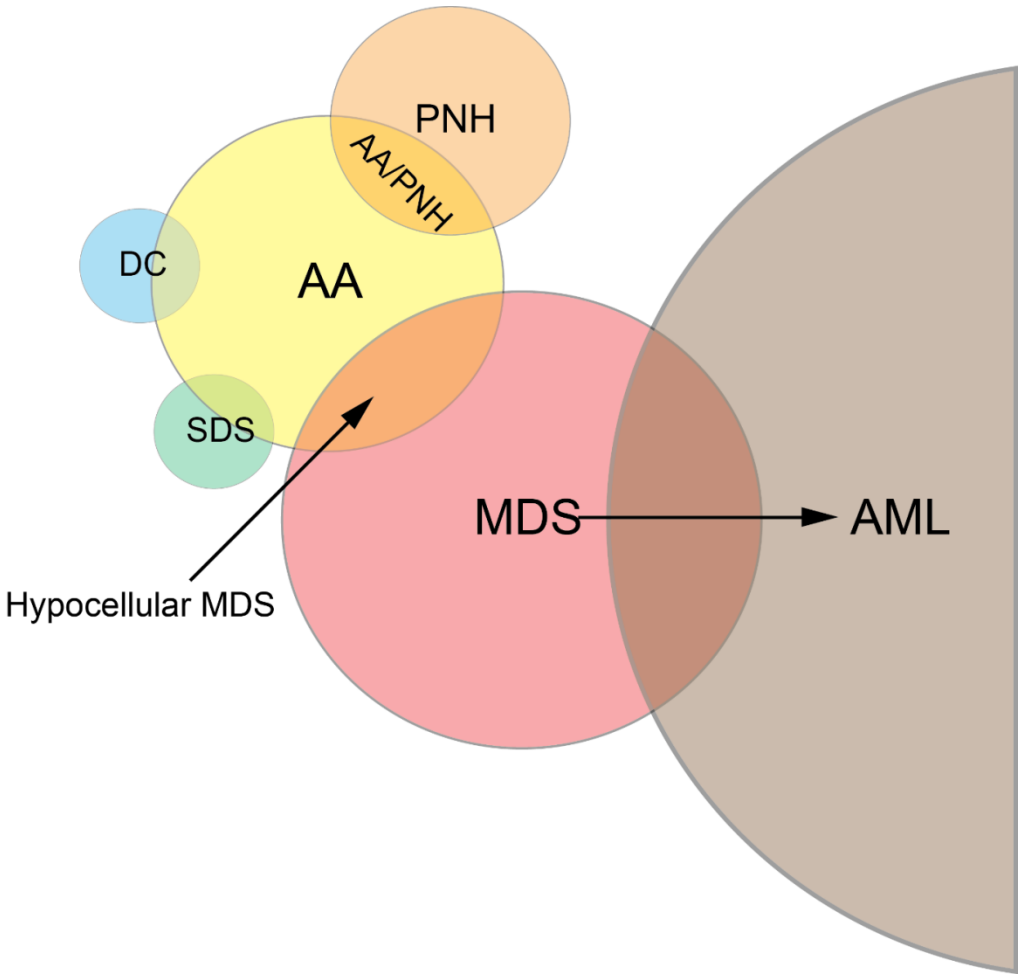


Figure 2. Clinical association of AA with inherited aplastic anaemia and clonal disorders.
DC, dyskeratosis congenita; SDS, Schwanman-Diamond syndrome, AA, idiopathic aplastic anaemia; PNH, paroxysmal nocturnal haemoglobinuria; MDS, myelodysplastic syndrome; AML, acute myeloid leukaemia. Adapted from Young *et al.* 2006 (Young *et al.*, 2006)

AA is diagnosed by exclusion. The British Journal of Haematology published in 2009 the AA diagnosis and management guidelines as a series of tests recommended to confirm the clinical manifestation of AA and exclude other causes of pancytopenia and bone marrow hypocellularity as well as assessment of the severity of the disorder (Marsh *et al.*, 2009) (**Figure 3**). Likewise, in order to rule out influence of drugs or chemicals that might be involved in the aetiology of the AA, careful drug and occupational exposure history would be required during diagnosis.

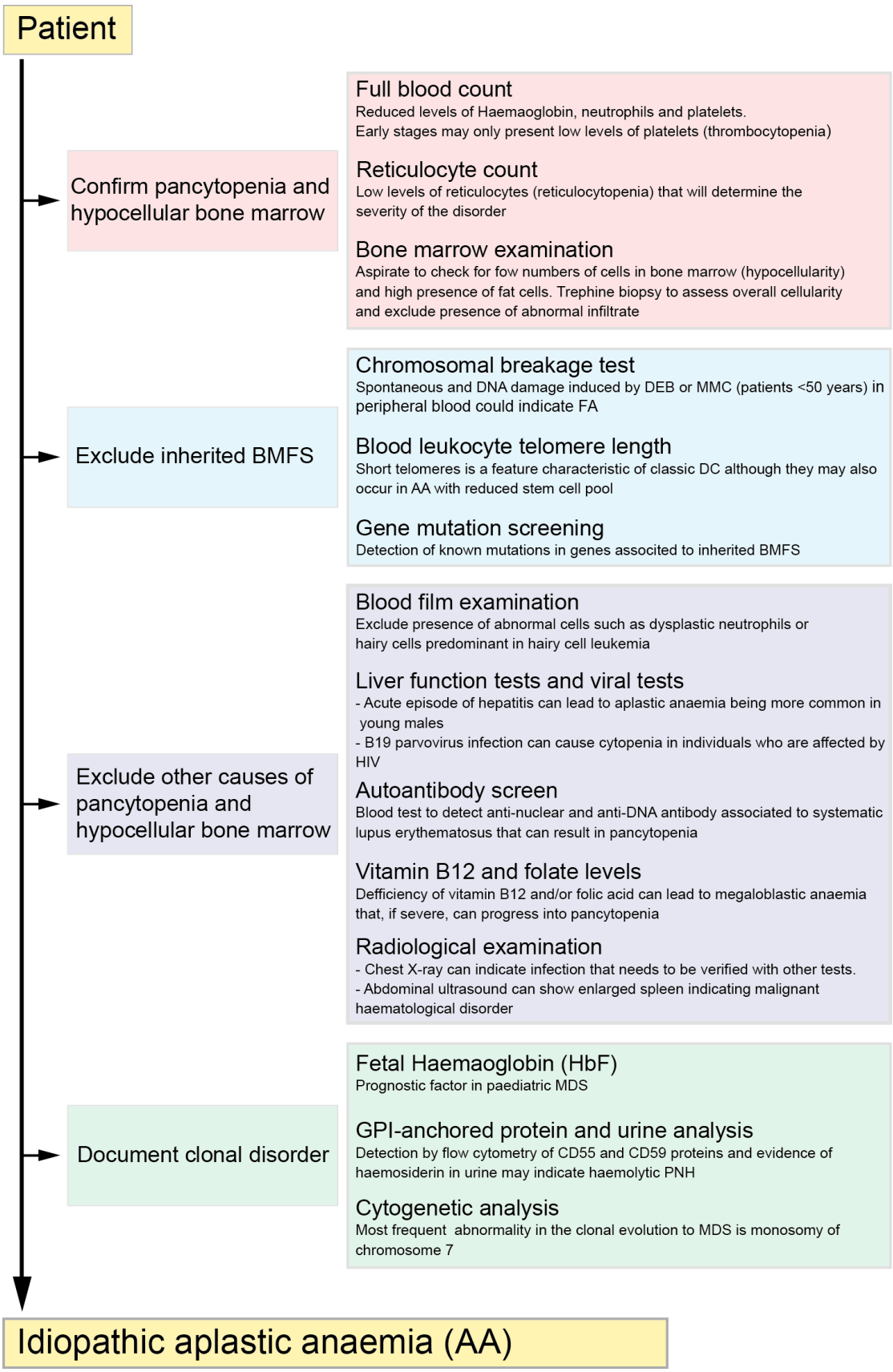


Figure 3. Diagnosis of AA.

DEB, diepoxybutane; MMC, mitomycin C; FA, Fanconi anaemia; DC, dyskeratosis congenital; BMFS, bone marrow failure syndrome; HIV, human immunodeficiency virus; MDS, myelodysplastic syndrome; GPI, glycerophosphatidylinositol; PNH: paroxysmal nocturnal haemoglobinuria

Patients with BMFS usually present physical features such as short stature, skin areas with hyper/hypo pigmentation and skeletal abnormalities in FA and dystrophy, oral leucoplakia and reticular skin pigmentation in DC (Shimamura and Alter, 2010). Clinical assessment for these characteristic features together with the required family history for potential existence of other family affected members can confirm the diagnosis of BMFS. However, BMFS patients present a high degree of heterogeneity in the disease phenotype with different genes potentially associated with the aetiology of the disorder (Vulliamy *et al.*, 2006). Additionally, physical features associated with BMFS may present a late onset or may remain absent even in adult life making necessary the use of additional tests for diagnosis (Zhang *et al.*, 2015). This makes the distinction of AA and BMFS rather difficult leading to misdiagnosis of BMFS cases as AA and inappropriate treatment. Thus, Fogarty *et al.* reported in 2003 that five percent of AA patients are undiagnosed cases of BMFS where the established abnormal phenotype characteristic of these patients was not apparent (Fogarty *et al.*, 2003).

Alternatively, AA may present or evolve to clonal disorders such as MDS, PNH and AML (**Figure 2**). Hypocellular MDS patients can present a profound cytopenia and extreme hypocellular bone marrow making it difficult to distinguish from AA in clinical diagnosis. However, there are features characteristic of hypocellular MDS such as dysplastic megakaryocytes, increased blasts in the bone marrow or blood and presence of reticulin fibrosis in trephine samples that are not common in AA patients helping in the diagnosis (Bennett and Orazi, 2009). In recent years, different studies have shown that around 20-25% of AA patients present acquired somatic mutations in genes such as *ASXL1* and *DNMT3A* which could predispose to clonal evolution to MDS (Kulasekararaj *et al.*, 2014; Yoshizato *et al.*, 2015). Around 40-50% of AA patients have PNH clones resulting for the somatic *PIG-A* mutations in HSC (Young, 2005). However, the presence of these PNH clones does not necessarily imply the clinical manifestation of PNH since the number of these clones can reduce, remain stable or even disappear. In contrast, an expansion of the PNH clones in the multipotent HSC population can lead to the evolution of AA into the classic PNH (AA/PNH) with clinical manifestation of the characteristic haemolysis of PNH (Pu *et al.*, 2012). Mechanisms leading to this clonal expansion and advantage of the PNH clones in the HSC compartment of AA patients remain unknown although it has been hypothesized that

the absence of GPI proteins in PNH clones provides a mechanism to escape the immune attack associated with AA pathogenesis (Murakami *et al.*, 2002).

1.1.4.2 Management of AA

AA patients undergo supportive care such as packed red blood cell transfusions in order to alleviate anaemia symptoms and platelet transfusions when platelet levels are below $10x10^9$/L or in case of frequent bleeding and bruising to improve symptoms associated with thrombocytopenia (Miano and Dufour, 2015). Prevention of infection is highly considered in patients with severe neutropenia as well as granulocyte transfusions for those affected by infections (Quillen *et al.*, 2009) since it is the major cause of mortality in AA patients (Dezern and Brodsky, 2011). However, more curative treatments have been developed to overcome the disorder and restore permanently normal levels of blood cells in AA patients. The choice of treatment to apply to AA patients depends on several factors such as the severity of the disease, the age of the patient and the availability of a suitable human leukocyte antigen (HLA)-matched HSC donor (Marsh *et al.*, 2009). Patients with non-severe AA are advised regular follow-ups if they are not transfusion dependent (Kwon *et al.*, 2010). For patients with severe AA (SAA) and very severe AA (VSAA) two main options of treatment are considered depending on the factors mentioned above: syngeneic or allogeneic haematopoietic stem cell transplantation (HSCT) and immunosuppressive therapies (IST) (Marsh *et al.*, 2009) (**Figure 4**).

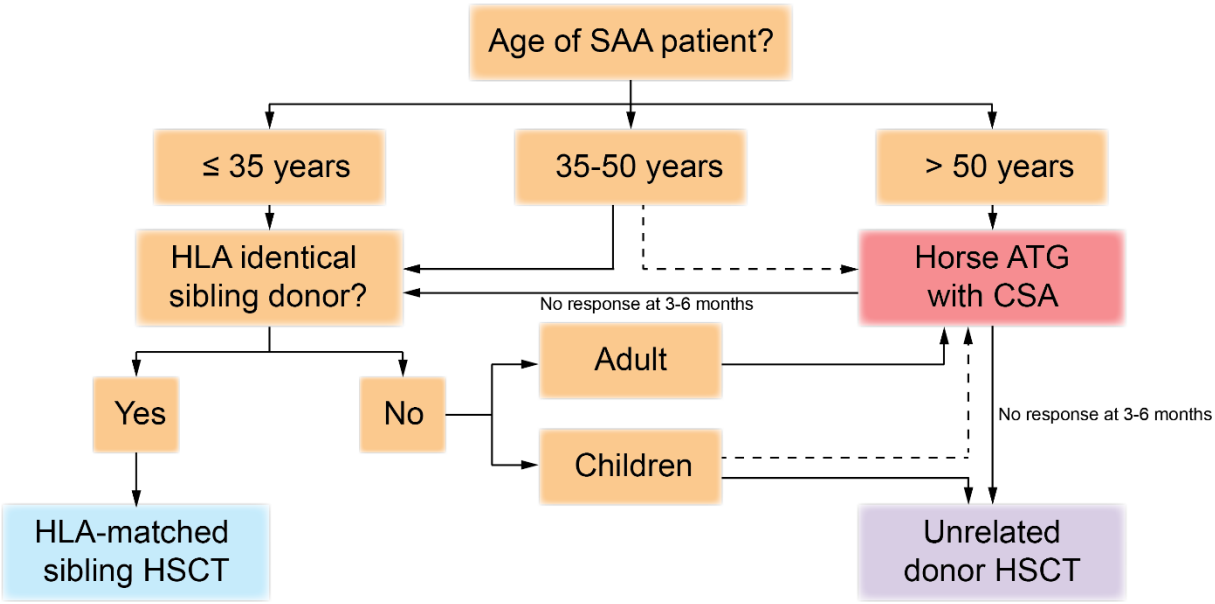


Figure 4. Proposed algorithm for treatment of SAA patients.

Solid lines indicate first-line treatment whereas dashed line indicates alternative treatment. SAA, severe idiopathic aplastic anaemia; HLA, human leukocyte antigen; HSCT, haematopoietic stem cell transplantation; ATG, anti-thymocyte globulin; CSA, cyclosporine. Adapted from Killick *et al.* 2016 (Killick *et al.*, 2016)

The first-line of treatment for SAA/VSAA patients <35 years is HSCT from HLA-matched sibling donor if available with 70-80% survival rate reported (Gupta *et al.*, 2010). This approach has serious complications such as rejections and chronic graft-versus-host disease (Young *et al.*, 2010). IST is the treatment of choice for SAA/VSAA patients >50 years and second-line treatment for adult patients 35-50 years who do not have a HLA-matched sibling donor (Sureda *et al.*, 2015). Combination of horse anti-thymocyte globulin (ATG) and cyclosporine (CSA) represents the first line of IST with a response of 50% in the first three months and around 75% in 6 months (Young *et al.*, 2010). However, AA patients might show refractory to IST treatment and cytopenia-associated symptoms might persist after 3-6 months. In the cases with lack of IST response, HSCT from HLA-matched or alternatively unrelated donor is recommended if available. Likewise, if no suitable donor can be found, a second course of IST is advised using rabbit or horse ATG (Killick *et al.*, 2016). Alternate approaches can be used for patients who failed to respond to a second round of IST including the use of experimental drugs such as eltrombopag (EP) and danazol.

EP is a non-peptide molecule mimetic to thrombopoietin (TPO). It has an additive effect to TPO since it does not bind at the same domain of the TPO receptor (MPL). Activation

of TPO signalling leads to megakaryocyte expansion and maturation as well as platelet release (Erickson-Miller *et al.*, 2009). Haematopoietic stem and progenitor cells (HSPCs) harbour TPO receptors (Zeigler *et al.*, 1994) and TPO signalling has been shown to be critical for HSC homeostasis and expansion (Alexander *et al.*, 1996; Qian *et al.*, 2007). EP has been originally used to treat immune thrombocytopenia purpura (ITP), an autoimmune disorder characterized by a destruction of platelets and inhibition of platelet production mediated by antibodies (Zhang and Kolesar, 2011). Recently, Desmond *et al.*, in a recent update of the Phase II study originally published by Olnes *et al*, confirmed the efficacy of EP in stimulating bi- and trilineage haematopoiesis by increasing platelet and neutrophil counts and haemoglobin levels with 40% of SAA patients showing response at 3-4 months (Desmond *et al.*, 2014). Likewise, an increase in the bone marrow cellularity in some of the AA responders to EP was observed. Interestingly, patients that were withdrawn of EP showed normal stable blood counts and normocellular bone marrow after 24 months off drug. The authors of the study hypothesized that EP favours the generation of certain number of HSC that can sustain normal levels of blood cells once the patients are removed from treatment (Desmond *et al.*, 2015). Likewise, it has been suggested that EP might increase regulatory T cell activity in AA patients restoring immune tolerance as demonstrated by the inhibition of autologous activated T cells by TPO-receptor agonists in ITP patients (Marsh and Mufti, 2014). However, there is a general concern regarding clonal evolution since around 20% of the patients treated with EP developed clonal cytogenetic abnormalities. Stimulation of HSPC proliferation by EP might lead to accelerated telomere attrition that can cause destabilization of the genome and emergence of clonal transformation has been proposed as a mechanism to explain clonal evolution of treated patients (Desmond *et al.*, 2015). Thus, how EP is promoting the generation of blood cells and impacting HSPC from AA patients remains undetermined.

1.1.5 Pathophysiology of AA

Very reduced numbers of HSPC is a consistent finding observed in the bone marrow of AA (Scopes *et al.*, 1994; Maciejewski *et al.*, 1996; Manz *et al.*, 1996; Schrezenmeier *et al.*, 1996; Rizzo *et al.*, 2002; Matsui *et al.*, 2006). Likewise, haematopoietic progenitors of AA patients have shown an impaired capacity to form haematopoietic

progenitor colonies when assayed in *in vitro* clonogenic assays (Marsh *et al.*, 1990; Bacigalupo *et al.*, 1992; Rizzo *et al.*, 2004). Several potential mechanisms leading to increased apoptosis and/or reduced proliferation and differentiation capacity have been traditionally linked to pathogenesis of AA including immune suppression of the HSC in the bone marrow, underlying dysfunction of the HSPCs and defective bone marrow mesenchymal stem cells (MSC) (**Figure 5**).

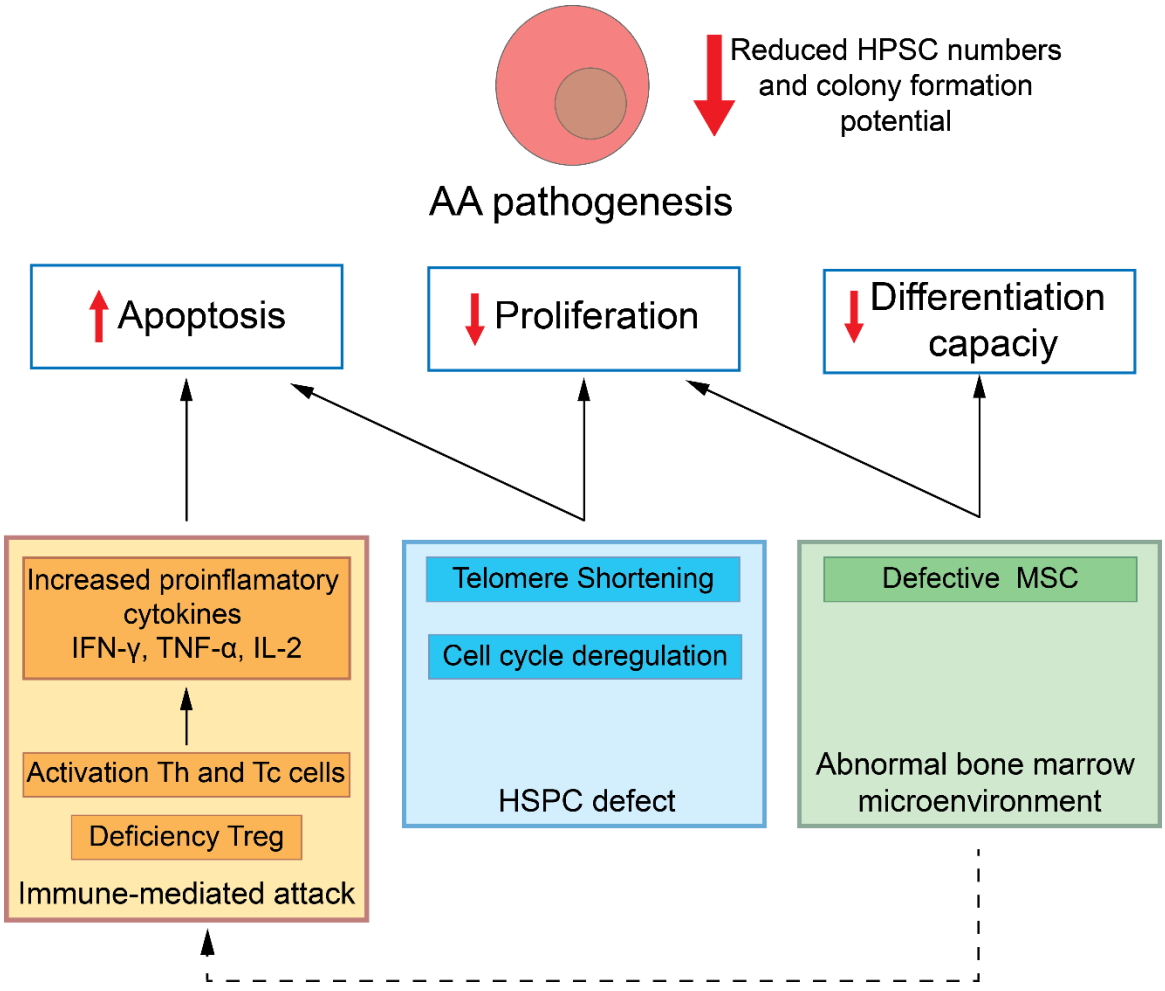


Figure 5. Proposed mechanisms and aetiologies associated with AA pathogenesis

1.1.5.1 Immune destruction of HSPC in the bone marrow

The strongest evidence supporting the immune mediated destruction of bone marrow in AA is the fact that around 80% of AA patients show response to IST (Young *et al.*, 2006). There is compelling evidence suggesting that HSCs in the bone marrow of AA patients suffer an autoimmune suppression by cytotoxic T lymphocytes. Elevated levels of interferon gamma (IFN-γ) in serum of 30% of AA patients (Nistico and Young, 1994), the presence of tumour necrosis factor alpha (TNF-α) in the bone marrow of a large number of AA patients as well as detection of specific T helper lymphocytes and

oligoclonal T cytotoxic lymphocytes in AA patients support the autoimmune basis associated with AA pathogenesis (Risitano *et al.*, 2004; Kordasti *et al.*, 2012). Thus, this immune-related marrow failure in AA is based on the induction of apoptosis of multipotential haematopoietic progenitors by inhibitory cytokines secreted by cytotoxic T lymphocytes (Sloand *et al.*, 2002). Activation of cytotoxic T lymphocytes by antigen recognition leads to the production of interleukin-2, (Giannakoulas *et al.*, 2004), clonal polyclonal expansion of T lymphocytes (Kordasti *et al.*, 2012) and up-regulation of T lymphocytes receptors (Ren *et al.*, 2014) and Fas receptor in haematopoietic progenitor cells by IFN-γ and TNF-α (Kakagianni *et al.*, 2006) (**Figure 6**).

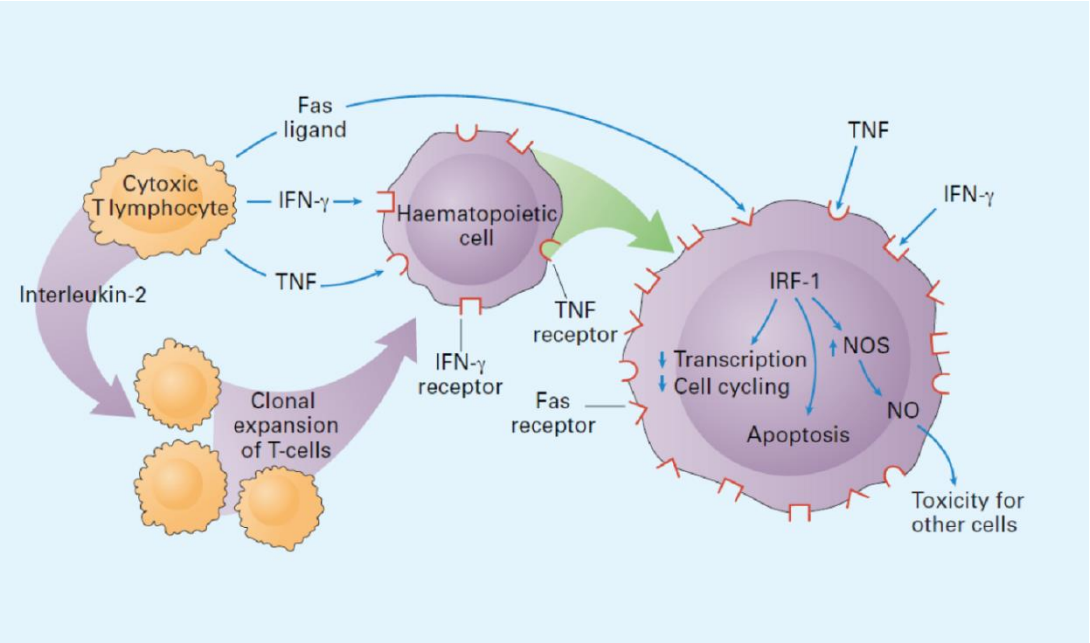


Figure 6. Immune-mediated destruction of haematopoietic progenitor cells in AA.
IFN-γ, interferon gamma; TNF, tumour necrosis factor; IRF-1, interferon regulatory factor-1; NOS, nitric oxide synthase; NO, nitric oxide. Reproduced from Marsh 2005 (Marsh, 2005)

IFN-γ secreted by cytotoxic T lymphocytes induces activation of interferon regulatory factor-1 (IRF-1) which inhibits the transcription of cellular genes and cell cycle progression and induces the production of nitric oxide synthase (NOS) and nitric oxide (NO) ultimately leading to cell death by apoptosis of the haematopoietic progenitor cells in the bone marrow (Young *et al.*, 2006). Likewise, decreased levels of regulatory T lymphocytes might explain excessive presence of cytotoxic and helper T lymphocytes, therefore supporting an immune-related destruction of haematopoietic progenitor cells (Solomou *et al.*, 2007). What triggers the activation of T lymphocytes

remains unclear. Young *et al* hypothesized that this aberrant activation of T cells in AA might have a genetic basis since it has been reported that the frequency of human leukocyte antigen (HLA) DR2 haplotype is significantly increased in AA patients and specific polymorphisms in TNF2, IFN-γ and IL-6 genes are associated with a high production of these cytokines in AA patients (Young *et al.*, 2006). A microarray gene expression analysis of the CD34+ haematopoietic progenitors from AA bone marrow patients revealed that genes associated with apoptosis, cell death and defence/immune regulation were the most differentially up-regulated compared to CD34+ progenitors from healthy volunteers (Zeng *et al.*, 2004). IFN-γ and TNF-α inhibit *in vitro* haematopoietic colony formation capacity of bone marrow progenitors by inducing apoptosis through increased expression of Fas receptor (Maciejewski *et al.*, 1995). Thus, the increased levels of proinflammatory cytokines present in AA patients would explain this significantly induced apoptosis observed in the AA haematopoietic progenitors indicating that it could be secondary to T cell-mediated destruction. Likewise, similarly to the study published in 2004, Zeng *et al.* used microarray technologies in 2006 to analyse the effect of IFN-γ on CD34+ haematopoietic progenitors from healthy volunteers. They observed that IFN-γ-induced CD34+ haematopoietic progenitor cells from healthy volunteers showed an upregulation of apoptotic and immune response genes in a similar fashion than the CD34+ from AA patients providing more evidence of the role of IFN-γ in the pathogenesis of AA (Zeng *et al.*, 2006).

1.1.5.2 HSPC defect

No response or relapse after IST observed in a substantial number of AA patients might also indicate an underlying defect of the HSPC in these patients. Several intrinsic deficits have been observed in AA-HSPC that might be associated with the pathophysiology of AA, including increased apoptosis and reduced proliferation and differentiation capacity. Excessive telomere shortening and defective cell cycle checkpoints could explain this aberrant behaviour observed in AA HSPC.

Telomere shortening. Telomeres are structures located at the end of the chromosomes that protect them from being recognized by cellular DNA damage response mechanisms as double-strand breaks that otherwise could lead to end-to-end chromosome fusions and nonreciprocal translocations (Blasco, 2005). In each cell

division the telomere length of the daughter DNA strand is shortened about 50-100 base pair due to what is called the end-replication problem (Huffman *et al.*, 2000) (**Figure 7**).

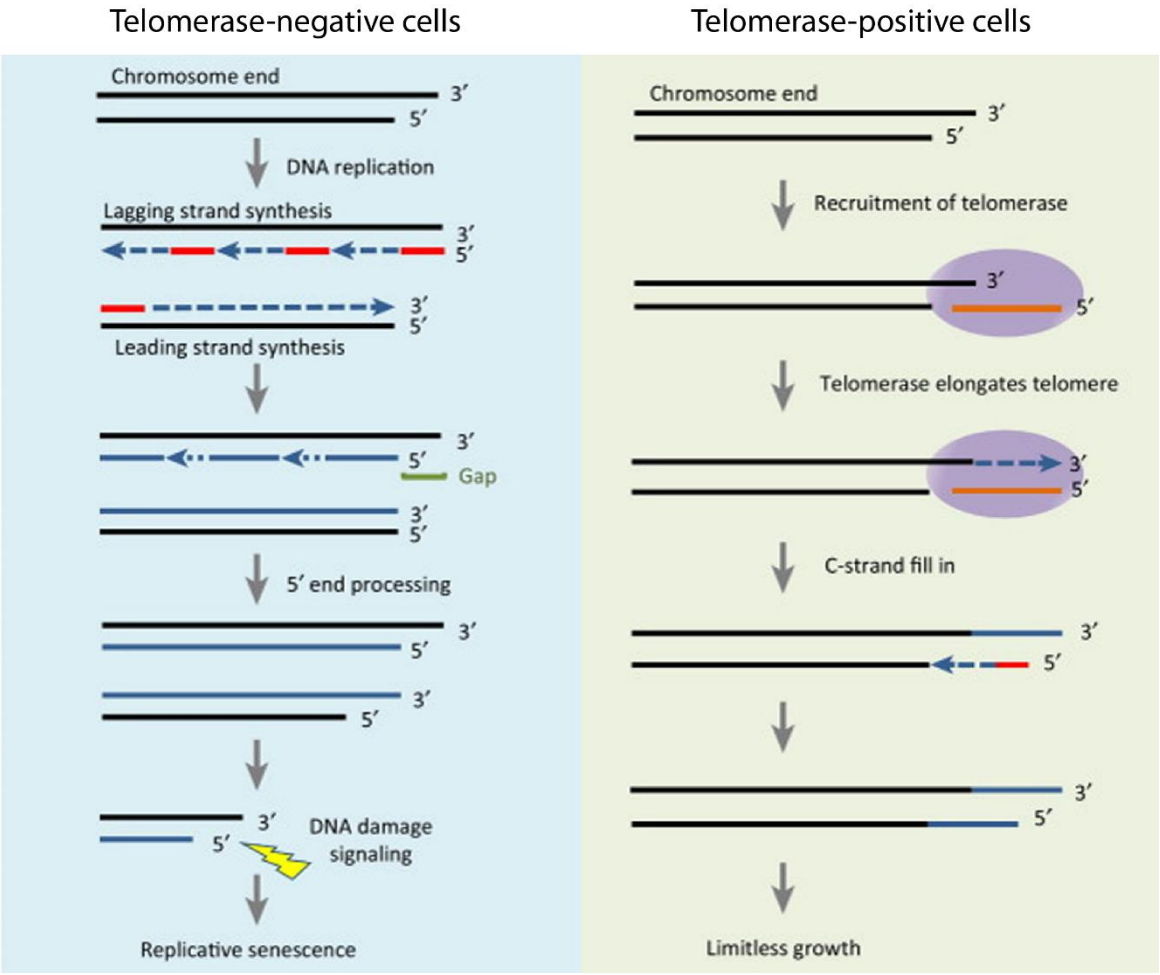


Figure 7. End replication problem.

In telomerase-negative cells, telomeres shorten in the 5' end of the lagging strand in every division due to incomplete replication when RNA primers (in red) are removed. Once the telomeres become critically short, a DNA damage signalling response is activated leading to replicative senescence. In telomerase-positive cells, telomerase complex uses its RNA component (in orange) to elongate the 5' end of lagging strand maintaining telomere length and unlimited proliferative capacity. Reproduced from Wong *et al* 2014 (Wong *et al.*, 2014)

Telomere shortening is a mechanism developed to avoid indefinite proliferation of cells suppressing risk of tumorigenesis associated with aberrant replication (Hackett and Greider, 2002). When telomeres become critically short, cellular mechanisms that lead to cell proliferation arrest or apoptosis are activated protecting the cell from chromosomal instability that could ultimately lead to malignant transformation (Bernardes de Jesus and Blasco, 2013). In humans, telomeres are formed by

hundreds of repetitions of a specific sequence, TTAGGG in the sense strand and CCCTAA in the antisense strand. In order to protect the end of chromosomes from being recognized as double-strand breaks, telomeres are structured forming a terminal loop structure (T loop) (Griffith *et al.*, 1999) together with a telomere-binding protein complex named shelterin (de Lange, 2005) (**Figure 8A**). To overcome the end replication problem, cells with high proliferative capacity are able to extend the telomere length by activating the telomerase complex (Collins and Mitchell, 2002) (**Figure 7**). Excessive telomere shortening has been linked to AA pathogenesis since leukocytes with significantly short telomeres have been identified in one third of AA patients, especially those who are refractory to IST (Ball *et al.*, 1998; Brummendorf *et al.*, 2001). To date several components have been involved in human telomere maintenance including telomerase complex, RNA chaperone TCAB1, CST complex, DNA helicase RTEL1 and the shelterin complex (**Figure 8B**).

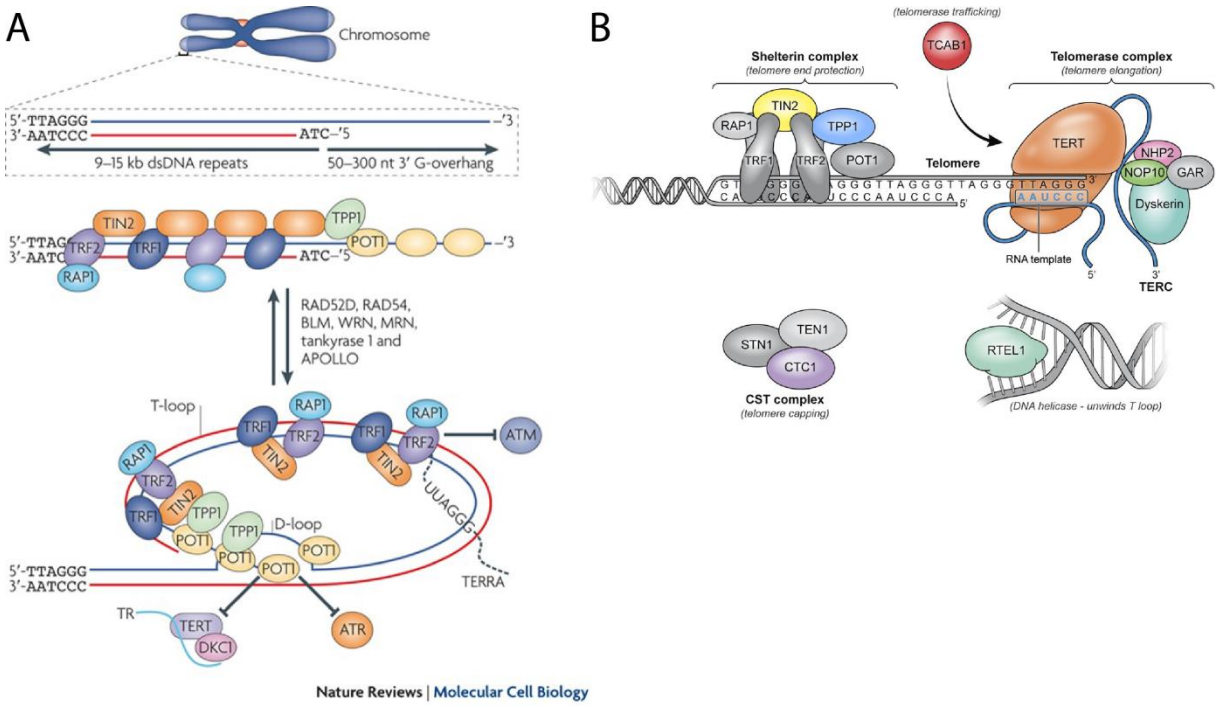


Figure 8. Telomere protection and maintenance.

(A) Telomere protection. The 3' G-overhang present at the end of the leading strand in human telomeres invades the double-stranded TTAGGG repeats forming the telomeric loop (T-loop) with the help of the shelterin complex (TRF1, TRF2, TIN2, RAP1, TPP1 and POT1). Reproduced from O'Sullivan *et al.* 2010 (O'Sullivan and Karlseder, 2010). (B) Telomerase maintenance components. Telomerase complex consists of reverse transcriptase telomerase (TERT), an RNA template (TERC) and a protein scaffold complex involved in the stability and recruitment of telomerase to telomeres (dyskerin, NOP10, NHP2 and GAR). TCAB1 is involved in the trafficking of the telomerase complex along the telomeric ends. CST complex is associated with telomerase activity inhibition and lagging strand synthesis. RTEL1 unwinds the T loop needed for telomere elongation and shelterin complex is also involved in recruitment and regulation of the telomerase complex. Reproduced from Townsley *et al.* 2014 (Townsley *et al.*, 2014)

Mutations in any of the genes involved in telomere maintenance and protection might result in accelerated telomere attrition in high proliferative cells such as HSPCs leading to low proliferative capacity and reduced haematopoietic function (Calado and Young, 2008). In DC patients, mutations in genes encoding for telomerase complex components such as *TERT (Marrone et al., 2007), TERC (Vulliamy et al., 2001), DKC1 (Mitchell et al., 1999)* and *NOP10 (Walne et al., 2007),* genes encoding for a DNA helicase *RTEL1* (Walne *et al.*, 2013) and shelterin complex component *TINF2* (Savage *et al.*, 2008) have been described. In AA, approximately 4% of patients present mutations in *TERT* (Yamaguchi *et al.*, 2005) and *TERC* (Yamaguchi *et al.*, 2003) genes and less of 1% have mutations in genes *TERF1* and *TERF2* encoding for shelterin

components (Savage *et al.*, 2006). Interestingly, not all AA patients with mutations in *TERT/TERC* genes show DC-associated phenotypic features. This highly variable phenotypic expression in patients with mutations in *TERC* and *TERC* suggests that other factors might also be involved in bone marrow failure (Yamaguchi *et al.*, 2005). Many AA patients have relatives with the same mutation in telomere-associated genes displaying short telomeres but normal blood counts (Fogarty *et al.*, 2003). In order to explain this, Calado *et al* suggested that HSPC with short telomeres and mutations in telomere-associated genes might be able to maintain normal haematopoiesis but are unable to cope with the stress induced by immune attack or environmental factors due to their reduced telomere length (Calado and Young, 2008). Therefore, mutations in telomere-associated genes should be considered risk factors of bone marrow failure and not genetic determinants.

Nevertheless, only 10% of AA patients displaying short telomeres present mutations in previously characterised telomere-associated genes (Young *et al.*, 2006). First, excessive telomere shortening has been suggested to be the result of increased proliferative stress in HSPC as compensatory mechanism to replenish the reduced bone marrow stem cell compartment as reported in patients following allogeneic HSCT (Gadalla and Savage, 2011). However, telomere attrition rate observed in patients with mutations in telomere-associated genes is more pronounced, more than 3kb, than the shortening produced due to 'regenerative stress' after transplantation, less than 1kb (Calado and Young, 2008). Excessive telomere shortening observed in AA patients could be also explained by the presence of mutations in telomere pathway-associated genes uncharacterised to date due to the complexity of the telomere maintenance, regulation and repair processes. Difficulties identifying these genetic defects could be also explained by the hypothesis that manifestation of the abnormal phenotype is not entirely originated by mutations in these genes but also environmental factors inducing bone marrow failure in genetically predisposed AA patients as suggested by Calado *et al.* (Calado and Young, 2008).

As discussed in **section 1.1.4** of this introduction, evolution of AA into clonal disorders such as MDS and PNH due to the reduced stem cell compartment in the bone marrow has been described in some AA patients. Presence of short telomeres in AA patients would provide an explanation to this increased probability to develop clonal disorders

and risk of relapse observed in these patients due to the chromosomal instability associated with critically reduced telomere length (Scheinberg *et al.*, 2010; Dumitriu *et al.*, 2015).

Cell cycle deregulation. Potential role of cell cycle deregulation in AA pathogenesis was recently proposed by Zeng *et al.* on the basis that defective proliferation capacity might explain insufficient generation of haematopoietic cells despite the abundance of haematopoietic growth factors present in AA patients (Zeng and Katsanis, 2015). As described by these authors in 2004, besides displaying up-regulation of genes associated with apoptosis, CD34+ haematopoietic progenitor from AA patients also showed a significant up-regulation of genes associated with inhibition of cell cycle entry and down-regulation of cell proliferation and cell-cycle progress-enhancing genes such as cyclin-dependent kinase (CDK) genes and cell division cycle (CDC) genes (Zeng *et al.*, 2004). Likewise, it is also hypothesized that down-regulation observed in the same study of other cell cycle "checkpoint" genes such as *FANCG, c-myb and c-myc* might also explain susceptibility of AA patients to develop clonal disorders. It remains undetermined if these cell cycle defects observed in AA CD34+ progenitors might be associated with telomere dysfunction.

1.1.5.3 Defective bone marrow MSCs

Bone marrow stroma is formed by adipocytes, fibroblasts, osteoblasts, osteoclasts and endothelial cells, all derived from mesenchymal stem cells (MSC), providing a suitable microenvironment for haematopoiesis, protecting stem cells from damage and restraining them from differentiation (Muguruma *et al.*, 2006). Likewise, MSCs play an important role regulating immune cells function (Uccelli *et al.*, 2008). There are conflicting results in the literature regarding the role of MSC in the pathogenesis of AA. Some studies have shown that MSC from AA patients had low proliferation activity, were more prone to differentiate into adipocytes than osteoblasts and deficient in suppressing T cell activation/proliferation (Chao *et al.*, 2010; Li *et al.*, 2012). However, this hypothesis seems unlikely since functionality of MSC in AA patients was demonstrated in an elegant crossover experiment carried out by Marsh *et al.* showing that AA stromal cells support normal growth of CD34+ cells from healthy volunteers whereas CD34+ cells from AA patients still showed poor growth properties when cultured on normal stromal cells (Marsh *et al.*, 1991). Additionally, other studies have

also shown normal functionality and immunosuppressive properties of the MSC of AA patients (Xu *et al.*, 2009; Bueno *et al.*, 2014). Not sufficiently large populations and differences in the degree of severity and the age of AA patients at diagnosis in these studies might explain the divergences in the results with regards to the role of bone marrow MSCs in AA pathogenesis.

1.1.6 Current problems in the investigation of AA

Several studies have reported the use of CD34+ from AA patients in *in vitro* clonogenic assays to investigate the numbers of HSPC in the bone marrow and their clonogenic properties. In these studies CD34+ progenitors from AA patients appeared in extremely low numbers in the bone marrow and also showed a reduced ability to generate haematopoietic progenitor colonies (**see section 1.1.5**). Thus, acquisition of HSPC from AA patients to evaluate stem cell function and properties is extremely difficult due to the paucity of these cells in the bone marrow of these patients. Likewise, use of HSPC from AA patients for disease modelling studies is also restricted by the absence of valid protocols to properly expand HSC *in vitro* (Walasek *et al.*, 2012). As an example, the microarray gene expression analysis of CD34+ from AA patients reported by Zeng *et al.* needed to pool RNA from several AA patients in order to provide sufficient material to perform the gene expression analysis of AA CD34+ progenitors (Zeng *et al.*, 2004). Currently, there are no animal models suitable for the *in vivo* study of AA (Elbadry *et al.*, 2017). Animal models have been generated to study the bone marrow failure induced by different toxic chemicals as well as to investigate the role of the different factors involved in immune-mediated mechanisms associated with AA pathogenesis (Chen, 2005; Scheinberg and Chen, 2013). This involves infusing haploidentical parental lymphocytes into offspring (Chen *et al.*, 2004), administration of toxic drugs such as chloramphenicol (Turton *et al.*, 2000) or busulfan, exposure to ionizing radiation (Meng *et al.*, 2003) or generation of genetically modified mice (Trf-1 and Tert-deficient) displaying short telomeres (Bar *et al.*, 2016). However, these models do not recapitulate the entire spectrum of AA. Additionally, animal models have been also used to model BMFS such as FA, DC and Diamond-Blackfan anaemia (DBA) among others providing valuable insights into the pathological mechanisms of these syndromes. Thus, Beier *et al.* generated a conditional and bone marrow-specific Trf1 mouse mutant to address the possible role of the shelterin component protein Trf1 in

the pathogenesis of BMFS (Beier *et al.*, 2012). Trf1-deleted haematopoietic progenitors displayed increased cellular senescence resulting in telomere shortening of wildtype haematopoietic progenitors by hyper-proliferation and high turnover to compensate for constant progenitor loss. This study highlights the importance of the different components associated with telomere pathway and how mutations in those components might lead to bone marrow failure. Despite the existence of these animal models, important physiological differences between animal and humans can prevent the recapitulation of the disease phenotype in animal models (Adam *et al.*, 2017). Disease causing mutations in humans might have the same mechanistic role in animals leading to differences in the abnormal phenotype. The need to create double knock-outs in the *FANC* genes in mice to recapitulate FA phenotype observed in humans or prolonged breeding of knock-out mice during several generations in order to erode sufficiently telomeres due to longer telomere length and shorter lifespan of mice are some of the problems that undermine the utility of animal models to investigate disease mechanisms and pathology of BMFS. Thus, there is an urgent need to generate a human AA disease model that can complement animal model studies and be used as a platform to provide insight into the underlying dysfunction of AA-HSPCs.

1.2 Induced Pluripotent Stem Cell Technology (iPSC) technology

Pluripotent stem cells (PSCs) are capable of unlimited self-renewal capacity and have the potential to differentiate into tissues of all three germ layers contributing to the generation of all somatic cell types of the human body (De Los Angeles *et al.*, 2015) (**Figure 9**).

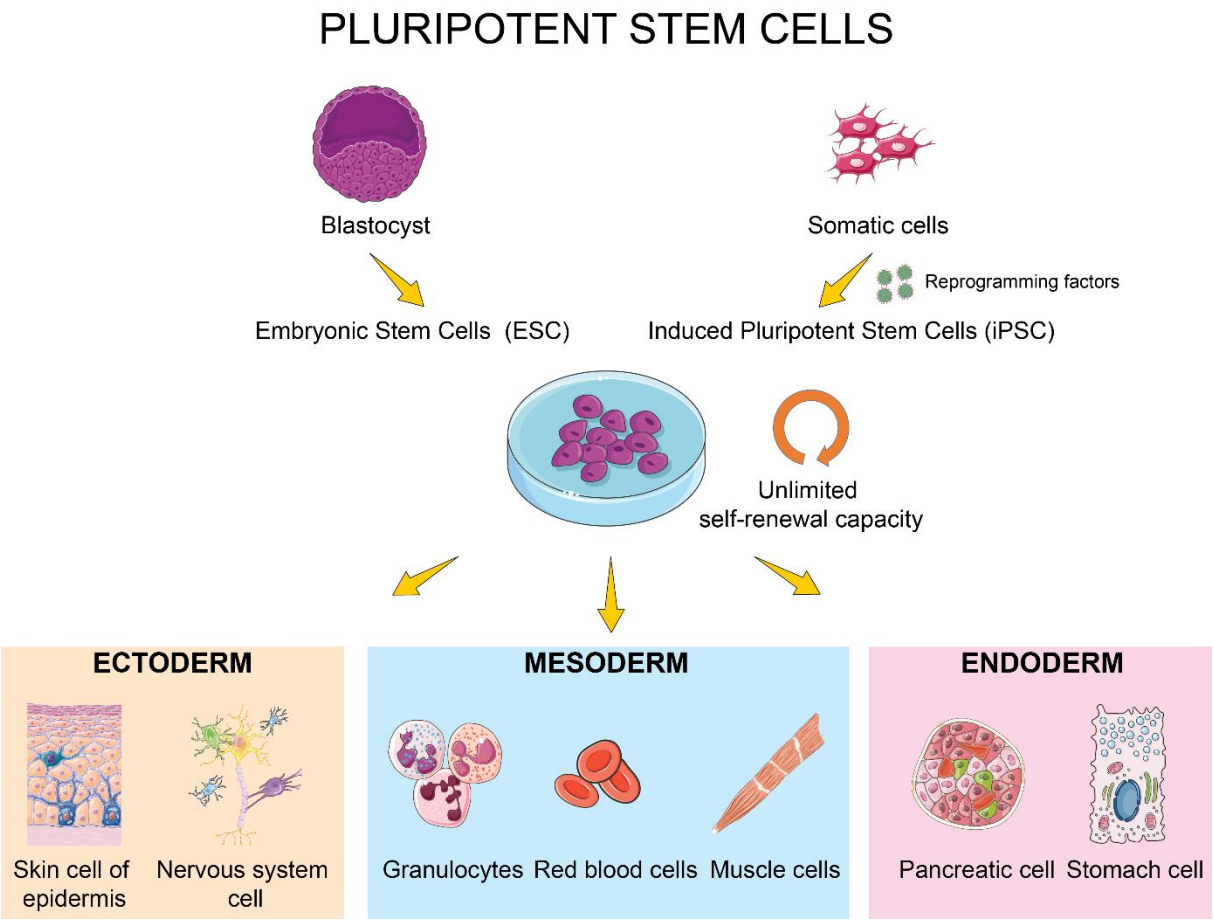


Figure 9. Pluripotent stem cells (PSCs).

PSCs are commonly obtained after isolation of the inner cell mass of human blastocyst or nuclear reprogramming of somatic cells. PSCs can proliferate indefinitely and give rise to any foetal cell type or any of the 200+ cell types present in the adult body

1.2.1 Origin of Pluripotent Stem Cells (PSCs)

The interest in PSCs began in 1954 with the study in mouse of teratocarcinomas which are germ cell tumours (Stevens and Little, 1954). Subsequent studies of these tumours showed the presence of cells with the ability to differentiate into almost any cell type of the developing embryo (Stevens, 1967). Embryonic carcinoma cells (ECCs) exhibit similar characteristics to the cells present in the inner cell mass (ICM) of the early embryo (Solter, 2006). Studies with ECCs provided the basis for the process of

isolation of these ICM cells from mouse blastocyst and the derivation of the first embryonic stem cell (ESC) line. The firsts derivations of ESCs were performed in 1981 from the ICM of a mouse embryo by Martin and Evans and Kauffman (Evans and Kaufman, 1981; Martin, 1981). However, it was not until 1998 when Thompson *et al* successfully derived the first human ESC line from surplus IVF-produced human embryos (Thomson *et al.*, 1998). There is also a further PSC type that can be obtained naturally in humans; embryonic germ cells (EGCs). While human ESCs are derived from the cells of the ICM of the early embryos, human ECCs and EGCs have a shared origin in primordial germ cells (PGCs). These human PGCs are unipotent cells that are the founder cell population of the gametes. PGCs can be identified in humans around day 22 of gestation by expression of tissue non-specific alkaline phosphatase (TNAP). Thus, human PGCs can give rise *in vivo* to ECCs, whereas generation of human EGCs requires the *in vitro* culture of PGCs with a specific cocktail of growth factors (Matsui *et al.*, 1992; Resnick *et al.*, 1992; Shamblott *et al.*, 1998; Donovan and de Miguel, 2003) (**Figure 10**).

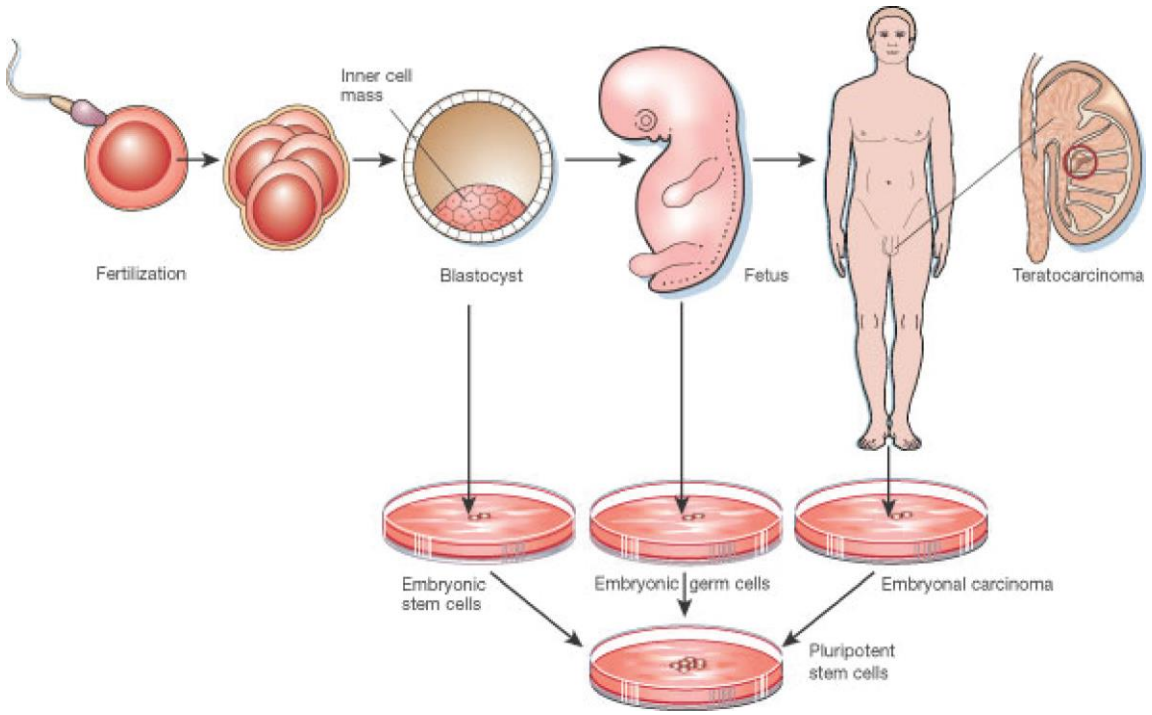


Figure 10. Types of PSCs existing in humans naturally.

Reproduced from Donovan (2001). (Donovan and Gearhart, 2001)

ECCs are considered the malignant counterpart of ESC, as they share the same pluripotency markers, but ECCs are usually aneuploid and the malignancy of ECCs is highly dependent on their microenvironment. However, PGCs rapidly lose the ability to give rise to cells that form teratomae after 12.5 day *post coitum* in mice, suggesting that at this time they lose developmental potential. Despite their pluripotency, human PGCs show many disadvantages for their use in regenerative medicine. As well as their noted tumorigenic nature, they also have a reduced capacity for multi-lineage differentiation, so the use of ECCs is usually limited to models for investigation of cell differentiation in a manner pertinent to early vertebrate embryogenesis, especially in mouse (Martin, 1980). However, human ESCs are not identical to ICM cells in the human embryo, showing distinct global DNA methylation profile to the cells found in blastocysts (Smith *et al.*, 2014). Human ESCs spontaneously adopt a 'primed' state similar to the ones found in cells of the late epiblasts of post-implantation. Conversely, mouse ESCs represent a 'naive' state that is considered more similar to the cells found in earlier stages of the development in pre-implantation embryos displaying higher differentiation potential than primed PSCs. Thus, mouse 'primed' PSCs are more heterogeneous and show limited *in vivo* differentiation potential compared to 'naïve' mouse PSCs as they show low contribution to the formation of chimeric embryos in post-implantation embryos (Huang *et al.*, 2012)

1.2.2 Features of PSCs

Human ESCs express specific markers or characteristics similar but not identical to the pluripotent cells present in the ICM of an embryo. These characteristics include stage specific embryonic antigens (SSEA), enzymatic activities such as alkaline phosphatase (AP), telomerase activity and expression of "stemness" genes that are rapidly down-regulated upon differentiation, which include *POU5F1* (*OCT4)* and *NANOG* among others (De Los Angeles *et al.*, 2015). Under specific conditions, human ESCs can proliferate indefinitely maintaining an undifferentiated phenotype with self-renewing properties due to a high telomerase activity (Thomson *et al.*, 1998). Furthermore, ESCs retain their ability to differentiate into any cell-type present in the adult body as shown when ESCs are injected in severe combined immunodeficiency disease (SCID) mice resulting in the formation of tumours (teratomae) which contain cells from the three major germ layers: endoderm, mesoderm and ectoderm (Mitalipov

and Wolf, 2009). This huge potential for differentiation and their potential therapeutic effect has been reported in animal models of spinal cord injury, retinal dystrophies and Parkinson´s disease (Keirstead *et al.*, 2005; Yang *et al.*, 2008; Lamba *et al.*, 2009). Currently, there are several clinical trials involving human ESC-derived differentiated cells approved by the U.S. Food and Drug Administration (FDA) to treat disorders such as spinal cord injury, Stargardt´s Macular Dystrophy and type 1 diabetes (Alper, 2009; Liras; Takahashi and Yamanaka, 2016). The primary objective of these Phase I/II studies is to assess the safety and tolerability of these human ESC-derived cells in patients.

However, there are concerns with the use of human ESCs as a therapeutic. The immune response initiated by transplantation of human ESC into genetically unrelated patients that may result in rejection and the ethical issues associated to the use of human embryos are issues that need to be considered when using human ESC (Dawson *et al.*, 2003; Fischbach and Fischbach, 2004).

1.2.3 Inducing Pluripotency in somatic cells

Nuclear reprogramming of somatic cells allows the generation of pluripotent cells resembling ESCs. Nuclear reprogramming has been defined as the reversal of the differentiation state of a mature cell to one that is characteristic of the undifferentiated embryonic state (Gurdon, 2006; Hochedlinger and Jaenisch, 2006). Traditionally, normal embryonic development has been considered an irreversible state, a one way-street where the process of cellular differentiation of an undifferentiated cell was pictured by Conrad Waddington on the mid-20th century as a ball rolling downhill reaching the physiologically mature state at the bottom of the hill (**Figure 11**).

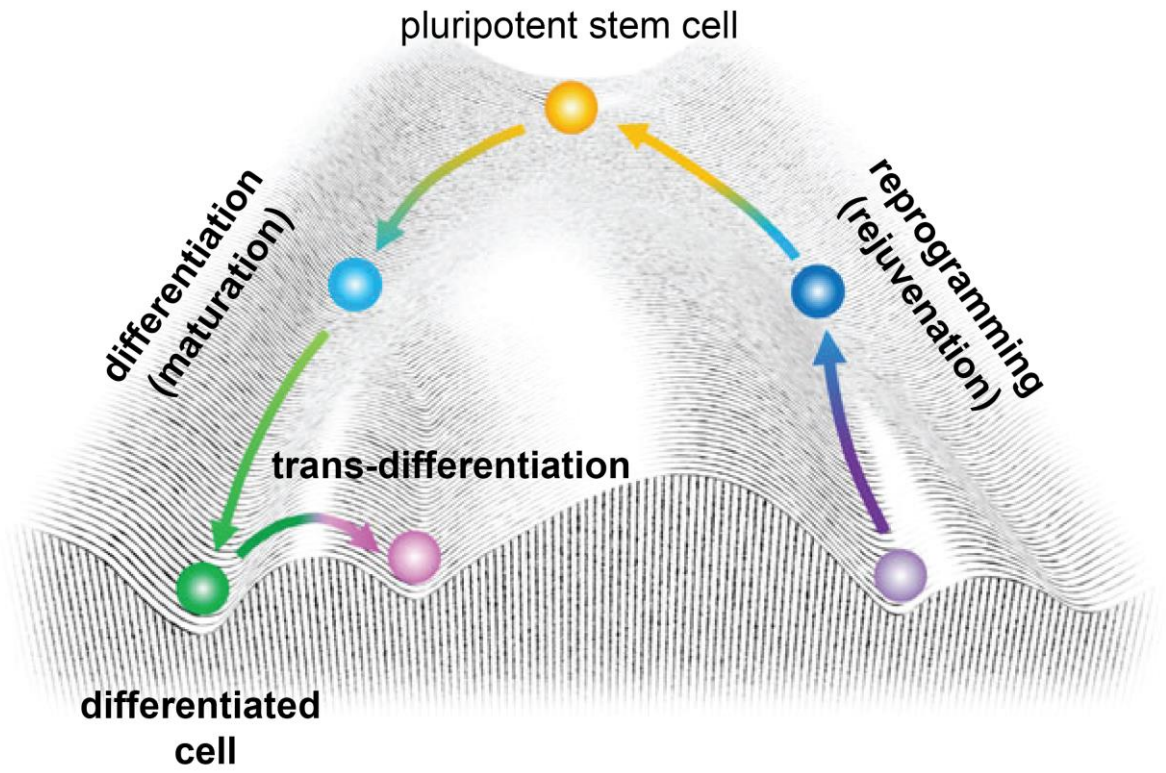


Figure 11. Conrad Waddington's epigenetic landscape.

In this model, the ball on the top of the hill represents a PSC that remains in an undifferentiated state. As the ball (PSC) rolls down the hill the ball can take different paths, representing the different lineages that a PSC can take upon differentiation. The ball reaching the bottom of the hill represents the acquisition of the mature state of the differentiated cell, such as a blood cell or a neuron. Differentiated cells can be differentiated to another cell type (trans-differentiation) pictured as moving the ball from one hill to other hill. Likewise, the re-acquisition of the undifferentiated state of a differentiated cell (reprogramming/rejuvenation) is represented as taking the ball back to the top of the hill. Modified from Ohnuki *et al* 2015 (Ohnuki and Takahashi, 2015).

However, Sir John Gurdon demonstrated in 1962 that differentiated intestinal cells of frogs were capable of developing into adult frogs following somatic cell nuclear transfer (SCNT) of the nucleus of the intestinal cell into the ooplasm of the frog oocyte, although at low efficiency (approximately 1%) (Gurdon, 1962). These experiments were the basis for the later mammalian cloning experiments that gave rise to the first cloned sheep, "Dolly", by Wilmut and colleagues (Wilmut *et al.*, 1997) and recently the derivation of the first human ESCs lines from human cloned embryos via nuclear transfer (Tachibana *et al.*) (**Figure 12**). Fusion of somatic cells with ESCs to form tetraploid hybrids or exposing them to ECC extracts can also generate cells with pluripotent phenotype (Cowan *et al.*, 2005) (**Figure 12**).

Additionally, studies with somatic cells have demonstrated that cell fate can be modified by forcing the expression of a single lineage-specific transcription factor, leading to the trans-differentiation or dedifferentiation of the somatic cell (**Figure 12**). Early research in this field by Weintraub *et al.* showed that fibroblasts can be converted into muscle cells by the overexpression of the muscle specific transcription factor *MyoD* while recent research has shown that mature B-cells can be reprogrammed into macrophages by the overexpression of C/EBPα or C/EBPβ (Davis *et al.*, 1987; Xie *et al.*, 2004). These studies led to the publication of the ground breaking work by Takahashi and Yamanaka first in 2006 and later in 2007 that demonstrated that forced expression by retroviral transduction of four known ESC factors *(OCT4, SOX2, KLF4* and *c-MYC*) in murine fibroblasts can reprogram them to a pluripotent state (Takahashi and Yamanaka, 2006; Okita *et al.*, 2007). These reprogrammed cells were named induced pluripotent stem cells (iPSC) and, within months, the same group succeed in generating human iPSC by introducing the human orthologues of the four transcription factors into human fibroblast (Takahashi *et al.*, 2007). Concurrently, Yu *et al.* achieved a similar reprogramming of human somatic cells using slightly different combinations of genes (*OCT4, SOX2, NANOG* and *LIN28*) (Yu *et al.*, 2009). The generated iPSCs, both human and mouse, were similar to corresponding ESCs in morphology, growth characteristics and expression of phenotypic markers. These cells maintained a normal karyotype and a pluripotential developmental capacity for extended periods and mouse iPSC appear to be competent for germline transmission in mice (Okita *et al.*, 2007). Moreover, mouse iPSC have also succeeded the most stringent test of pluripotency, tetraploid complementation, showing their ability to contribute to the generation of an entire living mouse (Kang *et al.*, 2009).

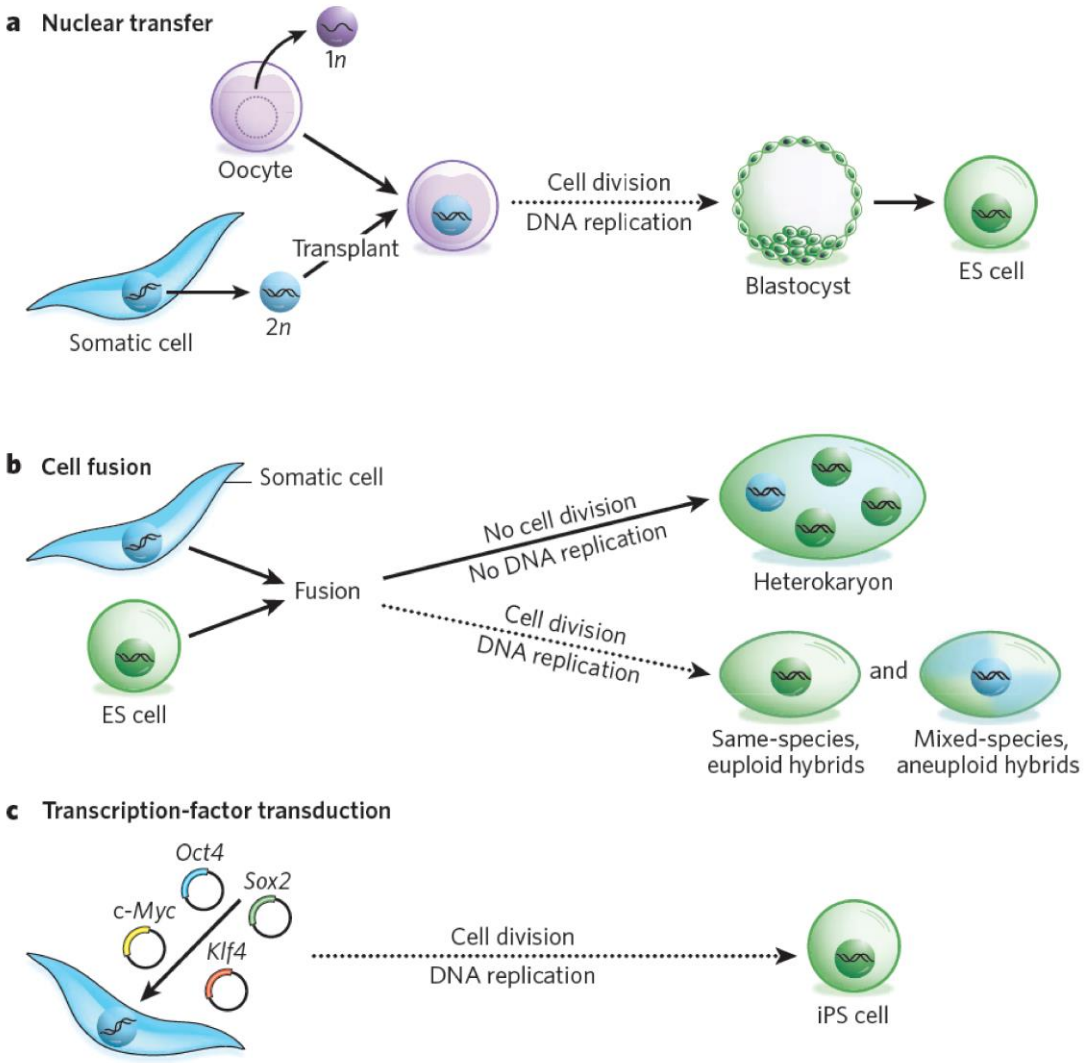


Figure 12. Strategies to induce pluripotency by nuclear reprogramming.

Reproduced from Yamanaka *et al* 2010 (Yamanaka and Blau, 2010)

1.2.4 Advantages of using iPSC technology

Human iPSC have the same features/properties as human ESC including expression of human pluripotent factors, self-renewal capacity and potential to differentiate into three germ layers. However, iPSC present additional features that make them more appealing for basic and clinical studies:

1.2.4.1 Patient specific

Cells from patients carrying genetic mutations can be reprogrammed into iPSC, opening a new window of opportunity for applications of iPSC technology in disease modelling, drug screening and cell-based therapy (**Figure 13**).

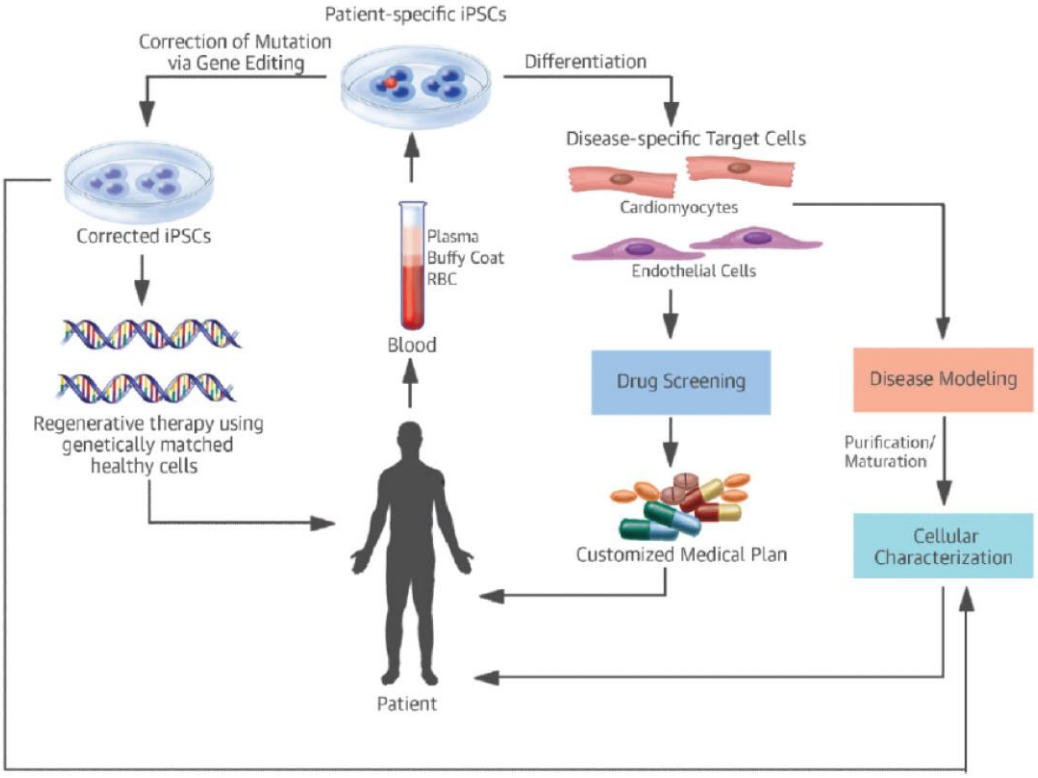


Figure 13. Short-term and long-term applications of iPSC technology.

Patient specific iPSC and iPSC-derived differentiated cells generated from blood cells can be used for disease modelling and drug screening. Genetic correction of patient specific iPSC allows the generation of corrected differentiated cells that could be potentially used in cell therapy. RBC,red blood cells. Reproduced from Sayed *et al* 2016 (Sayed *et al.*, 2016)

Disease modelling. Differentiation of patient-specific iPSC into the disease-relevant cell type makes possible to recapitulate specific disease phenotypes and pathologies in a dish. This disease-in-a-dish model would allow the identification of pathological mechanisms associated to those disorders and reveal disease aetiology (**Figure 13**). To date, numerous studies have reported the generation of disease-specific iPSC lines recapitulating the phenotype observed in patients including BMFS (Adam *et al.*, 2017). Additionally, iPSC technology in combination with gene editing technologies has proved to be a powerful tool to confirm the pathogenic nature of the identified genetic defects by the correction of disease-causing mutations in patient iPSC as wells as introduction of specific mutations into control-iPSC (Hotta and Yamanaka, 2015). This is especially relevant for disorders caused by a single mutation and an early onset (Ebert *et al.*, 2009; Lee *et al.*, 2009). For disorders classified as idiopathic, where the aetiology is unknown and genetic risk variants and environmental factors may contribute to the pathogenesis of the disease, is important to minimize the genetic

background variations present in order to confidently identify the disease-associated phenotype in the patient iPSC when comparing to unaffected controls (Shi *et al.*, 2017). Thus, by using iPSC technology, specific gene mutations were identified in several idiopathic cases of Alzheimer disease exhibiting the same phenotype leading to improved diagnosis and re-classification of this condition (Kondo *et al.*, 2013).

Drug screening. Generation of specific disease-relevant cell types from patient iPSC at high scale in an *in vitro* setting is one of the main advantages that iPSC technology offers in drug screening to identify and test the efficacy of drug candidates (**Figure 13**). Thus, drug screening in iPSC-based cells have been used to identify candidate drugs in disorders including spinal muscular atrophy, amyotrophic lateral sclerosis and progressive supranuclear palsy that are currently being tested in clinical trials by pharmaceutical companies such as Roche and GlaxoSmihKline (Shi *et al.*, 2017).

Cell-based therapy. Although it is considered by experts as a long term goal, the most promising application of iPSC technology is the possibility of creating personalized therapies to treat patients with their own cells avoiding immunological rejections associated to allogeneic therapies and reducing costs and secondary effects associated to IST. Combination of gene editing and iPSC technologies may allow the correction of the disease-causing genetic defect in patient-specific iPSC, differentiation into the cell type of interest and transplantation of autologous healthy cells to treat the patient (**Figure 13**). As a proof-of-concept of the therapeutic use of iPSC, a study using a mouse model of sickle-cell anaemia reported in 2007 the correction of the mutation in the *β-globin* gene in mutant iPSC using gene editing approach (Hanna *et al.*, 2007). The mutation-free mouse iPSCs were differentiated into haematopoietic progenitors and transplanted to the affected mouse allowing for the disease to be cured. In 2014, the first clinical trial using human iPSC was launched by Masayo Takahashi to treat patients with age-related acute macular degeneration (AMD) (Ohnuki and Takahashi, 2015). The therapy consisted in autologous transplantation of iPSC-derived retinal pigment epithelium (RPE) cells resulting in promising results since the patients had a reduction of macular degeneration and improvement of vision.

1.2.4.2 Telomere rejuvenation

iPSC have self-renewal capacity due to the activation of the telomerase activity during reprogramming that leads to the elongation of the telomeres and maintenance of those telomeres during long-term culture (Marion *et al.*, 2009; Rivera *et al.*, 2017). Firstly, this ability to self-renew makes iPSC technology a very attractive tool to be used as unlimited source of disease-relevant cells, overcoming difficulties of using primary cells from patients which can be difficult to expand, hard-to-access or difficult to obtain due to low numbers as in the case of AA. Secondly, ability to elongate telomeres during the reprogramming process gives iPSC the potential to be used as a disease model to investigate the pathological mechanisms involved in telomeropathies or uncover uncharacterised genetic defects associated to telomere maintenance. Thus, different studies have reported the use of iPSC technology to model telomeropathies associated to mutations in genes such as *TERT, TERC* and *DKC* showing that defective iPSC failed to elongate telomeres and showed a reduced haematopoietic differentiation capacity (Batista *et al.*, 2011; Winkler *et al.*, 2013; Gu *et al.*, 2015)

1.2.4.3 Avoidance of ethical issues

iPSC do not have the ethical problems associated to human ESCs and SCNT since they can be generated from differentiated cells without the need for human embryos or oocytes.

1.2.4.4 Accessibility of cell source for reprogramming

Generation of iPSC lines from easily accessible cell types such as skin fibroblasts and peripheral blood cells have been widely reported (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Loh *et al.*, 2009; Staerk *et al.*, 2010). Thus, a simple blood sample or skin biopsy from a donor can usually provide with enough material to establish different donor-specific iPSC lines. This availability and accessibility in the source of cell for reprogramming confers iPSC technology a huge advantage compared to disease modelling approaches using primary cells that are difficult to isolate or access in the human body such as brain or heart cells or the use of oocytes to induce reprogramming such as in SCNT approaches.

1.2.5 Challenges in iPSC technology

Although a very promising future lies ahead for the iPSC field, several challenges need to be addressed in order to get the full potential from this technology. ESC are considered the gold standard of pluripotency (De Los Angeles *et al.*, 2015). Currently, the degree of similarity between ESC and iPSC is still a matter of debate. Several studies have reported transcriptional and epigenetic differences between ESC and iPSC, which can lead to a reduced iPSC differentiation potential, attributable to a an inefficient reprogramming to pluripotency (Chin *et al.*, 2009; Deng *et al.*, 2009; Ghosh *et al.*, 2010). However, more recent studies using high-throughput sequencing analysis indicate that ESC and iPSC are indistinguishable and that the differences observed in ESC and iPSC are most likely due to genetic background differences rather than reprogramming artefacts present in iPSC (Newman and Cooper, 2010; Bock *et al.*, 2011; Choi *et al.*, 2015). Moreover, by comparing 4 ESC and 14 iPSC lines reprogrammed using Sendai virus-based vector, Feraud *et al.* reported that ESC and iPSC showed similar haematopoietic differentiation potential, and that differences in the differentiation potential were attributable to donor cell of origin (Feraud *et al.*, 2016). To date, these are the challenges described in the use of iPSC technology:

1.2.5.1 Viral integration

Integration of provirus transgenes into the host genome and silencing of the provirus transgenes in the reprogrammed cells are problems associated with the use of integrative vectors in reprogramming such as retroviral and lentiviral vectors. The use of retroviruses and lentiviruses to generate iPSC leads to the random integration of viral transgenes into the host genome (up to 40 viral integration sites in their genomes). This transgene integration could lead to mutation within the host genome or could alter expression of neighbouring host genes in a similar manner than gene therapy induced oncogenesis in children with SCID following transplantation of retroviral gene-modified HSCs (Hacein-Bey-Abina *et al.*, 2003). Likewise, although integrated proviruses are usually silenced during iPSC generation, risk of reactivation of these viral transgenes, including potent oncogenes, could result in formation of tumours. Additionally, if the viral transgenes are not silenced properly, leaky expression may inhibit proper iPSC differentiation and maturation, leading to a greater risk of immature teratoma formation (Chan *et al.*, 2009; Ramos-Mejia *et al.*, 2012). These problems are especially relevant

for the first generation of iPSC since they were generated via integrative viral vectors. However, many methods have been developed during the last years to overcome these problems. Thus, in order to promote reprogramming without transgene integration, different strategies have been designed including use of adenoviral vectors based on a transient expression of exogenous factors without integration (Stadtfeld *et al.*, 2008), Cre/LoxP recombination system to excise integrated transgenes after transfection of target cells (Kaji *et al.*, 2009), use of DNA-based vectors (Yu *et al.*, 2009), reprogramming factors in the form of proteins instead of DNA (Kim *et al.*, 2009), synthetically modified messenger RNA (mRNA) (Warren *et al.*, 2010), ectopic introduction of microRNA (miRNA) (Judson *et al.*, 2009; Miyoshi *et al.*, 2011) and use of RNA viruses such as Sendai virus (SeV) (Fusaki *et al.*, 2009). All these new strategies are on the right track to avoid problems associated to transgene integration, with episomal DNAs, synthetic mRNA and SeV being the most commonly applied due to their high reprogramming efficiency. SeV stands out by both its relative simplicity of use and its high efficiency of reprogramming compared to other integration-free approaches. This type of virus is a RNA virus that replicates its genome in the cytoplasm of the host cell and can stably express reprogramming factors with a high reprogramming efficiency with no need of multiple transductions (Fusaki *et al.*, 2009). However, Sendai-based iPSC tend to carry the virus genome even after long-term culture. Therefore, it is necessary, in some cases, to subclone part of the iPSC culture or use a temperature-sensitive mutant in order to eliminate remaining SeV particles that could be present in the culture. Nonetheless, the high reprogramming efficiency together with the lack of genomic integration and the easy-to-use performance showed by SeV appears as one of the best methods to reprogram somatic cells to pluripotency developed to date.

1.2.5.2 Incomplete reprogramming

Reprogramming to pluripotency is a complex process where many stages have to be completed in order to obtain fully-reprogrammed cells. In the first stages of reprogramming, there is an induction of proliferation and down-regulation of cell-of-origin specific genes. In the following stages of reprogramming acquisition of epithelial characteristics and induction of pluripotency network by up-regulation of pluripotency-related genes are crucial (Plath and Lowry, 2011). Thus, absence/defective transcriptional and epigenetic changes during these stages can lead to an incomplete

reprogramming of the cell and the appearance of partially-reprogrammed clones with different degrees of reprogramming status within the same iPSC line, leading to clonal heterogeneity. Although these partially-reprogrammed clones may present similar characteristics to fully-reprogrammed iPSC clones such as morphology, pluripotency gene expression and even ability to form teratomae in SCID mice, they exhibit impaired differentiation potential (Chan *et al.*, 2009; Koyanagi-Aoi *et al.*, 2013). Thus, it is necessary to identify these partially-reprogrammed clones in order to avoid confusion and enable the identification of true biological phenotypes when analysing the differentiation potential of patient-specific iPSC lines. Better understanding of the reprogramming process may help to thoroughly characterize and select fully-reprogrammed iPSC. Thus, Nishizawa et al., carried out a comprehensive study using 35 human iPSC lines generated by different methods and reported the identification of genes (*IGF2* and *TRIM58*) as indicators of the haematopoietic differentiation potential of human iPSC (Nishizawa *et al.*, 2016). Differences in the expression of these genes in iPSC due to aberrant DNA methylation patterns acquired during reprogramming can lead to impaired differentiation potential within clones from the same iPSC line providing with a method to faithfully select the best iPSC lines for downstream applications.

1.2.5.3 Epigenetic memory

Currently, the existence of somatic memory in iPSC is a matter of debate. The fact that iPSC originated from peripheral blood cells have a higher differentiation capacity to generate haematopoietic cells than neuronal cells might suggest that iPSC retain some memory from the cell of origin (Kim *et al.*, 2010). Several studies reported incomplete erasure of histone modifications and existing epigenetic marks in DNA of tissue of origin that might lead to changes at transcriptional and proteomic levels in different iPSC lines ultimately resulting in impaired differentiation potential and clonal heterogeneity (Bar-Nur *et al.*, 2011; Kim *et al.*, 2011; Ohi *et al.*, 2011; Nishizawa *et al.*, 2016). Interestingly, Polo *et al.* reported that prolonged passaging of iPSC seems to contribute to the erasure of the epigenetic memory associated to the cell of origin, indicating that reprogramming process is prolonged over time (Polo *et al.*, 2010). However, high-throughput sequencing studies showed recently that iPSC lines generated from different tissues are transcriptionally similar suggesting no retention of memory associated to cell type of origin (Rouhani *et al.*, 2014; Kyttala *et al.*, 2016;

Carcamo-Orive *et al.*, 2017). Likewise, accumulation of repressive marks such as DNA methylation has been reported in both ESC and iPSC (Bock *et al.*, 2011) suggesting that the reprogramming process has no influence in the presence of epigenetic marks in iPSC. In fact, this global increased in DNA methylation has been associated to the primed state characteristic of human PSC described in **section 1.2.1**. Acquisition of naïve pluripotency is associated with the removal of epigenetic repressive marks that would explain the greater differentiation potential of naïve PSCs over primed PSCs (Gafni *et al.*, 2013). Conversion of human PSC from primed to naïve state has been achieved by a combination of transgenes, inhibitors and growth factors (Takashima *et al.*, 2014; Theunissen *et al.*, 2014) or use of inhibitors and growth factors only (Gafni *et al.*, 2013; Theunissen *et al.*, 2014; Theunissen *et al.*, 2016). Conversion to naive state would provide a means to improve quality of iPSC by facilitating the removal of residual epigenetic memory and minimize clone heterogeneity (Weinberger *et al.*, 2016). Use of epigenetic modifiers have been suggested by different authors to increase reprogramming efficiency and erasure potential epigenetic memory of the tissue of origin (Nashun *et al.*, 2015). Interestingly, Hou *et al.* reported in 2013 the generation of iPSC through the use of epigenetic modifiers and manipulation of signalling pathways using solely small-compounds, highlighting the importance of chromatin manipulation for establishment of reprogramming (Hou *et al.*, 2013).

1.2.5.4 Genomic instability

Presence of genetic variations in iPSC, potentially attributed to the reprogramming-induced stress or long-term *in vitro* culture, might alter the differentiation capacity of the ESC/iPSC and increase risk of tumorigenicity (Ben-David and Benvenisty, 2011). Thus, genetic mutations were identified in the iPSC used in one of the patients in the first clinical trial evaluating human iPSC-derived RPE cells started in 2014, although there was no clear evidence that these mutations could lead to adverse effects (Shi *et al.*, 2017). Genetic variations found in iPSC can be present in parental somatic cells, induced by reprogramming or by prolonged passaging and include chromosomal aberrations such as aneuploidy, copy number variations (CNV), and single nucleotide variations (SNV) (Liang and Zhang, 2013). Trisomy 12 is the chromosomal abnormality most commonly found in both ESC and iPSC and it has been suggested to provide a selective advantage by increasing proliferation and reprogramming due to the presence of cell-cycle related genes and pluripotency gene *NANOG* (Taapken *et al.*,

2011). Early studies using comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) array analysis reported the presence of CNV in iPSC that were not detected in parental cell of origin, leading to the hypothesis that the reprogramming process generates de novo genetic variations (Elliott *et al.*, 2010; Hussein *et al.*, 2011; Martins-Taylor *et al.*, 2011). However, with the use of most recent technologies such next-generation sequencing (NGS) technologies, deeper genetic analysis revealed that most of the CNV detected in iPSC were present in low frequency in the parental cell population (Abyzov *et al.*, 2012; Kwon *et al.*, 2017) indicating that the iPSC reprogramming process does not induce variations in the number of copies. Such discrepancy in the conclusions has been attributed to the limited dynamic range to detect low-frequency SNP by array-based analysis compared to the higher resolution offered by the NGS technologies (Liang and Zhang, 2013). Likewise, by using NGS, different studies have identified no more than a dozen SNV in protein-coding genes in iPSC with half of them already present in the subpopulation of the parental population and hundreds of mutations considered as benign distributed throughout the genome (Young *et al.*, 2012; Bhutani *et al.*, 2016), suggesting that the reprogramming process is not mutagenic. Nonetheless, extensive screening for meaningful genetic alterations is necessary in order to ensure the safety, quality and purity of the iPSC cultures for downstream applications, especially for clinical application of iPSC-derived cells (Yoshihara *et al.*, 2017).

1.2.5.5 Use of proto-oncogenes.

Use of proto-oncogenes such as *cMyc* in the reprogramming cocktail of factors have raised concern regarding the potential therapeutic use of iPSC due to the potential tumorigenic transformation of the reprogrammed cells (Nakagawa *et al.*, 2008). Although not essential to generate iPSC, *cMyc* seems to increase the reprogramming efficiency significantly by regulating cellular proliferation (Nakagawa *et al.*, 2008; Sridharan *et al.*, 2009). Interestingly, it has been found that the use of a transformation-deficient variant of the Myc family, *L-myc*, resulted in the generation of mouse iPSC even with a higher efficiency than with *cMyc* and a much lower rate of tumour formation (Nakagawa *et al.*, 2010).

The iPSC field is progressing rapidly and increased knowledge of the reprogramming process and development of improved reprogramming strategies is helping to

overcome these challenges, ultimately leading to the generation of *bona fide* patient-specific PSCs for disease modelling studies and therapeutic applications.

1.3 Differentiation of human PSCs into haematopoietic cells

Human PSCs provide a useful means to study human blood development due to their ability to differentiate into cell lineages of all three germ layers. Several studies have shown that *in vitro* haematopoietic differentiation of PSCs similarly mirrors *in vivo* haematopoiesis making them very useful for the study of haematopoiesis during embryonic development (Lensch and Daley, 2006).Likewise, numerous studies have demonstrated the ability of PSCs to generate specifically all mature blood cell types under suitable conditions, from haematopoietic progenitors to mature blood cells (Vodyanik *et al.*, 2006; Kennedy *et al.*, 2007; Woll *et al.*, 2008; Galic *et al.*, 2009; Saeki *et al.*, 2009; Tseng *et al.*, 2009; Carpenter *et al.*, 2011; Dias *et al.*, 2011; Mills *et al.*, 2013) . Thus, PSCs can be an excellent source for large-scale production of blood cells components because of their ability of being expanded indefinitely without losing pluripotency (Olivier *et al.*, 2006).

1.3.1 Haematopoietic ontogeny in the human embryo

Understanding the process of *in vivo* haematopoiesis will help to design *in vitro* strategies to generate functional transplantable haematopoietic cells. Haematopoietic cells have been detected in the human yolk sac (YS) between days 16 and 19 of embryonic development, and in the placenta, aorta-gonad-mesonephros (AGM) and liver from days 24 and 28 (Luckett, 1978; Tavian *et al.*, 1999; Robin *et al.*, 2009). These cells subsequently enter the circulation and colonize the foetal liver, where they mature from week 6 to 16 and migrate to the bone marrow, the major haematopoietic organ from the second half of the gestation (Lensch and Daley, 2004). *In vitro* culture of cells from both YS and AGM generate haematopoietic derivatives, indicating the independent origin of haematopoietic cells in different sites. However, only cells from AGM give rise to B- and T-cells and HSC (referred to as definitive haematopoiesis), whereas YS predominantly generates nucleated erythroid cells, macrophages and monocytes (referred to as primitive haematopoiesis) (Tavian *et al.*, 2001) (**Figure 14**).

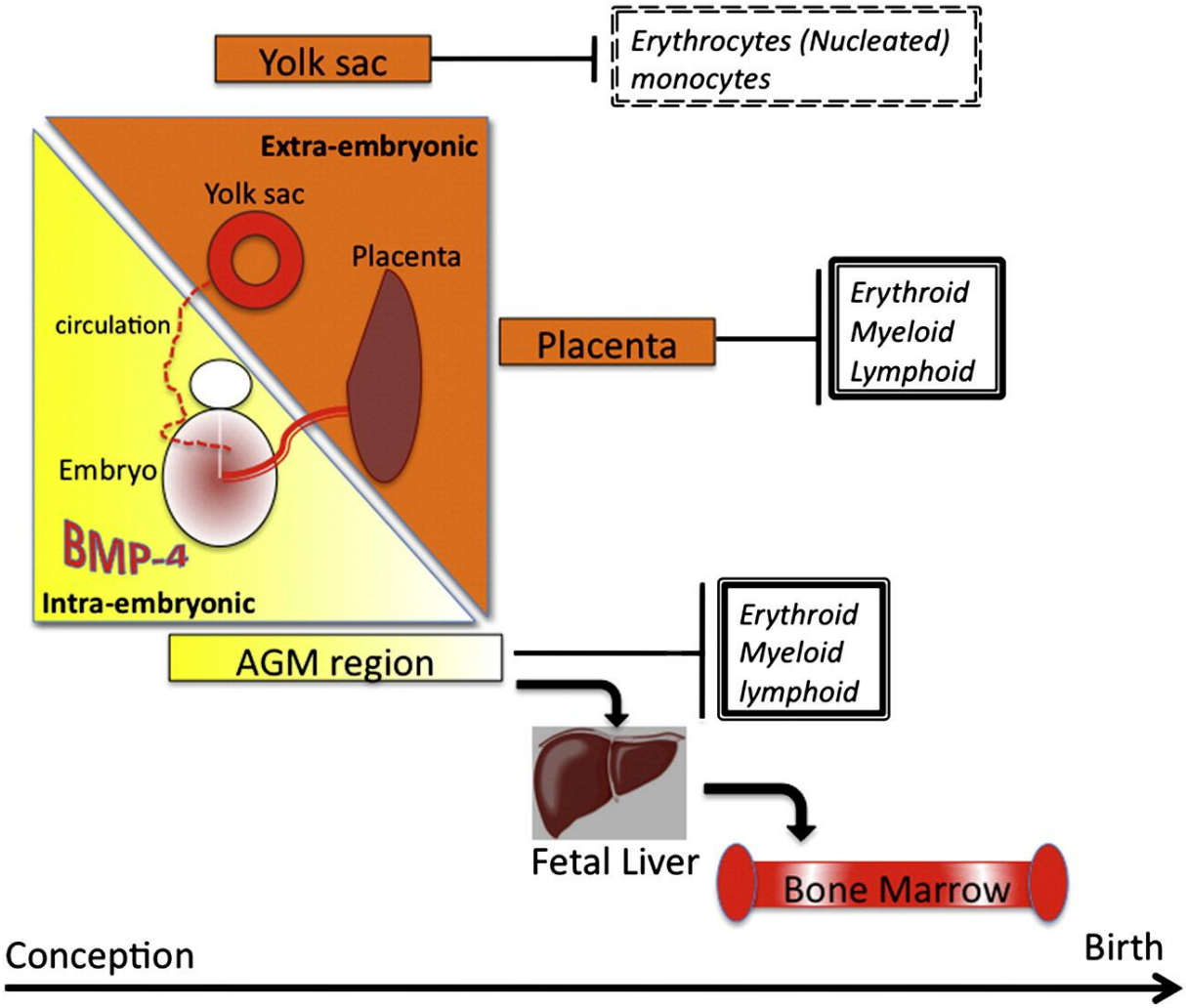


Figure 14. Human embryonic haematopoiesis

Primitive erythrocytes and monocytes (primitive haematopoiesis) are generated in the yolk sac. With onset of circulation, yolk-sac derived blood cells enter embryonic tissues. Definitive erythrocytes, myeloid and lymphoid progenitors and HSC emerge on the floor of the AGM region and migrate to foetal liver where they undergo expansion before colonizing the bone marrow where they will reside throughout adult life. AGM, aorta-gonad-mesonephros. Reproduced from Dravid and Crooks, 2011. (Dravid and Crooks, 2011)

Medvinsky and Dzierzak reported that definitive haematopoiesis in the mouse emerges in the AGM region prior colonization of foetal liver of haematopoietic stem cells (Medvinsky and Dzierzak, 1996). This common origin between haematopoietic and endothelial cells could reflect two intrinsic processes to produce haematopoietic cells, i.e., either via a cell without evidence endothelial or haematopoietic differentiation (haemangioblast) or a cell that already displays endothelial features but able to produce haematopoietic cells (haemogenic endothelium). Studies based on the *in vivo* haematopoiesis in the mouse show that haemangioblast cells migrate to the YS and

could be responsible for the generation of primitive haematopoietic cells, whereas the vascular endothelial cells, located in the dorsal aorta in the AGM region, forming the haemogenic endothelium, may be responsible for the generation of the adult-definitive haematopoietic cells (Dzierzak and Speck, 2008). The existence of the haemangioblast has been identified in the mouse, but not yet in human due to the inaccessibility to human embryonic tissue. Keller and his colleagues demonstrated that a comparable haemangioblast population exists by generating blast cells from mouse with both haematopoietic and vascular potential (Choi *et al.*, 1998). On the other hand, studies based on lineage tracing and live imaging in the mouse and zebra fish proved that endothelial cells can transform into haematopoietic cells that will form the HSCs in the adult animal (Zovein *et al.*, 2008; Eilken *et al.*, 2009; Boisset *et al.*, 2010; Kissa and Herbomel, 2010) Thus, the concept of haemangioblast is still controversial since most of the data published so far can be explained by the concept of haemogenic endothelium (Bautch, 2011).

1.3.2 In vitro differentiation of PSC to haematopoietic cells

Haematopoiesis is an intricate process resulting from a cascade of specific events determined by the expression of a specific group of genes that encode surface antigens. The expression of these antigens can change as the cells get further through the differentiation process. Understanding of the *in vivo* process is extremely important to establish the culture conditions to *in vitro* differentiate PSC into haematopoietic cells, identify the stage-specific phenotypes of the cell through the differentiation process and establish methods to distinguish the cells as they are specified.

1.3.2.1 Molecular mechanisms and factors promoting haematopoiesis

Different signalling cascades are activated during the *in vitro* generation of HSPCs trying to mimic the molecular events occurring in the haematopoietic development *in vivo*. Similarly to early embryonic development, activation of signalling pathways by bone morphogenetic protein 4 (BMP4), WNT, fibroblast growth factor-2 (FGF2) and vascular endothelial growth factor (VEGF) is required in the haematopoietic specification of PSCs (Chadwick *et al.*, 2003; Kennedy *et al.*, 2007; Pick *et al.*, 2007). Formation of primitive streak (PS) is characterized by up-regulation of transcription factors such as *Brachyury (T)* and *MIXL1* induced by BMP4, Activin A and FGF2 during

the early phases of PSCs differentiation (Davis *et al.*, 2008). Formation of posterior/anterior mesendoderm requires the activation of WNT signalling, whereas specification to posterior PS (mesoderm) or anterior PS (endoderm) is regulated by the balance between BMP4 and Activin signalling pathways (Sumi *et al.*, 2008) (**Figure 15**).

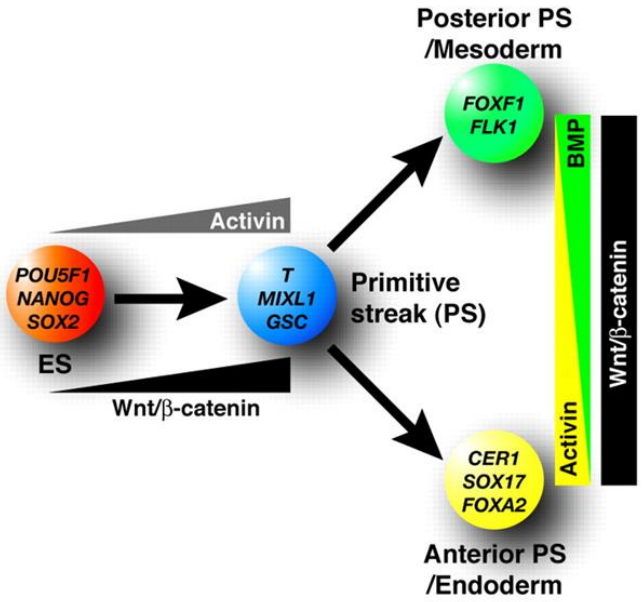


Figure 15. Early differentiation of PSCs into primitive streak (PS).
Wnt/β-catenin signalling pathway is essential to establish the generation of PS-mesendoderm progenitors from PSCs. Balance of Activin/BMP signalling pathways dictates specification into endoderm and mesoderm. ES, embryonic stem cells; T, Brachyury; PS, primitive streak. Reproduced from Sumi *et al.* 2008 (Sumi *et al.*, 2008)

Primitive posterior mesodermal cells can be identified by the expression of markers such as apelin receptor (APLN), platelet-derived growth factor receptor (PDGFR)α/CD140a and KDR (Vodyanik *et al.*, 2010) (**Figure 16**). Likewise, WNT signalling induces the formation of haematovascular mesodermal progenitors (HMVP) from the posterior PS population inducing up-regulation of angiohaematopoietic genes such as T cell acute lymphocytic leukaemia 1 *(TAL1), HHEX, LMO2,* GATA protein *2 (GATA2)* and ETS variant 2 (*ETV2)* and high expression of *KDR* and low expression of *PDGFR* markers (Choi *et al.*, 2012) (**Figure 16**). Likewise, it has been described the existence of intermediate progenitors at the mesodermal stage, referred as haemangioblasts, that, unlike HMVP, have potential to generate haematopoietic colonies through endothelial intermediates in the presence of FGF2 (Kennedy *et al.*, 2007) (**Figure 16**). These progenitors define the onset of haematopoiesis since they

consist of vascular and haematopoietic progenitors; however, their potential is restricted to primitive haematopoietic cells similarly to their *in vivo* counterparts found in the posterior PS (Huber *et al.*, 2004).

Following *in vitro* differentiation, the first population of endothelial cells, characterized by the expression of VE-cadherin, CD31 and CD34 markers, emerge from the HMVP population (**Figure 16**). Within this population of endothelial cells, *Choi et al.* identified two distinct subpopulations of cells based on the expression of CD73, leukosialin (CD43) and CD235a markers: haemogenic endothelium (HE) (CD73-/CD43-) and non-HE (CD73+/CD43-) (Choi *et al.*, 2012). Both HE and non-HE display endothelial features and lack of haematopoietic colony-forming potential when cultured in methylcellulose-based media supplemented with haematopoietic cytokines However, HE cells have the ability to generate both endothelial cells and definitive haematopoietic progenitors upon cultivation on OP9 mouse stromal cells (Choi *et al.*, 2012). Interestingly, Kennedy *et al.* reported that HE cells display T lymphoid potential when cultured on OP9 cells (Kennedy *et al.*, 2012). Various subpopulations of HE cells can be defined by runt-related transcription factor 1 (*RUNX1)* expression (Ran *et al.*, 2013). *Runx1* is required for definitive haematopoiesis but not for primitive and endothelial cells from HE for it has been shown to be critical in the endothelial to haematopoietic transition (EHT) during mouse embryonic development (Chen *et al.*, 2009). SRY-box 17 (SOX17) is another transcription factor that has been involved HE regulation and EHT in human PSCs (Clarke *et al.*, 2013; Nakajima-Takagi *et al.*, 2013).

First haematopoietic progenitors arising from HE population can be detected by surface expression of CD43 (Vodyanik *et al.*, 2006; Kennedy *et al.*, 2012) (**Figure 16**). This is important because use of this marker allows the separation of haematopoietic progenitor cells, characterized by colony-forming potential in semi-solid medium supplemented with haematopoietic cytokines, from non-haematopoietic cells such as endothelial and mesenchymal cells. Emerging from this CD34+CD43+ haematopoietic progenitor population, different subpopulations of progenitors can be found. The first to emerge are CD235a+ and CD41a+ cells within the CD43+ cells with potential to generate erythroid (EryP) and megakaryocytic progenitors (MkP) in colony-forming assays (Vodyanik *et al.*, 2006; Klimchenko *et al.*, 2009) (**Figure 16**). These progenitors display features of primitive haematopoiesis since they mainly express embryonic

haemoglobins and contain large nucleated cells. Finally, CD43+ haematopoietic progenitors with potential to generate myeloid lineage colonies appear shortly after emergence of erythroid/megakaryocytic progenitors (Ery/MkP) and are characterized by CD41a-CD235a- phenotype (Vodyanik *et al.*, 2006) (**Figure 16**). These CD34+CD43+CD41a-CD235a- myeloid progenitors (MyeP) have been associated with initial stages of definitive haematopoiesis since show expression of haematopoietic-regulator genes *GATA-2*, *GATA-3*, *RUNX1* and *c-MYB* (Rodrigues *et al.*, 2012; Frelin *et al.*, 2013; Ran *et al.*, 2013; Sankaran and Orkin, 2013) and display potential to generate cells of lymphoid lineage such as natural killer cells and cells from B-lymphoid lineage (Carpenter *et al.*, 2011; Knorr *et al.*, 2013). Expression of the pan-haematopoietic marker CD45 is acquired in later stages and it has been associated to commitment to more mature myeloid progenitors (Vodyanik *et al.*, 2006).

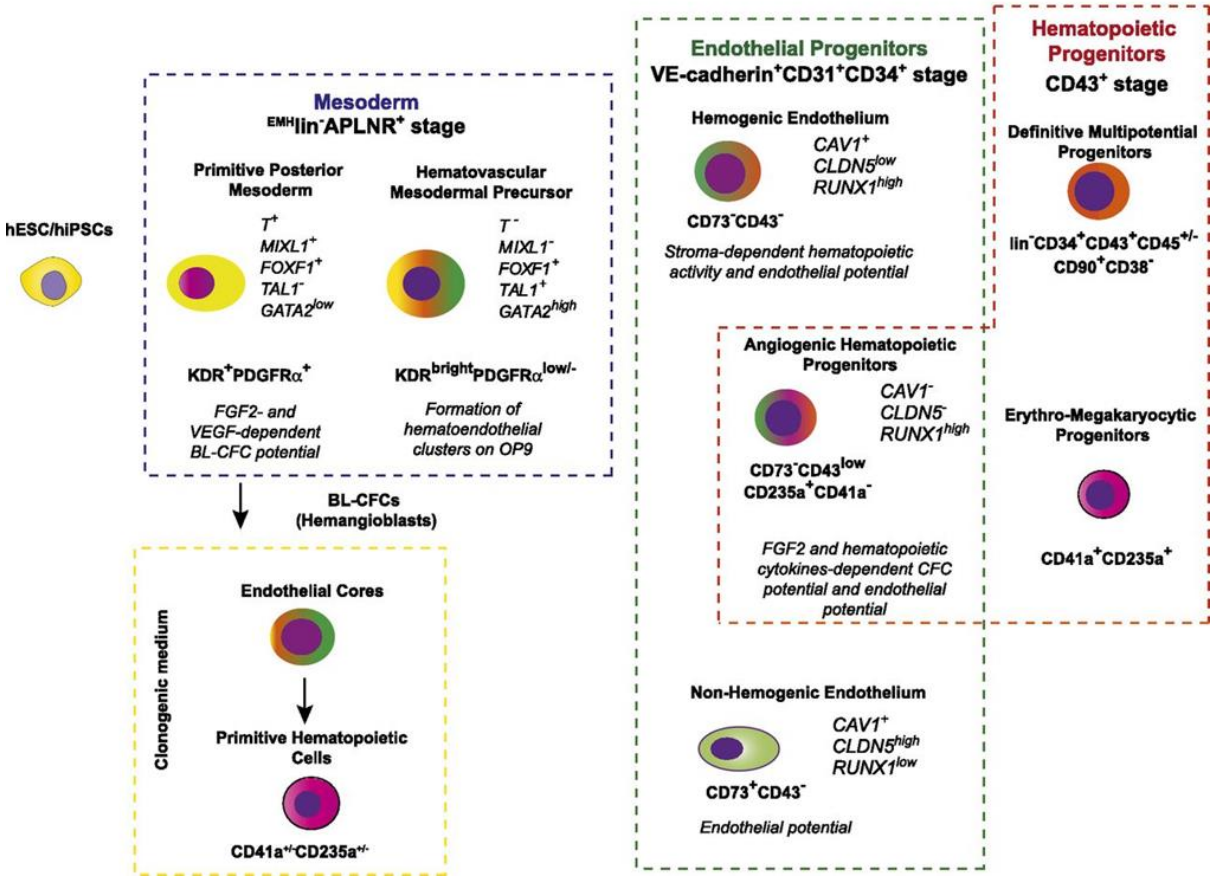


Figure 16. Haematopoietic differentiation from PSCs.

Generation of mesoderm from PSCs is defined by expression of mesodermal markers APLNR and KDR. Most primitive mesodermal precursors with haematopoietic potential emerge from the primitive posterior mesoderm cells and are able to generate primitive haematopoietic cells ($CD41a^{+/-}CD235a^{+/-}$) in clonogenic medium. Endothelial commitment of mesodermal precursors is characterized by up-regulation of KDR in haematovascular mesodermal precursors (HVMP). HVMP give rise to endothelial progenitors identified by expression of VE-cadherin, CD31 and CD34 endothelial markers. Within this endothelial, haemogenic endothelium (HE) cells display endothelial features but are capable of forming haematopoietic cells when cultured under specific conditions. Up-regulation of CD43 expression defines the emergence of the haematopoietic progenitor cells including erythroid-megakaryocytic ($CD41a^{+}CD235a^{+}$) and multipotent myelolymphoid progenitors ($CD34^{+}CD43^{+}CD45^{+/-}lin^{-}$). Lin: CD41aCD235a. Modified from Slukvin, 2013. (Slukvin, 2013)

1.3.2.2 Methods to generate haematopoietic cells from PSC

Directed differentiation. Two distinct differentiation approaches have been most commonly used for the differentiation of PSCs into haematopoietic progenitors: co-cultivation of PSC on feeder cells mimicking the HSC niche or timed addition of key cytokines and morphogens to induce activation of specific signalling pathways.

Several cell lines have been reported as stromal feeder cells such as OP9, S17 and MS5 from murine bone marrow, mFLSC from murine foetal liver derived stromal line, FH-B-hTERT from immortalized human foetal liver hepatocyte line and primary human stromal cell lines derived from AGM, foetal liver and foetal bone marrow (Vodyanik *et al.*, 2005; Moore *et al.*, 2006; Ledran *et al.*, 2008). Stromal cells create an *in vitro* microenvironment, similar to that *in vivo*, that seems to help survival, proliferation and haematopoietic differentiation of PSC. Likewise, stromal cell lines can be modified to produce specific factors that can help to reproduce specific environment for the generation of specific haematopoietic lineages such as T and B cells by using OP9 stromal cells constantly overexpressing the Notch ligand Delta-like 1 or 4 (Timmermans *et al.*, 2009; Roundy *et al.*, 2010; Kennedy *et al.*, 2012). However, the need for serum-containing media to grow these stromal cell lines distances this method from a future clinical application.

Use of cytokines and morphogens for the derivation of *in vitro* HSPCs is an alternative method that enables the differentiation of PSC in a serum-free and defined media. Early differentiation of PSCs into mesodermal and endothelial lineages relies on the addition BMP4, VEGF, WNT and stem cell factor (SCF) (Pick *et al.*, 2007; Sumi *et al.*, 2008; Niwa *et al.*, 2011; Sturgeon *et al.*, 2014). Likewise, use of cytokines such as SCF, TPO, fibroblast-growth factor (FGF), Fms-related tyrosine kinase 3 ligand (FLT3-L), granulocyte colony-stimulating factor (G-CSF), interleukin-6 (IL-6) and interleukin-3 (IL-3) at specific time points promote the haematopoietic differentiation and expansion from PSCs *in vitro* (Chadwick *et al.*, 2003; Zambidis *et al.*, 2005; Uenishi *et al.*, 2014). These methods are usually combined with the formation of 3 dimensional spheres, embryoid bodies (EB), by plating the PSCs on ultra-low attachment surface. The lack of adhesion will promote the formation of these structures and the differentiation of the PSCs into all three germ layers. The use of serum-free defined media helps to the study of the roles of specific morphogens and cytokines in the haematopoietic

differentiation of PSC as well may make it the method of choice for a possible clinical use of the cells. Likewise, differentiation recipes can include erythropoietin (EPO) to further differentiation of PSCs to red blood cells (Chang *et al.*, 2006) or combined with stromal cells for driving differentiation to B and T cells (Kennedy *et al.*, 2012; Sturgeon *et al.*, 2014).

However, these approaches used to differentiate *in vitro* PSCs into haematopoietic progenitors have several limitations. Studies using these strategies have reported the generation of *in vitro*-derived haematopoietic progenitor populations but lacking robust engraftment and lymphoid potential (Ackermann *et al.*, 2015). Additionally, the haematopoietic progenitors resemble those generated in the yolk sac during *in vivo* primitive wave of haematopoiesis producing erythrocytes mostly nucleated and containing embryonic and foetal globins (Wahlster and Daley, 2016).

Directed conversion. Overexpression of critical transcription factors in PSCs is an alternative strategy developed to directly convert PSCs into angiohaematopoietic progenitors or HSPCs. First attempts inducing haematopoietic differentiation of human ESCs using transcription factors involved the overexpression of the homeobox B4 (*HOBX4)* gene, with multiple roles in haematopoietic development (Alharbi *et al.*, 2013). Different studies using ectopic expression of *HOXB4* are characterized by variability and lack of consistency in their findings (Forrester and Jackson, 2012), with one study reporting that *HOXB4* is dispensable for haematopoietic differentiation in human cells (Wang *et al.*, 2005), leading to researchers to focus on other transcription factors.

Cells with endothelial features displaying haematopoietic potential, HE cells, have been generated from human iPSCs using two combinations of transcriptions factors *ETV*)/*GATA2* and *TAL1*/*GATA2* (Elcheva *et al.*, 2014) (**Figure 17**). Haematopoietic progenitors generated from each population of HE cells displayed different potential depending on the combination of transcription factors used, with *ETV2*/*GATA2* resulting in progenitors with myeloid potential and *TAL1*/*GATA2* in progenitors with erythro/megakaryocytic potential, highlighting the complexity of haematopoietic specification, starting at the HE stage (Easterbrook *et al.*, 2016).

Screening of genes differentially expressed in umbilical cord blood HSC and haematopoietic myeloid progenitors enabled the identification of up-regulated genes in cord blood haematopoietic progenitors such as homeobox A9 (*HOXA9*), ETS transcription *factor (ERG)* and RAR-related orphan receptor A *(RORA)* (Doulatov *et al.*, 2013). Ectopic expression of all these genes combined conferred self-renewal and multipotential differentiation capacity to myeloid-committed haematopoietic progenitors. Likewise, addition of transcription factors SRY-Box 4 *(SOX4)* and MYB proto-oncogene *(MYB)* enabled engraftment of these progenitors *in vivo*, highlighting the relevance of these regulatory networks in haematopoietic development (**Figure 17**). However, engraftment of these converted haematopoietic progenitors was not maintained in time and no lymphoid cells were generated *in vivo* (Easterbrook *et al.*, 2016). Similar approach was very recently developed by Daley's group that reported the generation of HSPCs with multi-lineage haematopoietic engraftment potential in immunodeficient mouse by forcing expression of *ERG, HOXA5, HOXA9, HOXA10*, ligand dependent nuclear receptor corepressor (*LCOR*), *RUNX1* and Spi-1 proto-oncogene (*SPI1*) genes previously identified to facilitate *in vivo* haematopoietic engraftment (Sugimura *et al.*, 2017). Interestingly, PSCs were differentiated into HE cells via directed differentiation using cytokines and morphogens. PSCs-derived HE cells were transduced with the combination of transcription factors giving rise to HSPCs with multi-lineage differentiation potential and engraftment potential although not in a robust fashion.

Crucial genes for generation of HE and definitive haematopoiesis such as *RUNX1*, *SOX17* and *TAL1* have been used separately to induce the generation of haematopoietic progenitor cell generation from human PSCs. Upon *RUNX1A* isoform overexpression, haematopoietic progenitor cells with definitive features, such as ability to generate erythrocytes containing adult haemoglobin and *in vivo* multilineage engraftment, were generated (Ran *et al.*, 2013) (**Figure 17**). However, a high percentage of the engrafted cells retain a phenotype characteristic of undifferentiated progenitors questioning the ability of these progenitors to efficiently differentiate into mature haematopoietic cells (Easterbrook *et al.*, 2016). Likewise, *SOX17* overexpression led to the successful formation and expansion of HE cells from human PSCs, although it is necessary the release of HE cells from *SOX17* overexpression to enable the generation of haematopoietic progenitor cells (Nakajima-Takagi *et al.*,

2013). Forced expression of *TAL1* led to the formation of haematopoietic progenitors with megakaryocytic and erythroid potential from human ESCs although lacking of *in vivo* engraftment capacity (Real *et al.*, 2012). These studies using single transcription factors to induce formation of HSPCs from PSCs are characterized by the inability to produce fully *in vivo* multilineage engraftment, highlighting the complexity of the haematopoietic regulatory networks (Daniel *et al.*, 2016).

Likewise, transdifferentiation approaches involving the ectopic expression of *OCT4* and *SOX2* in human fibroblasts (Pulecio *et al.*, 2014) and *FOSB, GFI1, RUNX1* and *SPI1* in human endothelial cells (Sandler *et al.*, 2014) have resulted in the generation of haematopoietic progenitors with *in vivo* engraftment capacity, albeit with a reduced differentiation potential (Ackermann *et al.*, 2015) (**Figure 17**).

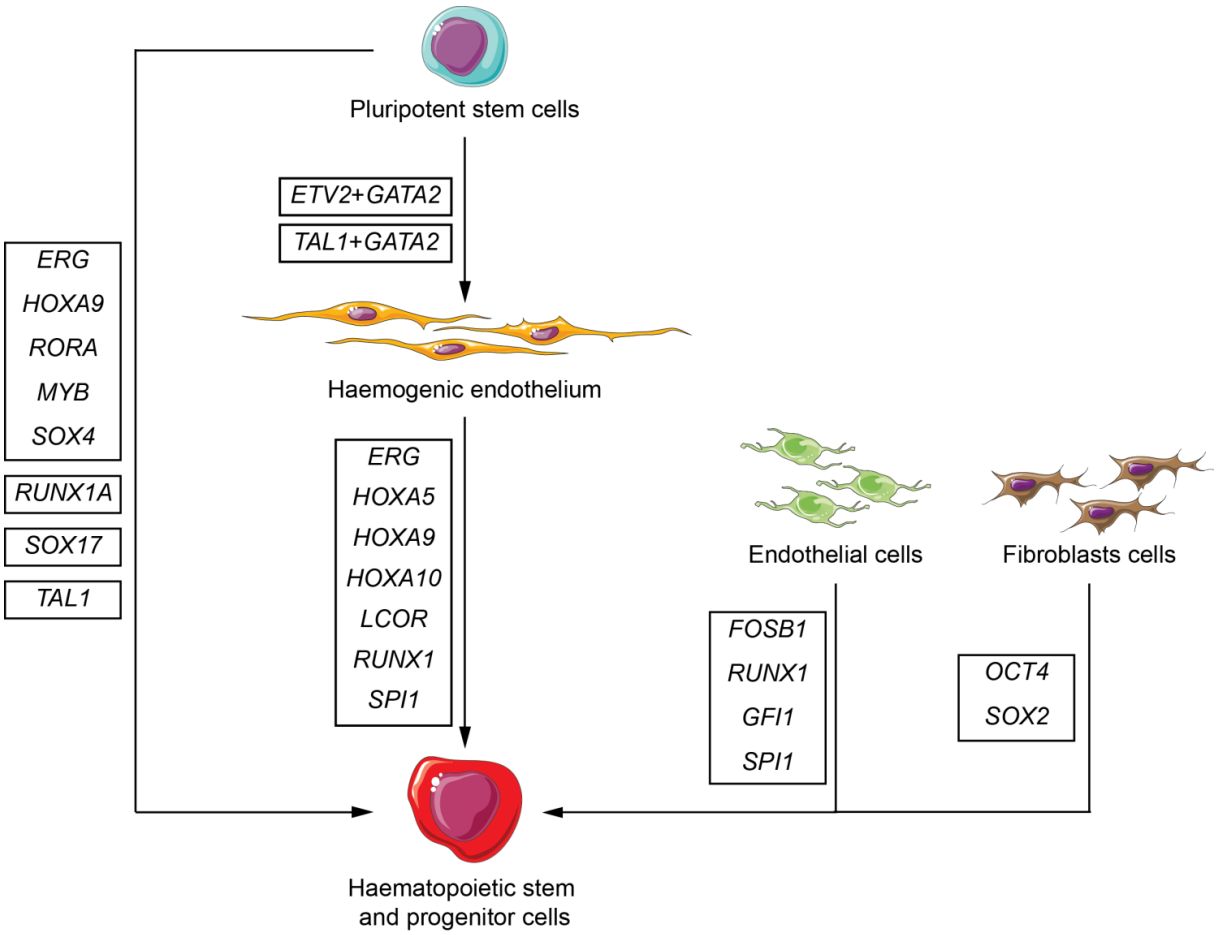


Figure 17. Strategies to generate HSPCs by in vitro direct conversion of PSCs or differentiated cells.

Boxes list combination of multiple transcription factors or single transcription factors used for direct conversion of PSCs or differentiated cells (endothelial or fibroblast cells) into HSPCs.

Isolation from teratoma. Recently, two different studies have reported the successful isolation of CD34+CD45+ cells from teratomae induced in immune-deficient mice by mouse and human iPSCs (Amabile *et al.*, 2013; Suzuki *et al.*, 2013). iPSC-derived haematopoietic progenitor cells isolated from teratomae were used in serial transplantation experiments showing engraftment and capacity for multi-lineage reconstitution in SCID mice. Although these studies are still displaying low induction efficiency and engraftment levels, they highlight the importance of the generation of a suitable microenviroment for HSPC generation provided in these *in vivo* studies by extracellular matrix or blood (Ackermann *et al.*, 2015).

1.3.3 Challenges in haematopoietic differentiation from PSC

Outstanding progress has been made regarding haematopoietic differentiation of PSC so far. However, there are several hurdles to overcome in regards to a future clinical use of the PSC-derived HSC.

1.3.3.1 Engraftment potential of PSC-derived HSC

The most stringent assay to evaluate the long-term reconstitution ability and lympho-myeloid capacity of the *in vitro* PSC-derived HSC is the use of *in vivo* transplantation. Several studies have shown the engraftment potential of human PSC-derived haematopoietic cells (Wang *et al.*, 2005; Narayan *et al.*, 2006; Tian *et al.*, 2006; Ledran *et al.*, 2008; Risueno *et al.*, 2012; Amabile *et al.*, 2013; Suzuki *et al.*, 2013; Sugimura *et al.*, 2017). However, these studies have shown a poor engraftment ability of the PSC-derived haematopoietic cells generated with bone marrow engraftment levels going from 0.1%-2% with predominant myeloid engraftment (Slukvin, 2013). The poor engraftment levels observed in these studies have been associated to the limited proliferative and colony-forming capacity of the PSC-derived haematopoietic cells mainly caused by an impaired haematopoietic differentiation (Tian *et al.*, 2009). These limitations are most likely due to the inability of these approaches to mimic the complexity of the *in vivo* HSC specification and expansion (Ackermann *et al.*, 2015). Therefore, it is necessary to develop better *in vitro* protocols in order to mimic the *in vivo* microenvironment needed to generate *bona fide* HSCs with robust long-term engraftment potential. Additionally, possible rejection of the host immune system, the mouse strain selected to test the cells or even the host age should be considered as factors that might improve the engraftment levels (Brehm *et al.*).

1.3.3.2 Primitive vs Definitive

As stated in **section 1.3.1**, primitive haematopoiesis is defined as the initial wave of haematopoiesis originated in the YS given rise to primitive erythrocytes, monocytes and megakaryocytes. Subsequent waves of haematopoiesis originated in the embryo, including HSCs produced in the AGM, are considered definitive haematopoiesis. Primitive and definitive waves are temporally and spatially separated in the embryo (**section 1.3.1**). However, both developmental processes co-exist during *in vitro* differentiation of PSCs towards haematopoietic lineages and their identification is

challenging, being recognizable only by functional criteria due to the lack of specific markers (**Figure 18**). Only primitive erythroid progenitors can be properly identified since they generate larger erythrocytes that express embryonic haemoglobin (Palis, 2014).

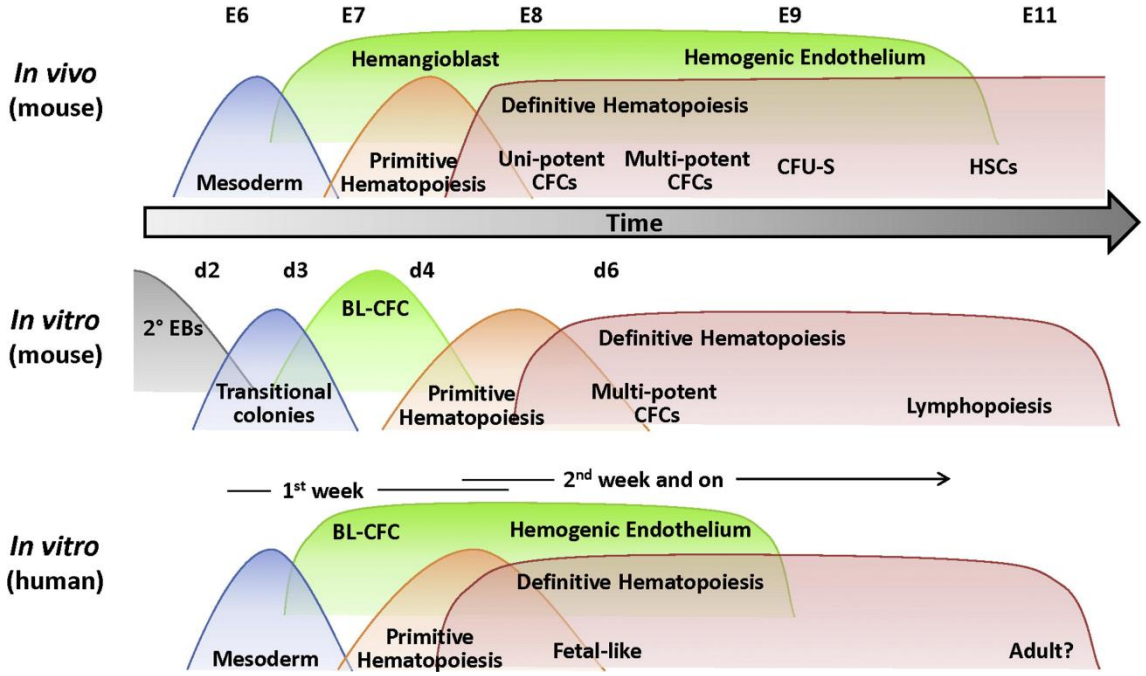


Figure 18. Schematic representation of in mouse and human in vitro haematopoietic development.

Timeline displaying the generation of the different types of haematopoietic precursors and emergence of the different waves of haematopoietic progenitors (primitive and definitive) in mouse embryos (*in vivo*) and from mouse and human PSCs (*in vitro*). CFCs, colony-forming cells; CFU-S, colony-forming spleen; HSCs, haematopoietic stem cells. Modified from Kardel and Eaves, 2012 (Kardel and Eaves, 2012)

Limitation in the identification and generation of HSPCs capable of long-term engraftment has been associated to the lack of protocols recapitulating exclusively the definitive wave of haematopoiesis in which *bona fide* HSC emerge. This is most likely due to the difficulty in mimicking the complexity of haematopoiesis ontogeny in an *in vitro* setting (Wahlster and Daley, 2016). Several studies have reported the importance of blocking the Activin/Nodal signalling pathway to induce definitive haematopoiesis (Kennedy *et al.*, 2012). Likewise, Sturgeon *et al.* reported the identification of a mesodermal population, characterized by the phenotype KDR+/CD235a-, as precursors of the definitive haematopoietic progenitor cells (Sturgeon *et al.*, 2014). These studies used T lymphocyte potential to distinguish definitive from primitive haematopoiesis since T cells clearly represent a definitive lineage that is not generated

during primitive haematopoiesis, unlike erythrocytes, macrophages and megakaryocytes. However, this has been recently challenged since it has been reported that T-cell progenitors are generated before emergence of the first HSCs, indicating that progenitors with T-lymphoid potential do not necessarily represent true HSCs (Boiers *et al.*, 2013).

1.3.4 iPSCs as source of haematopoietic cells

IPSCs stand out as an ideal source patient-specific haematopoietic cells when differentiated under specific conditions due to their pluripotency and unlimited self-renewal capacity (**Chapter 1, section 1.2.4**). Human iPSC, as human ESC, can give rise to cells of endodermal, mesodermal and ectodermal origin as described previously. The major advantage over human ESC, besides ethical dilemmas, is tailored therapy, since patients' own cells can be reprogram into iPSC that can generate histocompatible cells for transplantation as well as unparalleled short-term applications as unlimited source of haematopoietic cells for disease modelling and drug discovery (**Figure 19**) (Paes *et al.*, 2017).

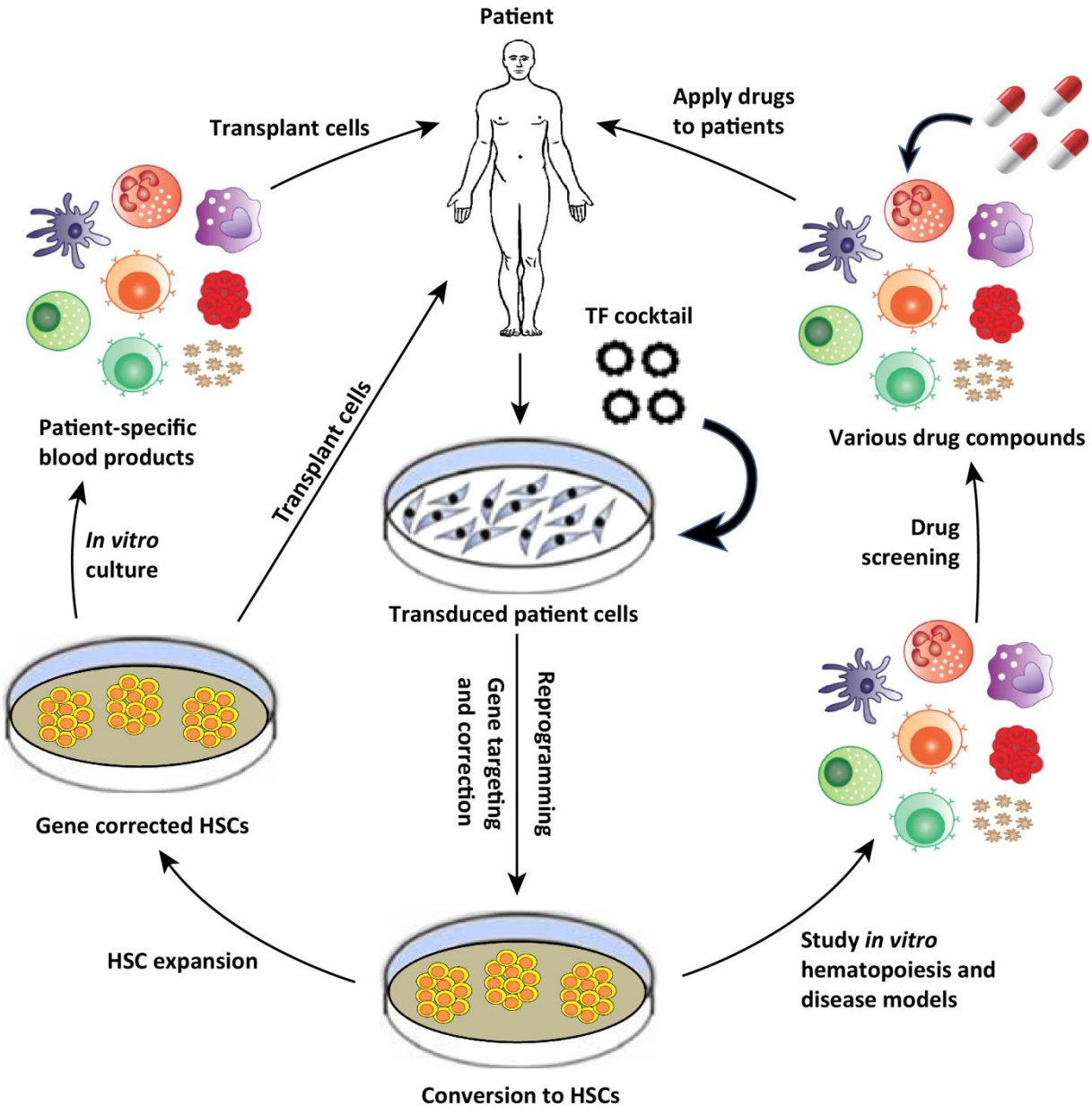


Figure 19. Application of human iPSC technology for haematopoietic diseases.

Patient-specific iPSCs can be potentially used as source of haematopoietic stem cells (HSCs) that can be subsequently differentiated into haematopoietic cells for disease modelling studies and drug screening. Alternatively, patient-specific iPSC of patients with monogenic genetic blood diseases can be gene corrected and disease-free iPSC-derived HSCs can be either transplanted into patients or used as source of patient-specific haematopoietic cells in cell therapy studies. Reproduced from Daniel et al. 2016. (Daniel *et al.*, 2016).

1.3.4.1 Haematopoietic cell differentiation of iPSC

Several studies have evaluated the haematopoietic differentiation potential of iPSC with some variation in the results. Choi *et al.* compared the haematopoietic differentiation potential between seven different human iPSC lines and five human ESC lines (Choi *et al.*, 2009b). The results shown some variation between both groups of cell lines, although the overall haematopoietic potential, based on the immunophenotypic expression of the haematopoietic markers as well as the production of colony-forming cells, was similar in both human iPSC and human ESC lines. However, Feng *et al.* showed that the haemangioblastic derivatives generated from human iPSC-derived haemangioblasts show decreased proliferation potential as well as apoptotic phenotype and early senescence compared to those from human ESC-derived haemangioblasts (Feng *et al.*, 2010). Variations between these studies may be related to a low transgene expression level resulting from the use of integrating vectors or variations in the degree of reprogramming of the target cells or even between clones of the same cell line. This highlights the need of generating transgene-free iPSC in order to exclude variability generated by the reprogramming process. Many studies have reported the generation of haematopoietic cells from human iPSC proving the ability of these cells to be used as source of haematopoietic cells disease model to study haematopoietic diseases (**Table 3**). Thus, production of human iPSC-derived blood cells, such as red blood cells, platelets and white blood cells, can help alleviate the high demand for blood products existing in transfusion medicine (Togarrati and Suknuntha, 2012).

Different studies have reported the production of erythrocytes from human iPSCs using combination of co-culture and cytokines or cytokines only (Lapillonne *et al.*, 2010; Dias *et al.*, 2011; Olivier *et al.*, 2016). However, low efficiency in the generation of enucleated erythrocytes expressing adult haemoglobin and use of mouse feeder layer are hampering the clinical application of these protocols (Dorn *et al.*, 2015). Similarly, Feng *et al.* reported the generation of mature megakaryocyte and platelet population from human iPSC, although with an efficiency still insufficient for clinical use (Feng *et al.*, 2014). Large numbers of neutrophils and eosinophils have been generated from human iPSC-derived myeloid progenitors (Choi *et al.*, 2009a; Lachmann *et al.*, 2015). These human iPSC-derived granulocytes displayed similar chemotactic, phagocytic functions and potential to generate reactive oxygen species similar to their *in vivo*

counterparts. Likewise, *in vitro* derived macrophages show functional and morphological properties than those found *in vivo* (Choi *et al.*, 2009a; Lachmann *et al.*, 2015). However, functionality in an *in vivo* setting of both human iPSC-derived granulocytes and macrophages remains to be evaluated (Ackermann *et al.*, 2015). *In vitro* differentiation of cells from lymphoid lineage from PSCs relies on the use of feeder cells such as OP9 or MS-5. Activation of Notch signalling pathway has been shown to be essential leading to the use of modified OP9 cells constitutively expressing Notch ligand Delt-1 and 4 (OP9-DL1, OP9-DL4). However, efficiency to generate natural killer cells, T and B cells from human iPSC has been shown even lower than for generation of myeloid and erythroid cells (Sturgeon *et al.*, 2014; Uenishi *et al.*, 2014; Ferrell *et al.*, 2015; French *et al.*, 2015). Thus, despite the feasibility showed by the iPSC model to generate all the different types of blood cells in an *in vitro* setting, there are some challenges to overcome such as large-scale production and *in vivo* functionality of the human iPSC-derived blood cells for its use as disease model and in transfusion therapies.

Cell type	Reprogramming strategy	Differentiation approach Co-culture Cytokines	Reference
Erythrocytes	Lentivirus	No BMP4, VEGF, SCF, TPO, Flt3-L, IL-3, IL-6, EPO	(Lapillonne *et al.*, 2010)
	Episomal vectors	OP9, MS5 TPO, IL-3, IL-6, Flt3-L, G-CSF, EPO	(Dias *et al.*, 2011)
	Retrovirus	No BMP4, VEGF, Activin A, WNT3a, Inhibitor VII, FGFα, SCF, Flt3-L, TPO, IL-3, IL-11, IGF1, EPO	(Olivier *et al.*, 2016)
Megakaryocytes	mRNA	No BMP4, VEGF, bFGF, TPO, SCF, Flt3-L, IL-3, IL-6, IL-9	(Feng *et al.*, 2014)
Granulocytes	Lentivirus	No IL-3, G-CSF, GM-CSF	(Lachmann *et al.*, 2015)
	Lentivirus	OP9 IL-3, IL-5, G-CSF, GM-CSF	(Choi *et al.*, 2009a)
Macrophages	Lentivirus	No IL-3, M-CSF, GM-CSF	(Lachmann *et al.*, 2015)
	Lentivirus	OP9 IL-1-b, M-CSF, GM-CSF	(Choi *et al.*, 2009a)
Dendritic cells	Lentivirus	OP9 M-CSF, GM-CSF IL-4, TNF-α	(Senju *et al.*, 2011)
	Lentivirus	OP9 GM-CSF, IL-4, TNF-α	(Choi *et al.*, 2009a)
NK cells	Retrovirus	OP9-DL4 BMP4, bFGF, Activin A, VEGF, IGF-1, IL-3, IL-6, IL-11, IL-13, IL-15, SCF, EPO, TPO, Flt3-L	(Sturgeon *et al.*, 2014)

	Retrovirus	OP9-DL1 BMP4, VEGF, SCF, IL-3, IL-6, IL-7, IL-15, SCF, EPO, TPO, Flt3-L	(Ferrell *et al.*, 2015)
T cells	Retrovirus	OP9-DL4 BMP4, bFGF, Activin A, VEGF, IGF-1, IL-3, IL-6, IL-7, IL-11, SCF, EPO, TPO, Flt3-L	(Sturgeon *et al.*, 2014)
	Episomal vectors	OP9-DL1 OP9-DL4 BMP-4, bFGF, VEGF, TPO, SCF, IL-3, IL-6, IL-7, Flt3-L	(Uenishi *et al.*, 2014)
B cells	Lentivirus	OP9, MS5 SCF, IL-3, IL-7, Flt3-L	(French *et al.*, 2015)

Table 3. Studies reporting generation of mature haematopoietic cells from human iPSC.
bFGF: basic fibroblast growth factor; BMP4: bone morphogenetic protein-4; DL: delta-like ligand; EPO: human erythropoietin; Flt3-L: human Fms-like tyrosine kinase 3 ligand; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor IGF: human insulin-like growth factor; IL: interleukin; M-CSF: macrophage colony-stimulating factor; SCF: human stem cell factor; TPO: human thrombopoietin; VEGF: human vascular endothelial growth factor.

1.3.4.2 iPSC as a disease model for inherited BMFS

Inherited BMFS are characterized by peripheral cytopenia and/or hypoplastic bone marrow (Shimamura and Alter, 2010). Use of HSPCs from BMFS patients in disease models is extremely challenging due to the low numbers of these progenitors in the patient's bone marrow. Thus, unlimited self-renewal capacity of iPSC makes them especially valuable as disease model providing extensive number of cells for the study of the complex pathophysiology underlying BMFS. Several studies have reported the generation of human iPSC from patients with BMFS (**Table 4**). Activation of DNA repair pathways during reprogramming to ensure genomic integrity have hindered the generation of human iPSC from FA patients. This role of FA pathway during reprogramming to pluripotency provides an excellent opportunity for the study of this defective pathway in FA patient cells. Generation of FA-iPSC has been achieved by correction of the defect in the affected *FANC* genes in patient fibroblasts prior to reprogramming (Raya *et al.*, 2009) or when reprogramming has been performed under hypoxic conditions (Muller *et al.*, 2012). Normoxic conditions have been also used to

generate FA-iPSC although at much lower efficiency (Yung *et al.*, 2013). Interestingly, Rio *et al.* reported the generation of disease-free FA-iPSC upon correction of *FANCA* defective fibroblasts that displayed resistance to exposure to DNA interstrand cross-link drugs and normal haematopoietic colony-forming potential (Rio *et al.*, 2014). As discussed in **section 1.2.4.2**, process of somatic cell-induced reprogramming is characterized by the rejuvenation of telomeres in iPSC cells due to up-regulation of telomerase activity (Marion *et al.*, 2009). This characteristic hallmark of reprogramming is appealing for the study of DC characterized by defects in telomere maintenance process. Human iPSC have been derived from DC patients with mutations in *DKC1, TERT, TERC* and *TCAB1* genes (**Table 4**). Despite initial conflicting results regarding the ability of DC-iPSC to elongate telomeres, several studies have reported the generation of human iPSC lines with mutations in *TERT, TERC, TCAB1* and *DKC1* displaying shortened telomeres, ultimately affecting self-renewal capacity, with severity of telomerase dysfunction depending upon the precise nature of the mutation (Batista *et al.*, 2011; Gu *et al.*, 2015; Woo *et al.*, 2016). DBA-iPSCs have been generated from patients with mutations in ribosomal-associated genes *RPS19* and *RPL5* (**Table 4**). This DBA-iPSC model successfully recapitulated the phenotype observed in patients showing an impaired generation of haematopoietic progenitors mainly affecting erythroid lineages (Garcon *et al.*, 2013). Likewise, DBA-iPSC displayed dysregulation of non-canonical TGFβ signalling pathway that may be associated to the defective phenotype observed in in DBA patients proving the usefulness of iPSC disease models to provide insights into disease pathogenesis (Ge *et al.*, 2015). IPSC generated from SDS patients showed increased levels of proteases in culture supernatant and reduced haematopoietic colony-forming potential when differentiated towards haematopoietic lineages. Use of protease inhibitors in the culture media rescued the defective phenotype providing an example of drug-reversible phenotype using human iPSC model (Tulpule *et al.*, 2013). Severe congenital neutropenia (SCN)-iPSC model was used by Nayak *et al.* to identify the mislocalization of the neutrophil elastase (NE) protein as the underlying cause of the dysfunctional myeloid differentiation and increased apoptosis observed in SCN-iPSC-derived neutrophils, successfully corrected by the addition of NE inhibitor sivelestat (Nayak *et al.*, 2015).

Thus, these studies highlight the utility of iPSC technology to model inherited bone marrow syndromes leading to think that iPSCs could be in a similar fashion to investigate a potential underlying HSPC dysfunction in SAA patients in order to shed light into the complex pathophysiology of this disorder.

Bone marrow failure syndrome	Reprogramming strategy	Defective genes	Genetic correction (system used)	Reference
Fanconi anaemia	Retrovirus	FANC-A, FANC-D2	Yes (lentiviral vectors)	(Raya *et al.*, 2009)
	Retrovirus, Lentivirus	FANC-A, FANC-C, FANC-D2 , FANC-G	No	(Muller *et al.*, 2012)
	Lentivirus	FANC-A, FANC-C, FANC-D2, FANC-G	No	(Yung *et al.*, 2013)
	Lentivirus	FANC-A	Yes (zinc-finger nucleases)	(Rio *et al.*, 2014)
Dyskeratosis congenita	Lentivirus	DKC1 (Q31E, ΔL37, A353V)	Yes (zinc-finger nucleases)	(Gu *et al.*, 2015)
	Lentivirus	TERT, TCAB1, DKC1	No	(Batista *et al.*, 2011)
	Retrovirus	DKC1, TERC	No	(Agarwal *et al.*, 2010)
	Lentivirus	DKC1	Yes (CRISPR/Cas9)	(Woo *et al.*, 2016)
Diamond-Blackfan anaemia	Lentivirus Sendai virus	RPS19, RPL5	Yes (zinc-finger nucleases)	(Ge *et al.*, 2015)

Shachman-Bodian-Diamond	Retrovirus	SBDS	Yes (lentiviral vectors)	(Tulpule *et al.*, 2013)
Severe Congenital Neutropenia	Retrovirus	ELANE	No	(Hiramoto *et al.*, 2013)
	Lentivirus	ELANE	Yes (CRISPR/Cas9)	(Nayak *et al.*, 2015)
	Retrovirus	HAX1	Yes (lentiviral vectors)	(Morishima *et al.*, 2014)

Table 4. Disease modelling studies of BMFS using iPSC technology.

1.4 Aims

The aims of this thesis were:

1. To generate and characterize iPSC from SAA patients' cells.

2. To assess the capacity of the SAA-iPSC to differentiate into haematopoietic progenitor cells, including megakaryocytic, erythroid and myeloid subpopulations and its haematopoietic colony-forming potential.

3. To study the telomere dynamics of SAA cells lines during reprogramming to pluripotency and subsequent differentiation into haematopoietic progenitors.

4. To assess the proliferation, apoptosis and DNA repair capacity of SAA-iPSC derived haematopoietic progenitors under normal and replicative stress conditions.

5. To assess the effect of EP on colony-forming potential, proliferation, apoptosis and DNA repair capacity of SAA-iPSC-derived haematopoietic progenitor cells.

Chapter 2. Material and Methods

2.1 Human dermal fibroblast (HDF) cell culture

Neonatal and adult human dermal fibroblast (HDF) cells were used to generate control-iPSC lines (Lonza, CC-2509, CC-2511). Skin biopsies from paediatric and young adult SAA patients were taken with informed consent by Dr Sujith Samarasinghe, Consultant Paediatric Haematologist at the Royal Victoria Infirmary, Newcastle upon Tyne. Fibroblasts from these biopsies were isolated and analysed by the Cytogenetics department of the Institute of Genetic Medicine (IGM) of Newcastle University. Human fibroblast cultures were maintained in T25/T75 culture flasks in HDF media comprised of Advanced Dulbecco's Modified Eagle Medium (Thermo-Fisher, Waltham, MA, USA) containing 10% foetal bovine serum (FBS) (Thermo Fisher Scientific), 1% Glutamax™ (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific) at 37°C and 5% CO_2 in a humidified incubator. Fibroblasts were passaged using a 0.05% trypsin/EDTA solution (Thermo Fisher Scientific) at 37°C + 5% CO_2. All fibroblast culture was performed in a Class II biosafety cabinet laminar air flow tissue culture hood. For long-term storage, human fibroblasts were cryopreserved in freezing media comprised of 90% FBS+ 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MI, USA) and stored in specialised cryovials. These were transferred to a pre-cooled isopropanol-containing freezing container which was stored in an 80°C freezer for 24 hours prior transferring to liquid nitrogen for long-term storage.

2.2 Mouse embryonic fibroblasts culture and feeder cell layer preparation

Mouse embryonic fibroblasts (MEFs) were isolated from 12.5-13.5 day old embryos via their dissection and mechanical digestion from Swiss MF1 Pregnant mice. Following isolation and MEFs were cultured and passaged as described for human dermal fibroblast culture. The MEFs were subjected to irradiation using the cabinet X-ray cell irradiator CP-160 (Faxitron, Tucson, AR, USA) at a dose of 120kV, 4.0mA for 7 minutes. The irradiated MEFs were then plated onto gelatine-coated 4/6-well tissue culture plates (20.000 cells/cm^2). The MEF-coated plates were then ready for iPSC seeding after a 24-hour incubation period at 37°C + 5% CO_2. For conditioned media preparation, MEFs (seeded at a density of 5.5 x 10^4 cells/cm^2) were irradiated as described above. The inactivated MEF culture was then submerged in iPSC media

comprised of Knock-Out Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific), 20% Knock-Out Serum Replacement (Thermo Fisher Scientific), 1% Non-Essential Amino acids (Thermo Fisher Scientific), 1% Glutamax™ (Thermo Fisher Scientific), 1% penicillin-streptomycin Solution (Thermo Fisher Scientific) and 8ng/ml basic Fibroblast Growth Factor (bFGF) (Thermo Fisher Scientific) and this was left to condition for 24 hours at 37°C + 5% CO_2 in a humidified incubator before collection. This was repeated for 7-9 days before exhaustion of the MEFs. Following collection, the MEF-conditioned iPSC (CM-iPSC) media was then supplemented with 1% insulin transferrin selenium-A (Thermo Fisher Scientific) and 8 ng/ml bFGF and filtered with a 0.20 µM filter before use for iPSC generation.

2.3 iPSC generation from SAA patients and healthy volunteers

Human dermal fibroblasts were transduced using the Cytotune™-iPS Reprogramming Kit (Thermo Fisher Scientific, A13780-01) according to manufacturer's instructions. Briefly, once fibroblast cultures reached 80% confluency SeV including the reprogramming transgenes separately were added. Transduced HDF were plated on mitotically-inactivated MEF feeder cells on day 9 (26.000 cells/cm^2). HDF media was replaced by iPSC media to enhance reprogramming. First iPSCs showing characteristic human ESC-like morphology emerged around day 20 and were manually picked on day 24 and transferred to fresh mitotically-inactivated MEF feeder cell wells. Due to feeder cell exhaustion, culture media was replaced by CM-iPSC media from day 20.

2.4 iPSC culture

iPSC colonies were established on inactivated primary mouse embryonic fibroblasts feeder layer and then adapted to a feeder-free system, cultured on recombinant Vitronectin (Thermo Fisher Scientific) and in StemPro human ESC SFM® media (Thermo Fisher Scientific) supplemented with 8ng/ml basic Fibroblast Growth Factor (Thermo Fisher Scientific), 1% penicillin/streptomycin and 0.1mM 2-Mercaptoethanol (Thermo Fisher Scientific). iPSCs cultures were maintained for approximately 4/5 days before passage or induction of differentiation. The white, thicker, heterogeneous differentiated areas of iPSCs colonies were removed daily and prior to passage using a 200µl micropipette tip. Passaging was carried out mechanically using the STEMPRO® EZpassage™ tool (Thermo Fisher Scientific). For long-term storage,

iPSCs were cryopreserved in freezing media comprised of 90% FBS+ 10% Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich) and stored in specialised cryovials. These were transferred to a pre-cooled isopropanol-containing freezing container which was stored in an 80°C freezer for 24 hours prior transferring to liquid nitrogen for long-term storage.

2.5 Immunocytochemistry analysis of pluripotency markers

For immunocytochemistry analysis, iPSC colonies were fixed in 4% Formaldehyde (Sigma-Aldrich) and permeabilized with 0.25% Triton-X-100 (Sigma-Aldrich). Following treatment, cells were stained with mouse anti-human SSEA4-Alexa Fluor® 555 (Beckton Dickinson, BD; Franklin Lakes, NJ, USA) at 1:60 dilution, mouse anti-human TRA-1-60-FITC (Merck Millipore, Billerica, MA, USA) at 1:60 dilution, mouse anti-NANOG-AF647 (Cell Signalling Technologies, Danvers, MA, USA) at 1:150 dilution and goat anti-human OCT4 primary antibody (R&D, Minneapolis, MN, USA) at 1:60 dilution. Secondary staining was performed using anti-goat IgG FITC (Sigma-Aldrich) at 1:200 dilution. Following treatment with the DNA nuclear stain 4', 6-diamino-2-phenylindole (DAPI) (Partec, Munster, Germany), stained iPSC colonies were photographed using a Bioscience Axiovert microscope (Axiocam, CarlZeiss) in combination with the associated CarlZeiss software- AxioVision.

2.6 Flow cytometric analysis of pluripotency markers

To assess the percentage of cells expressing the pluripotent markers TRA-1-60 and SSEA-4, flow cytometric analysis was performed, iPSC colonies were dissociated using TrypLE™ Express (Thermo Fisher Scientific) for 5 minutes at 37°C. Dissociated cells were stained with the following antibodies: anti-human TRA-1-60-FITC (Millipore) at 1:60 dilution and mouse anti-human SSEA-4-PerCPCy™5.5 (BD) at 1:20. Cell population was identified based on cell size and cell granularity. Single cells were discriminated using Forward Scatter area (FSC-A) and Forward Scatter Height (FSC-H) and live cells were gated from single cell population using DAPI nuclear staining (Partec). The cells were acquired using the BD LSRII flow cytometer (BD) and data analysed using FlowJo software (Tree Star, Ashland, OR, USA). At least 10.000 events were collected for each analysis. Gating strategies for identification of positive cell population are shown in **Appendix A**.

2.7 *In vivo* test of pluripotency

For *in vivo* analysis of pluripotency via teratoma formation, $0.5x10^6$ iPSCs were resuspended in 50% Matrigel™ (Becton-Dickinson, Franklin Lakes, NJ, USA) in a total volume of 200 µl and injected subcutaneously into both flanks of adult severe combined immunodeficiency (SCID) male mice (*Mus musculus*, ICRF-Foxn1^{nu}). Two animals were injected in each group. Following a period of 10 weeks, the mice were euthanized and the teratomae were excised, fixed in 4% paraformaldehyde (PFA) for 12 hours. Fixed teratomae were processed and sectioned according to standard procedures and stained for Weigert's haematoxylin, Masson's trichrome and Mayer's haematoxylin and Eosin histological analysis. Sections (5-8 µm) were examined using bright field microscopy and stained tissue photographed as appropriate.

2.8 Genomic DNA extraction

Genomic DNA was extracted from the pelleted cultures of iPSC lines and corresponding parental fibroblast cell lines using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA) according to standard protocols of the manufacturer.

2.9 Karyotyping and Fingerprinting Analysis

Genomic DNA from iPSC and parental fibroblasts was analysed using Illumina CytoSNP analysis and the BlueFuse Multi 4.3 software (Illumina, San Diego, United States) according to standard protocols of the manufacturer.

2.10 iPSC differentiation into haematopoietic progenitors cells

iPSCs maintained on Vitronectin™ in StemPro™ media were cut in homogeneous pieces using a STEMPRO® EZpassage™ tool (Thermo Fisher Scientific). Aggregates were resuspended in Stemline® II (Sigma-Aldrich) differentiation media supplemented with 1% penicillin/streptomycin and cultured in ultra-low attachment culture plates at 37°C and 5% CO_2 in a humidified incubator for 3 days to allow the formation of EBs. On day 3, EBs were dissociated using TrypLE™ Express (Thermo Fisher Scientific) for 10 minutes at 37°C and transferred to tissue-culture treated wells at a density of 25.000 cells/cm^2 to allow culture under monolayer conditions at 37°C and 5% CO_2 in a humidified incubator. Recombinant human BMP4 (day 0-2 10ng/ml, day 2-16 20ng/ml), VEGF (day 0-2 10ng/ml, day 2-16 30ng/ml), Wnt3A (10ng/ml), GSK-3β

Inhibitor VII (2μM), Activin A (5ng/ml), FGFα (10ng/ml), SCF (20ng/ml), IGF-2 (10ng/ml), TPO (10ng/ml), β-estradiol (0.4ng/ml), Heparin (5μg/ml) and 3-isobutyl-1-methylxanthine (IBMX) (50μM) were added to the differentiation media as previously described (Olivier *et al.*, 2016). All cytokines and compounds were purchased from Peprotech (Rocky Hill, NJ, USA) except BMP4 and Wnt3A (R&D, Minneapolis, MN, USA), GSK-3β Inhibitor VII (Calbiochem, San Diego, CA, USA) and β-estradiol, Heparin and IBMX (Sigma-Aldrich).

2.11 Flow cytometric analysis of mesodermal, endothelial and haematopoietic markers

iPSC-derived differentiated cells were treated with 1X TrypLE™ Express for 5 minutes at 37ºC to obtain a single a cell suspension. Cell pellets were resuspended in FACS buffer and cells counted with a haemocytometer. A final amount of $1x10^5$ cells resuspended in 100μl of FACS buffer a dilution of 1:20 antibody was used for each analysis. The following cell surface antigens were analysed for this study: KDR-PE, CD34-APC, CD43-FITC, CD41a-APCH7 and CD235a-BV421. All antibodies were purchased from Becton-Dickinson except for CD43-FITC (Thermo Fisher Scientific). Cells were washed using the FACS Lyse/Wash assistant (Becton-Dickinson) and analysed using LSRII flow cytometer (Becton-Dickinson). Size and cell complexity were used to identify cell populations in a scatter graph representation. Single cells were discriminated using FSC-A and FSC-H and live cells were gated from single cell population using DAPI nuclear staining. Analysis of data was performed using FlowJo software (Tree Star Inc., Ashland, OR, USA). At least 10.000 events were collected for each analysis. Gating strategies for identification of positive cell population are shown in **Appendix A**.

2.12 Analysis of haematopoietic colony-forming potential of haematopoietic progenitors by CFU Assay

iPSC-derived haematopoietic progenitor cells were treated with 1X TrypLE™ Express for 5-10 minutes at 37°C to obtain a single a cell suspension. TrypLE™ Express is diluted in PBS and cells were pelleted by centrifugation at 300g for 3 minutes. Cell pellets were resuspended in FACS buffer and cells were counted with a haemocytometer. A final amount of $6x10^4$ cells resuspended in 300μl of FACS buffer and mixed with 3ml of Methocult™ methylcellulose media enriched with recombinant

cytokines (Stem Cell Technologies, Vancouver, BC, Canada) and 1.5 ml were plated in duplicate in 35-mm dishes. Colonies were scored after 14 days of culture using light microscope according to standard criteria (Coutinho, 1993; Eaves, 1995) and averaged between the duplicate dishes.

2.13 RNA isolation

RNA from iPSCs and fibroblasts at day-7 of SeV transduction used as positive Sendai control was extracted using the ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, MA, USA) including DNase I (Promega) treatment for degradation of genomic DNA according to the manufacturer's instructions.

2.14 Reverse Transcription (RT)

A total amount of 1 µg of RNA was reverse transcribed into cDNA using the GoScript™ Reverse Transcription System (Promega) and random primers according to the manufacturer's instructions. For each test sample a negative control sample in which GoScript™ Reverse Transcriptase was substituted by nuclease-free water (Promega) in order to detect genomic DNA contamination.

2.15 Polymerase chain reaction (PCR)

For the PCR reaction mixture, 1µl of cDNA previously generated from 1µg of RNA was amplified using 0.5µl 10µM dNTP mix, 5µl 5X Green GoTaq® Reaction Buffer and 0.2µl GoTaq® DNA polymerase (5u/µl), 17.3µl of nuclease-free water (Promega) and 0.5µl 10µM primers shown in **Table 5** made to a final volume of 25µl. The PCR consisted of a 35-cycle program and was carried out using a Mastercycler® thermal cycler. The machine was set up to carry out as a sequence of reaction temperatures for denaturation, annealing and amplification of reverse transcription enzymes. The sequence of 35 cycles begins with 95°C for 30 seconds, 95°C for 30 seconds followed by 72°C for 30 seconds and held at 4°C.

Product	Primer sequence (5' → 3')		Size (bp)
SeV-OCT4	Forward	CCCGAAAGAGAAAGCGAACCAG	483
	Reverse	AATGTATCGAAGGTGCTCAA	
SeV-SOX2	Forward	ATGCACCGCTACGACGTGAGCGC	451
	Reverse	AATGTATCGAAGGTGCTCAA	
SeV-Klf4	Forward	TTCCTGCATGCCAGAGGAGCCC	410
	Reverse	AATGTATCGAAGGTGCTCAA	
SeV-cMYC	Forward	TAACTGACTAGCAGGCTTGTCG	532
	Reverse	TCCACATACAGTCCTGGATGATGATG	
SeV	Forward	GGATCACTAGGTGATATCGAGC	181
	Reverse	ACCAGACAAGAGTTTAAGAGATATGTATC	
GAPDH	Forward	GGATCACTAGGTGATATCGAGC	151
	Reverse	ACCAGACAAGAGTTTAAGAGATATGTATC	

Table 5. List of specific primers used for detecting SeV genome and transgenes by RT-PCR

2.16 Agarose gel electrophoresis

Amplification products were visualized by electrophoresis on horizontal 2% agarose gels using fluorescent nucleic acid dye GelRed™ Nucleic Acid Stain (Biotium, Fremont, CA, USA). In order to monitor the size and intensity of DNA fragments 5µl of Gene Ruler 100bp plus DNA ladder (Thermo Fisher Scientific) was used. Gels were electrophoresed at 70-100 volts for 40-60 minutes. DNA bands were visualized and photographed by illumination of fluorescent nucleic acid dye by excitation using ultraviolet (UV) light, suing a GelDoc-It® 310 imaging system trans-illuminator (UVP, Upland, CA, USA) and VisonWorks™ LS software (UVP) for image analysis.

2.17 Quantitative PCR for Telomere Length measurement

Telomere length from previously extracted genomic DNA was measured as abundance of telomeric template versus a single copy gene (*36B4*) as previously described (Martin-Ruiz *et al.*, 2005) using the primers shown in **Table 6**.

Product	Primer sequence (5' → 3')	
TelA		CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT
TelB		GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT
36B4	Forward	CAG CAA GTG GGA AGG TGT AAT CC
	Reverse	CCC ATT CTA TCA TCA ACG GGT ACA A

Table 6. List of specific primers used for measurement of telomere length

Three internal control DNA samples of known telomere length (10.4 kb, 3.9 kb and 2 kb) were run within each plate to correct for plate–to-plate variation. Measurements were performed in triplicate. All PCRs were carried out on an Applied Biosystems 7900HT Fast Real Time PCR system with 384-well plate capacity. The intra-assay coefficient of variation was 2.7% while the inter-assay coefficient of variation was 5.1%.

2.18 Telomere Repeat Amplification analysis for Telomerase Activity detection

Telomerase activity in iPSC and iPSC-derived haematopoietic progenitors was measured as previously described (Ishaq *et al.*, 2016) using the *T*elo*TAGGG* Telomerase PCR ELISA kit (Roche, Basel, Switzerland) according to manufacturer's instructions. In brief, cell pellets were lysate using CHAPS lysis buffer (Roche). Total protein concentration was measured using Bradford Protein assay (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions and absorbance at 450 nm was measured using an FLUOstar Omega spectrophotometer (BMG Labtech, Ortenberg, Germany). Initial amount of 100 ng of protein lysate was used for primer elongation and PCR amplification steps using no protein/lysate samples as negative controls and 4 consecutive dilutions of Hela cells: 100, 10, 1 and 0.1ng as positive controls. Hybridization of amplified products to digoxigenin-(DIG)-labeled telomeric repeat-specific detection and detection of probe by enzyme-linked immunosorbent assay (ELISA) were performed according to manufacturer's instructions and absorbance at 450 nm was measured using an FLUOstar Omega spectrophotometer (BMG Labtech).

2.19 Analysis of DNA damage, Proliferation and Apoptosis by Flow Cytometry

Day 12 iPSC-derived haematopoietic progenitor cells were exposed to 2M Hydroxyurea (HU) for 24 hours and collected at different time points (0, 1, 3, 8 and 24 hours). DNA damage, proliferation and apoptosis induction after HU-treatment was

analysed using a flow cytometric kit according to manufacturer's instructions (Becton-Dickinson). Briefly, at the specified time points after HU treatment, the cells were labelled with 50 µM 5-bromo-2-deoxyuridine (BrdU) for 1 hour and stained later with antibody anti-human CD43-FITC. The labelled cells were then fixed, permeabilized and labelled with anti-human γH2AX-Alexa Fluor®647, anti-human BrdU-PerCPCy™5.5 and anti-human Cleaved PARP (Asp214)-PE according to manufacturer's instructions. DNA content for cell cycle analysis was determined by DAPI staining provided by the kit. Size and cell complexity were used to identify cell populations in a scatter graph representation and single cells were discriminated using FSC-A and FSC-H. The cells were acquired using the LSRII flow cytometer (Becton-Dickinson) and data analysed using FlowJo software. At least 10.000 events were collected for each analysis. Gating strategies for identification of positive cell population are shown in **Appendix A**.

2.20 Statistical Analysis

Data are shown as mean ± S.E.M. from at least three independent experiments. The significance between means was determined with Multiple *t*-test using Holm-Sidak method and One-way ANOVA when Gaussian distribution was assumed and with Kruskal-Wallis test when Gaussian distribution was not assumed. Multiple comparisons test for comparison between particular pairs of control and patient groups. Statistically significant values were judged as follows: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Statistical analysis was performed using GraphPad Prism version 7.0 software (GraphPad Software, La Jolla, CA, USA) and Minitab 17 statistical software (Minitab Inc., State College, Pennsylvania, USA).

Chapter 3. Generation of SAA induced pluripotent stem cells (SAA-iPSC)

3.1 Introduction

iPSC technology has proved to be a powerful tool in disease modelling and therapy development as discussed in **Chapter 1** (**section 1.2.3**). Particularly in the study of SAA, generation of iPSC lines from SAA patient's cells could provide an unlimited source of haematopoietic progenitors to investigate the contribution of these progenitors to the phenotype observed in SAA patients.

During reprogramming, induction of pluripotency is driven by the forced expression of the reprogramming transgenes. Transgene expression induces cell proliferation and downregulation of the genes specific to the cell type of origin. Later, erasure of the existing epigenetic somatic memory and upregulation of the endogenous pluripotency-associated genes, such as *OCT4, SOX2* and *NANOG*, results in the acquisition of the pluripotent state (Plath and Lowry, 2011) . Thus, a percentage of the starting population of somatic cells is fully reprogrammed showing established expression of the pluripotency transcriptional network and full differentiation potential defined as ability to give rise to cells from the three different germ layers (Chan *et al.*, 2009).

However, iPSC technology faces some limitations that may affect the iPSC differentiation ability. Constitutive expression of the reprogramming transgenes, incomplete reprogramming and introduction of genetic variations during reprogramming and/or subsequent *in vitro* culture are the main challenges that need to be addressed to ensure the generation of fully reprogrammed iPSC suitable for downstream applications (Liang and Zhang, 2013) (**Figure 20**). Additionally, lack of robustness observed in some protocols used to differentiate patients' iPSC introduces variation that should be considered when interpreting phenotypic differences, as it will be discussed in **Chapter 4** (**section 4.2.3**) (Soldner and Jaenisch, 2012) (**Figure 20**).

The aim of this chapter is to describe the process of generation and characterization of control and SAA-iPSC lines generated for this study as follows:

- Reprogramming to pluripotency of HDF from control and SAA cells by transduction with SeV containing reprogramming transgenes.
- Analysis of residual expression of reprogramming transgenes and SeV genome in control and SAA-iPSC lines generated for this study.
- Assessment of pluripotency of control and SAA-iPSC lines by detection of pluripotency marker expression and *in vivo* differentiation potential.
- Analysis of major chromosomal abnormalities present in control and SAA-iPSC lines
- Authentication of genetic identity of control and SAA-iPSC lines and parental fibroblasts

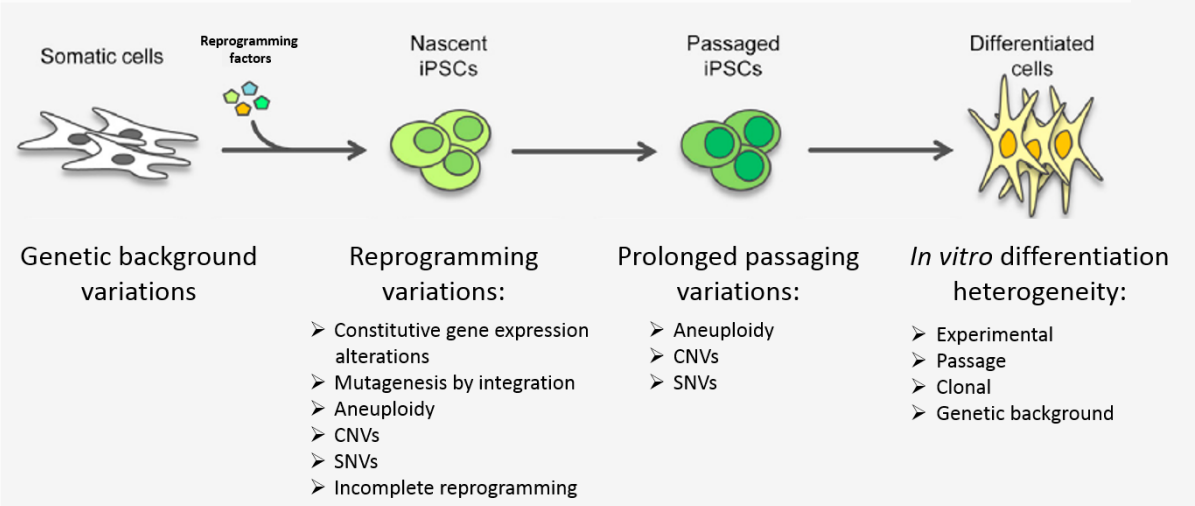


Figure 20. Sources of variation during the process of generation and differentiation of iPSC. Transduction of somatic cells with the reprogramming factors can lead to the integration of these RTs in the genome of the host cells leading to the transgene constitutive expression and mutagenesis in the genome. Likewise, incomplete reprogramming due to the inefficient removal of the somatic epigenetic marks the introduction of genetic variations at a chromosomal and nucleotide level can also impair the differentiation potential of the reprogrammed cells. CNVs: copy number variations, SNVs: single nucleotide variations. Adapted from Liang *et al* (Liang and Zhang, 2013).

3.2 Results

3.2.1 Reprogramming of HDF from SAA patients

Control and SAA-HDF were obtained from skin biopsy of healthy volunteers and SAA patients respectively with appropriate consent and approval from the Great Northern Biobank (GNB application GNB-ML4). **Table 7** shows the age, gender and clinical manifestation of both control (WT1, WT2 and WT3) and SAA patients used in this study. SAA patients' age range from 10 to 24 years old matching the first peak of incidence, classified as paediatric and young adult, characteristic in the biphasic distribution of SAA (10-25 and over 60 years) (Samarasinghe and Webb, 2012). All the SAA patients were classified as severe or very severe according to blood cells counts and bone marrow cellularity (Guinan, 2011) and showed different response to IST, which is likely to reflect an underlying heterogeneity in the aetiology of SAA. SAA1 patient successfully responded to IST whereas SAA2 patient relapsed after IST and failed with a second course. This relapse and failure to IST of the SAA2 patient might indicate the presence of an underlying defect in the HSPC of this patient. Likewise, response to IST in SAA patients does not necessarily imply that pathophysiology of the disease is solely due to immuno-mediated BM destruction since it has been previously reported that patients with HSC harbouring *TERT* mutations successfully responded to IST (Townsley *et al.*, 2014).

Patient ID	Age (years)	Gender	Phenotype, diagnosis and treatment	Reprogramming efficiency
WT1	Newborn	Male	Healthy	0.21%
WT2	51	Male	Healthy	0.29%
WT3	37	Female	Healthy	0.14%
SAA1	16	Male	Severe AA Negative for DEB test No clinical features of inherited BMFS No family history of SAA Responded to IST	0.11%
SAA2	24	Male	Very severe AA Negative for DEB test No clinical features of inherited BMFS No family history of SAA Responded to IST Developed PNH and relapsed Failed with second course of immunosuppressors (horse ATG) Successful matched unrelated HSCT	0.10%
SAA3	10	Female	Very severe AA and autism Negative for DEB test No clinical features of inherited BMFS No family history of SAA Successful matched unrelated HSCT	0.11%
SAA4	13	Male	Very severe AA Negative for DEB test No clinical features of inherited BMFS No family history of SAA Successful matched unrelated HSCT	0.14%

Table 7. Phenotype of control and SAA patients used in this study.

DEB, diepoxybutane; BMFS, bone marrow failure syndrome; SAA, severe idiopathic aplastic anaemia; IST, Immunosuppressive therapy; PNH, Paroxysmal Nocturnal Haemoglobinuria; ATG, Anti-Thymocyte Globulin; HSCT, Haematopoietic stem cell transplantation

HDF from unaffected donors (3 cell lines) and SAA patients (4 cell lines) were thawed and cultured for at least one passage before transduction. An outline of the

reprogramming process is shown in **Figure 21A**. **Figure 21B** depicts the transition from HDFs to iPSC. HDF showed characteristic long, elongated morphology when transduced with replication-incompetent SeV encoding the reprogramming factors-*OCT4, SOX2, KLF4, c-MYC* on day 2. Following a 7-day period, the cells began to form small colonies containing reduced cytoplasm and large nuclei cells (**Figure 21B**). Over the following three weeks of reprogramming, the cell cultures began to shift from a long, elongated conformation to a smaller structure consisting of higher nucleoli to cytoplasmic composition. First iPSC colonies showing cells with human ESC-like appearance (large nucleus with prominent nucleoli) emerged on day 24 (**Figure 21B**). Human iPSCs typically grow as a thin and flat monolayer forming colonies of cobblestone appearance as opposed to singularly existing HDF (**Figure 21B**). Colonies containing partially-reprogrammed cells were also observed during the throughout the reprogramming process. These partially-reprogrammed cells presented a heterogeneous differentiated morphology not resembling human ESC. (**Figure 21B**).

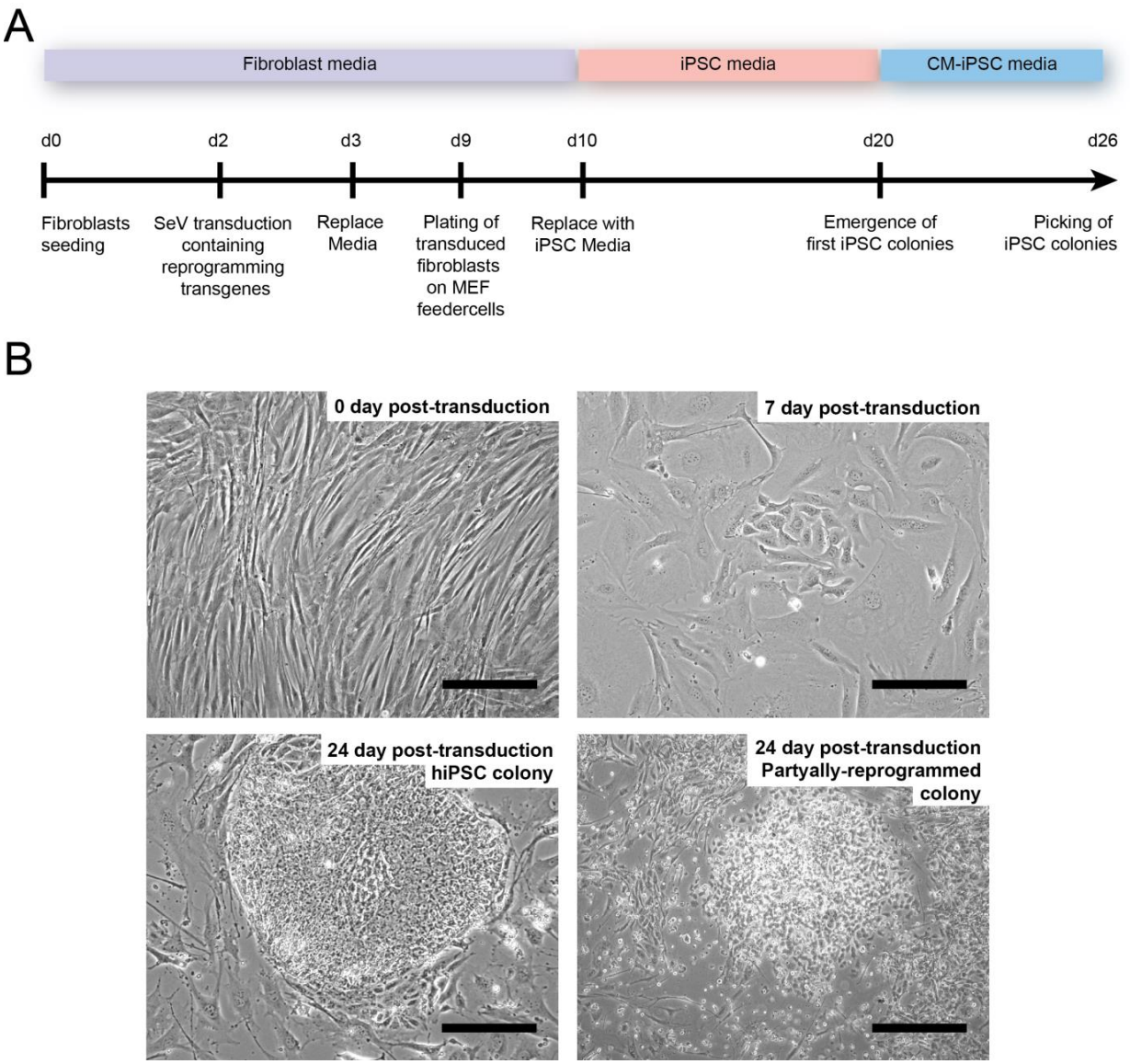


Figure 21. Description of the iPSC generation process.

(A) Timeline of iPSC generation from HDF cells. HDF were plated on day 0. Once fibroblast cultures reached 80% confluency (day 2) SeV including the reprogramming transgenes separately were added. Transduced HDF were plated on mitotically-inactivated MEF feeder cells on day 9. HDF media was replaced by induced pluripotent Stem Cell (iPSC) media to enhance reprogramming. First iPSCs showing characteristic human ESC-like morphology emerged around day 20 and were manually picked on day 24. Due to feeder cell exhaustion, culture media was replaced by MEF-conditioned iPSC (CM-iPSC) media from day 20. (B) Representative microscope images of the morphological changes observed in the transduced cells during the reprogramming process. Scale bars, 200 µm.

Single iPSC colonies containing characteristic human ESC-like cells were picked manually and transferred onto fresh MEF feeder cells. Each transferred colony represented a clone of the iPSC line. Different numbers of clones were picked and expanded in order to ensure the availability of different iPSC clones for each cell line (**Table 8**). Analysis of haematopoietic differentiation variability among clones of the same iPSC line will be thoroughly described in **Chapter 4**. The iPSC lines generated (7 total) were expanded mechanically on γ-irradiated MEF for at least 12 passages prior to PSC characterization. The reprogramming efficiency for each cell lines was calculated using the original number of cells that were transduced using SeV containing the reprogramming transgenes on day 0 and the total number of clones obtained after 30 days of transduction (**Table 8**).

Patient ID	Number of cells transduced	Number of clones generated	Reprogramming efficiency
WT1	100.000	210	0.21%
WT2	100.000	290	0.29%
WT3	100.000	142	0.14%
SAA1	50.000	63	0.11%
SAA2	100.000	102	0.10%
SAA3	100.000	114	0.11%
SAA4	100.000	141	0.14%

Table 8. Reprogramming efficiencies for control and SAA cell lines

Once control and SAA-iPSC lines were generated and maintained I proceeded to the characterization of the iPSC lines analysing the presence of SeV genome and transgenes in the iPSC lines and assessing the pluripotency, chromosomal stability and identity of the iPSC lines generated (**Table 9**).

Analysis	Aim	Method of detection
SeV expression	Detection of residual expression of SeV and/or reprogramming transgenes	RT-PCR
Assessment of pluripotency	Expression and localization of pluripotency-associated markers OCT4, NANOG, SSEA4 and TRA-1-60	Immunofluorescence
	Quantification of population of cells expressing pluripotency-associated markers SSEA4 and TRA-1-60	Flow cytometric analysis
	Induction of teratoma formation in SCID mice containing structures characteristic of the three germ layers: ectoderm, endoderm and mesoderm	*In vivo* growth of teratoma and histological analysis
Cytogenetic analysis	Detection of major chromosomal abnormalities present in iPSC and parental fibroblasts	SNP analysis
Genetic identity	Matching genetic identity between iPSC and parental fibroblasts	SNP analysis

Table 9. Overview of the iPSC characterization process.

SeV, Sendai virus; RT-PCR, reverse transcriptase- polymerase chain reaction, SCID, severe combined immunodeficiency mouse; SNP: single nucleotide polymorphism

3.2.2 Detection of SeV genome and reprogramming transgenes in control and SAA-iPSC generated

Detection of SeV genome (*SeV*) and transgenes (*SeV-OCT4, -SOX2, -KLF4* and –*cMYC*) can be achieved by reverse transcription PCR (RT-PCR), where the primers utilized are complementary to both the inserted reprogramming factor gene as well as to part of the viral vector genome. Analysis of the PCR products showed expression of SeV vector and reprogramming transgenes in 7-day transduced HDF (Sendai positive cells) but non-detectable residual expression of both *SeV* and/or reprogramming transgenes in the iPSC lines generated (**Figure 22**).

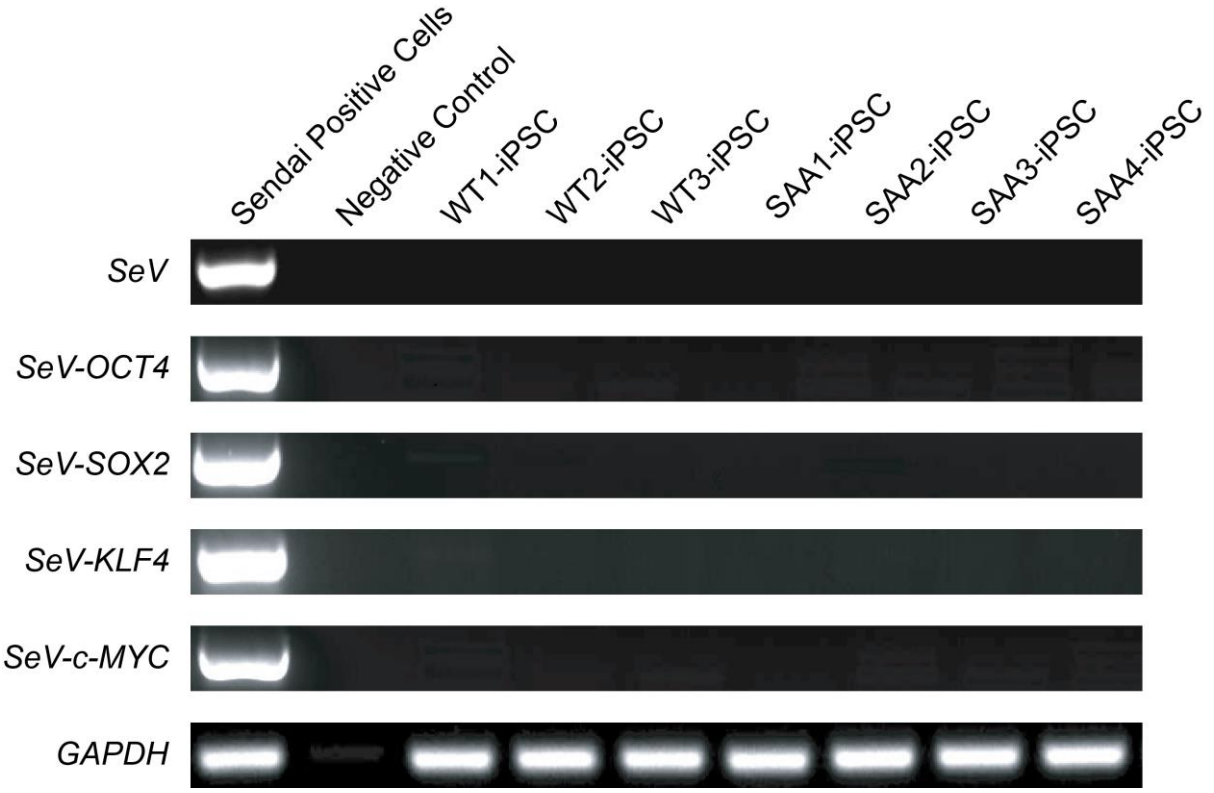


Figure 22. Detection of residual SeV genome and reprogramming transgene expression by RT-PCR.

Control and SAA-iPSC lines did not show detectable expression of backbone SeV (SeV) or the reprogramming transgenes included in the SeV vector (*OCT4, SOX2, KLF4* and *cMYC*). SeV-transduced HDF were used as a positive control (SeV Positive cells) as recommended by manufacturer and nuclease-free water as no-template control (negative Control) to assess the presence of primer-dimer formation or contamination in the PCR reagents.

3.2.3 Assessment of pluripotency of control and SAA-iPSC generated

Morphological analysis of iPSC colonies from both control and SAA-iPSC lines colonies revealed thin and flat monolayer cell colony with distinct borders characteristic of human PSC lines, as well as positive staining for pluripotency-associated markers OCT4, NANOG, stage specific embryonic antigen-4 (SSEA4) and tumour-rejection antigen 1-60 (TRA-1-60) (**Figure 23**). It is worth noting the localisation of the fluorescence staining for each marker, with staining for surface markers SSEA4/TRA-1-60 localised in the cell membrane and transcription factors OCT4/NANOG staining observed in the cell nucleus as concomitant with the DAPI nuclear stain (**Figure 23**).

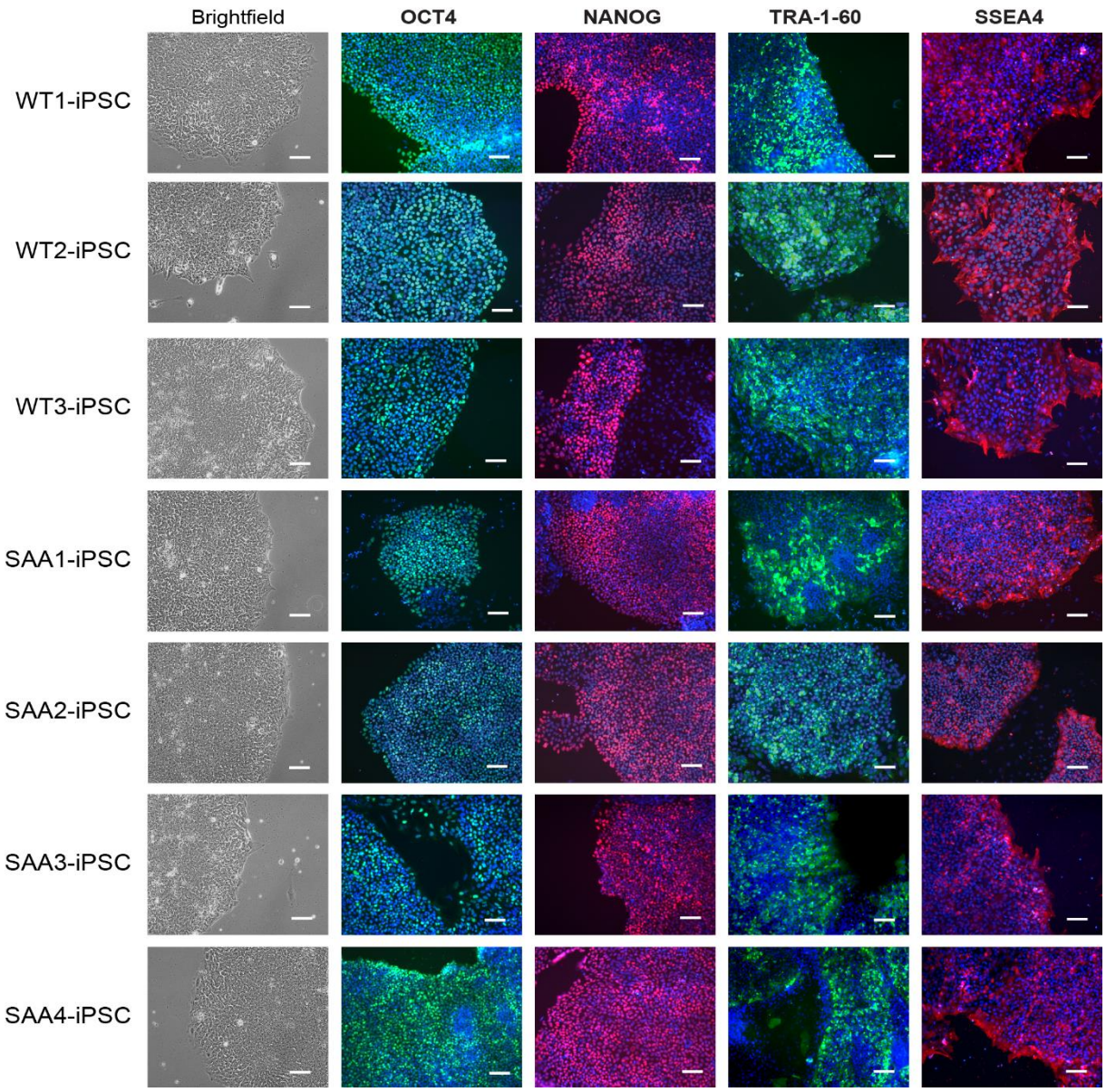


Figure 23. Detection of pluripotency-associated markers by immunofluorescence.

Representative brightfield and immunofluorescence images of control and SAA-iPSC lines showing expression of OCT4 (green), NANOG (red), TRA-1-60 (green) and SSEA-4 (red). Nuclei in immunofluorescence images were stained with DAPI (blue). Scale bars = 100 µm.

The quantification of the population co-expressing the pluripotency-associated surface markers SSEA-4 and TRA-1-60 was carried out by multicolour flow cytometric analysis. Control and SAA-iPSC lines analysed prior haematopoietic differentiation showed a high percentage of positive cell population co-expressing TRA-1-60 and SSEA-4 pluripotency-associated markers (**Figure 24A**) with no statistically significant differences in the percentage of SSEA-4+TRA-1-60+ cells between control and patient cell lines (**Figure 24B**).

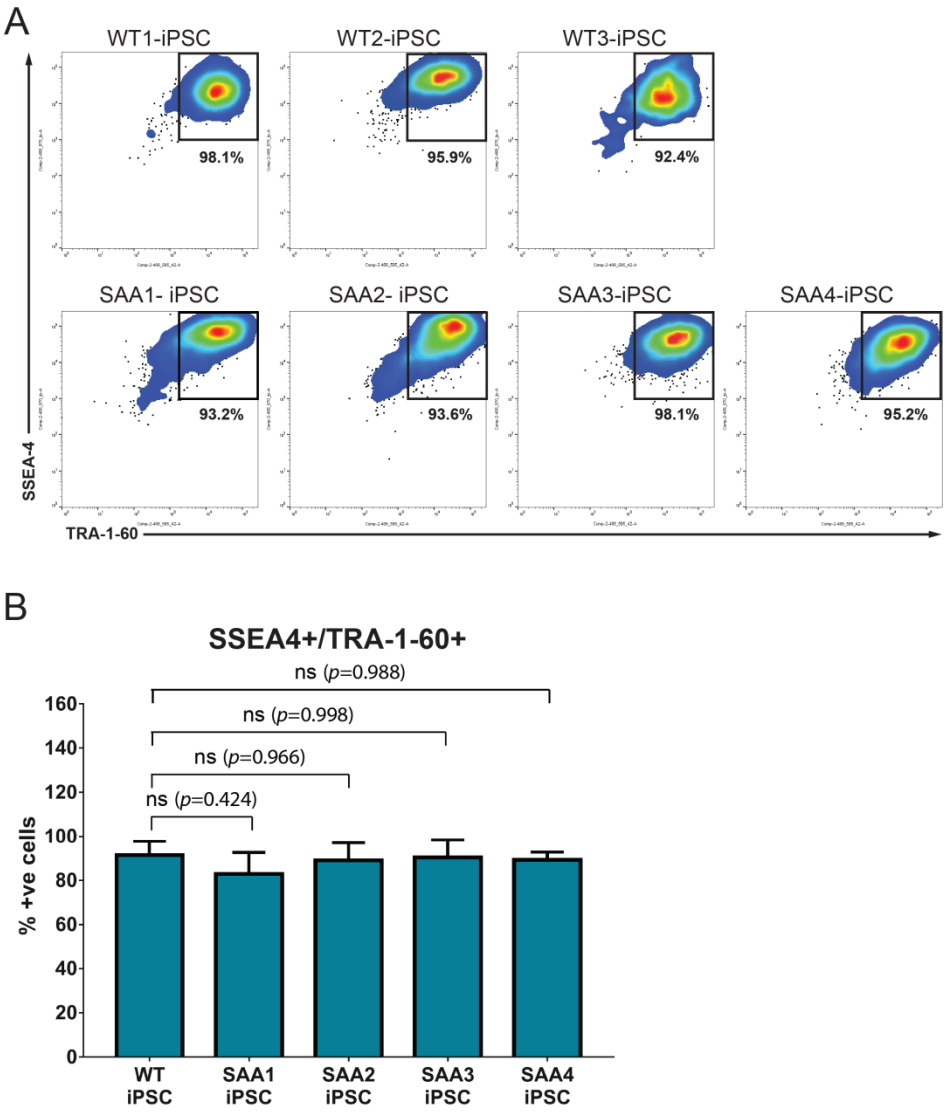


Figure 24. Detection of pluripotency-associated markers by flow cytometric analysis.

(A) Representative images of flow cytometric analysis of TRA-1-60/SSEA4 expression in control (WT1, WT2 and WT3) and SAA-iPSC lines. (B) Scatter plot showing percentages of SSEA4+TRA-1-60+ cells in grouped control (WT) and SAA-iPSC lines. One-way ANOVA with Dunnett's multiple comparison test was used for statistical comparison between grouped control (WT) and SAA-iPSC lines. Data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT)

Both control and SAA-iPSC lines used in this study successfully induced teratoma formation in SCID mice. **Table 10** provides detailed information of the number, description and time of formation of the teratomae induced by the iPSC lines used in this study. Most of the teratomae analysed were solid and presented formation of mature tissues. Histology of the teratomae was then taken and stained to reveal germ layer specific structures. Histological analysis of the teratomae from both control and SAA-iPSC lines revealed the formation of heterogenic tissues consisting of specific morphologies from ectoderm (neuroepithellium or pigmented epithelium), mesoderm (cartilage, kidney or connective tissue) and endoderm (glandular epithelium, gastrointestinal epithelium, primitive intestine or glomeruli structures) (**Figure 25**). However, teratomae from SAA3 and SAA4-iPSC lines failed to display tissues from ectoderm and endoderm/mesoderm germ layers respectively (**Figure 25**).

Patient ID	Passage number	SCID mice used	Number teratomae induced	Classification by content	Classification by clinical criteria	Time of growth (weeks)
WT1-iPSC	21	2	2	Solid/Solid	Mature/Mature	8
WT2-iPSC	20	2	2	Solid/Solid	Mature/ Inmature benign	12
WT3-iPSC	38	2	2	Solid/Solid	Mature/Mature	6
SAA1-iPSC	30	2	1	Solid	Inmature benign	7
SAA2-iPSC	9	2	2	Solid/Mixed	Mature/Mature	8
SAA3-iPSC	9	2	1	Solid (encapsulated)	Mature	8
SAA4-iPSC	36	2	1	Solid	Mature	6

Table 10. Methodology and description of the teratomae generated by control and SAA-iPSC lines

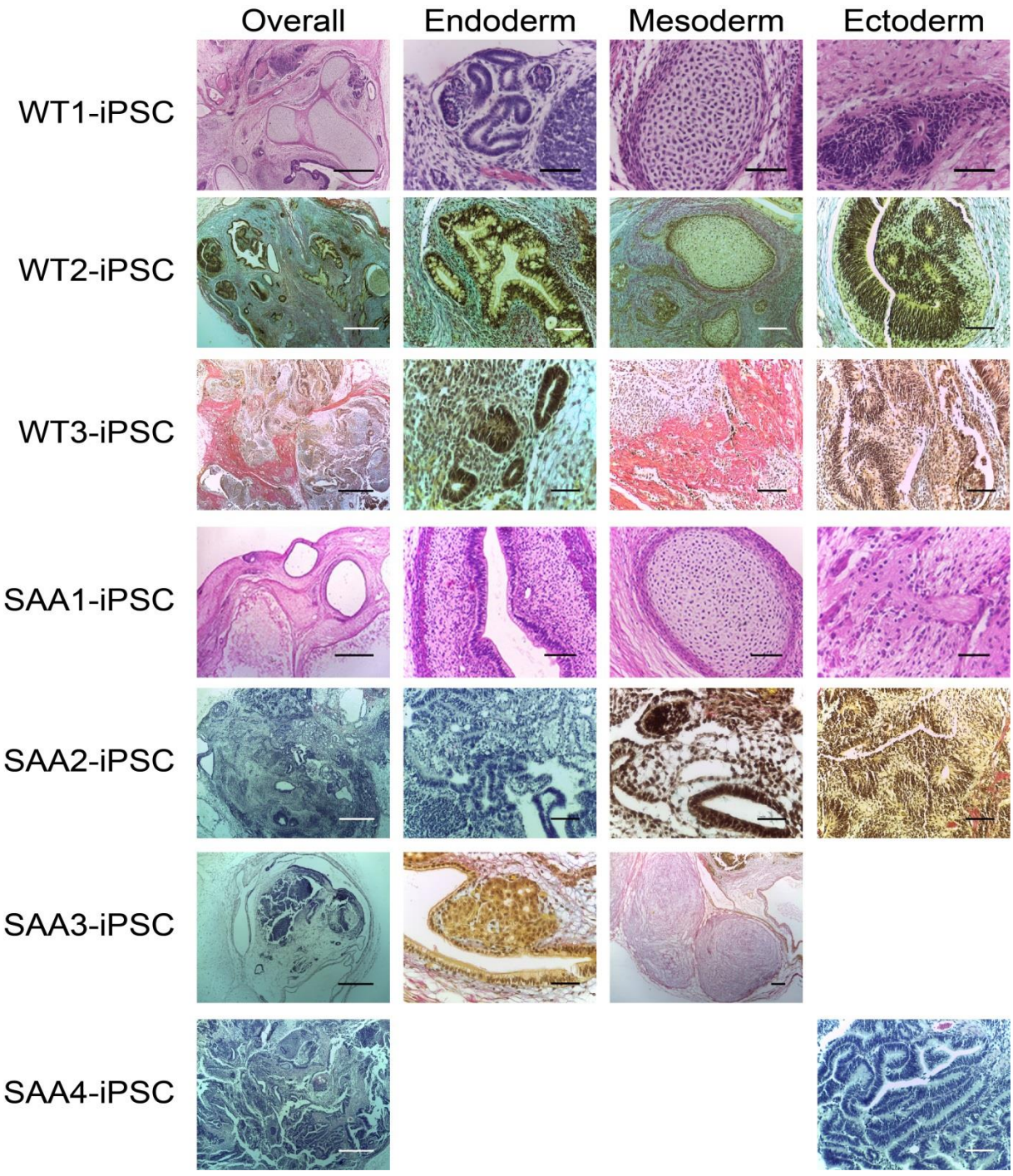


Figure 25. Induction of teratoma formation in SCID mice.

Representative histology images of tissue contained in the teratomae generated from control and SAA-iPSC lines showing tri-lineage differentiation. No ectoderm and endoderm/mesoderm structures were found for SAA3 and SAA4-iPSC lines respectively. Scale bars = 100 µm.

3.2.4 Cytogenetic analysis of control and SAA-iPSC and parental HDF

Cytogenetic analysis by using genomic SNP array was carried out on each set of iPSC/parental HDF for both control and SAA-iPSC lines. No chromosomal major alterations associated to reprogramming process or long-term culture when compared to a human reference genome (hg38) were detected in control-iPSC lines (WT1, WT2 and WT3) (**Table 11**). On the other hand, two regions of 1.3Mb and 1Mb with copy-neutral loss of heterozygosity (CN-LOH) in 3q11.2 and 7q22.1 locus respectively were noted in SAA1-iPSC. SAA2 and SAA3-iPSC lines presented different types of chromosomal alterations that were also present in the parental HDF (**Table 11**). SAA4-iPSC presented a small deletion of 0.14 Mb in locus 16p12.2 that was not present in the parental HDF (**Table 11**).

Patient ID	Cell type	Passage number	Alteration (size in Mb)	Locus	OMIM disease-causing genes	Karyotype
WT1	Fibroblast	4	-	-	-	46XY
	iPSC	24	-	-	-	
WT2	Fibroblast	7	-	-	-	46XY
	iPSC	20	-	-	-	
WT3	Fibroblast	7	-	-	-	46XX
	iPSC	11	-	-	-	
SAA1	Fibroblast	10	-	-	-	46XY
	iPSC	14	CN-LOH (1.3 Mb)	3q11.2		
			CN-LOH (1.1 Mb)	7q22.1		
SAA2	Fibroblast	4	CN-LOH (6.4 Mb)	11p11.12 -q11		46XY
	iPSC	23	CN-LOH (6.4 Mb)	11p11.12 -q11		
SAA3	Fibroblast	4	Del (1.5Mb)	15q13.3	*TRPM1, CHRNA7*	46XX
			Duplication (1.5 Mb)	16p13.11	*NDE1, MYH11, ABCC6*	
	iPSC	32	Del (1.5Mb)	15q13.3	*TRPM1, CHRNA7*	
			Duplication (1.5 Mb)	16p13.11	*NDE1, MYH11, ABCC6*	
SAA4	Fibroblast	9	-	-	-	46XY
	iPSC		Del (0.1Mb)	16p12.2	*OTOA*	

Table 11. Cytogenetic analysis of control and SAA cell lines by SNP array

Table showing karyotype, cytogenetic alterations observed in HDF and iPSC, location and OMIM disease-causing genes included in the altered region. Del, deletion

3.2.5 Genetic identity analysis

Genetic authentication of the iPSC lines is a critical step required to provide reliable and reproducible results. By confirming origin I guarantee the genetic identity of the iPSC used in the study in order to exclude undesired cross-contamination or switching with other iPSC lines therefore avoiding drawing erroneous conclusions derived from these mishandlings. For this study high-density SNP analysis of each set of iPSC and parental fibroblast cell line of control and SAA patient was carried out. SNP-based genetic identity analysis revealed correlation between parental HDF and iPSC lines indicating the same genetic profile and authenticity of all the iPSC lines used in this study (**Figure 26**).

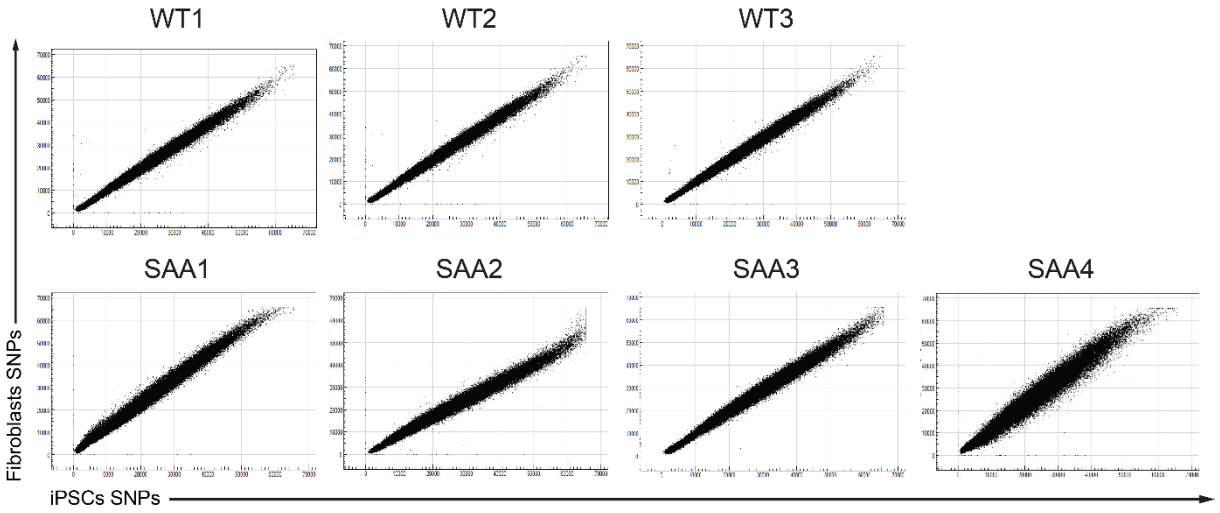


Figure 26. Authentication of genetic identity of iPSC and parental fibroblasts.

Global SNP patterns from parental HDF and iPSC were compared by whole SNP microarray indicating perfect genetic identity between iPSC and parental fibroblasts for control and SAA cell lines.

3.3 Discussion

The use of iPSC technology as a disease model represents an excellent opportunity to provide insights into the pathophysiology of SAA. Here I provide evidence of the successful generation and characterization of control and SAA-iPSC lines that will be the basis for further studies focused on investigating the existence of an underlying disease-causing genetic defect present in SAA cell lines. With this aim in mind iPSC from three control and four SAA HDF cell lines were generated. No dramatic differences were observed with regards to the iPSC generation efficiency of cell lines from control and SAA patients. These results suggest that neither the age of the patients nor the potential presence of a mutation carried by the patients' cells affected the reprogramming process. These results are particularly interesting since it has been previously reported that cells isolated from FA patients showed low reprogramming efficiencies (Yung *et al.*, 2013; Liu *et al.*, 2014). These low reprogramming efficiencies observed in FA cells are due to an impaired ability to repair DNA damage that is generated by cellular stress during the reprogramming process via FA DNA repair pathway. Thus, this observation might indicate that the potential genetic defect present in the SAA cells studied here may not be involved in this specific DNA repair pathway.

The successful generation and identification of iPSC from SAA patients is the first step towards the generation of a disease model for this disorder. However, as important as to generate SAA-iPSC is to provide proof of the fully reprogramming of the iPSC. To do so it is necessary to provide evidence of the pluripotency of the SAA-iPSC lines, absence of genetic abnormalities that can affect their differentiation potential and freedom from any residual expression of the ectopically applied reprogramming factors. As mentioned previously in **Chapter 1** (**section 1.2.5.1**), the generation of integration-free iPSC has become crucial in order to avoid genotoxicity, activation of any poised oncogenic activity via transgene integration and impairment of the differentiation potential via constitutive expression of the exogenous reprogramming factors. Vector based on SeV has been reported as a non-integrative method with a high efficiency induction of human PSCs (Fusaki *et al.*, 2009). The cytoplasmic-residing nature and infection-incompetent variant of the vector ensures reduced iPSC cytotoxicity and minimal genomic integration. However, even following the use of a non-integrating transgene vector such as SeV, the remaining possibility of residual mutagenesis/expression requires the transient nature of the vector to still be confirmed.

Thus, the generation of integration-free and transgene-free iPSC is essential for this project in order to provide a reliable disease model for identifying pathogenic variants in paediatric cases of SAA. Control and SAA-iPSC lines showed no amplification of the SeV vector or reprogramming transgenes by RT-PCR. Thus, the lack of detection of amplicon in the established iPSC lines indicates that SeV transgenes were transiently expressed during the first stages of reprogramming but were diluted during prolonged culture to undetectable levels or complete absence. These results indicate that the control and SAA-iPSC lines generated in this study successfully maintained stable regulation of the endogenous pluripotency-associated gene expression activated during reprogramming even with the loss of exogenous reprogramming transgene expression after several rounds of cell division.

To assess the pluripotent features of iPSCs, there are a number of tests used as standard practice originally with human ESCs and, currently, with iPSCs (Pera *et al.*, 2000; Hoffman and Carpenter, 2005; Takahashi *et al.*, 2007). Besides the morphological analysis of the cells and colonies formed, demonstration of the presence of pluripotency-associated surface antigens such as cell membrane-localised SSEA4 and TRA-1-60 as well as nuclear-localised transcription factors such as OCT4 and NANOG have been widely reported to validate the pluripotency of cells (Niwa *et al.*, 2000; Draper *et al.*, 2002; Silva *et al.*, 2009). The expression of these pluripotency-associated markers in both control and SAA-iPSC lines was assessed by both immunocytochemistry and multicolour flow cytometric analysis. Brightfield images confirmed the human ESC-like morphology of the control and SAA-iPSCs. Likewise, immunocytochemistry analysis to assess the localization of the pluripotency-associated markers expression revealed positive expression of both pluripotency-associated transcription factors OCT-4 and NANOG and surface markers SSEA-4 and TRA-1-60 of control and SAA-iPSC lines. Nuclear localization of the expression of OCT4 and NANOG transcription factors was observed by co-localization of fluorescence with DAPI nuclear staining. Likewise, cytoplasmic staining was observed in SSEA-4 and TRA-1-60 in accordance with its localization on the cell surface as surface antigens. Flow cytometric analysis was used to quantify the percentage of iPSC expressing the selected pluripotency-associated surface markers prior to every haematopoietic differentiation experiment as a check point to assess the quality of the iPSC lines. Control and SAA-iPSC showed high percentages of iPSC co-expressing

SSEA+4 and TRA-1-60+ (≥80%) with no significant differences between control and SAA-iPSC lines. Although previous work reported the expression of SSEA-4 and NANOG in partially-reprogrammed cells (Chan *et al.*, 2009) and the existence of an OCT4 alternative isoform B not related to pluripotency (Wang and Dai, 2010), the expression of the four pluripotency-associated markers shown by the control and SAA-iPSC has been previously described as consistently expressed in PSCs (Draper *et al.*, 2002; Henderson *et al.*, 2002; International Stem Cell *et al.*, 2007).

One of the most robust tests in human to test pluripotency is via demonstration of *in vivo* differentiation into tissue consisting of derivatives from all three germ layers (De Los Angeles *et al.*, 2015). Human PSC injected in SCID mouse induce the formation of benign tumours characterized by a rapid growth *in vivo* consisting of various disparate tissues that contain structures of all three germ layers (ectoderm, mesoderm and endoderm). This pluripotent ability of the injected cells to form a rich variety of mature tissue types from the three germ layers explains why *in vivo* teratoma-formation assay is regarded as the gold standard for validating the pluripotency of human PSCs (Zhang Wendy, June 10, 2012). The basic protocol for this assay begins with injection of the human iPSC/ESC of interest into a specific site of an SCID mouse. This will subsequently be followed by teratoma formation in over 8-12 weeks, derivative of the PSC type. Ectoderm, mesoderm and endoderm derived-tissue formation in the teratomae can then be examined by observation of the particular germ layer-specific microstructures after histological staining. Control and SAA-iPSC successfully generated teratomae in SCID mice with different degrees of maturation. Likewise teratomae generated by the three control and two of the SAA-iPSC lines displayed tissues from the three different germ layers. However two of the SAA-iPSC (SAA3 and SAA4) failed to display tri-lineage differentiation according to results from histological analysis. Absence of structures from a specific germ layer observed in these teratomae does not necessarily imply the lack of pluripotency of these cell lines since only two mice were used for each cell line in this assay. Unfortunately, as suggested by Muller *et al.*, the lack of an established standard for the parameters involved in the assay, such as the number of cells/injections/animals that should be used in order to establish if an iPSC line has functional pluripotent potential, makes difficult to interpret this data satisfactorily (Muller *et al.*, 2010). I therefore consider that additional teratoma assays most probably would induce teratoma formation displaying tissues from the germ

layers absent in this only experiment. Additionally, SAA3 and SAA4-iPSC lines successfully generated haematopoietic progenitors, as it will be thoroughly discussed in **Chapter 5**, thence proving the differentiation capacity of these iPSC lines. Likewise, it is also important to indicate that current tests are not sufficient and further rigorous quantifiable analysis are required to fully provide proof of pluripotency of a PSC line since even partially reprogrammed iPSC lines can show features of pluripotency including ability to induce formation of teratoma-like tumours (Chan *et al.*, 2009). Recently, different methods have been developed based on the analysis of molecular signature and expression levels of specific genes predicting differentiation potential of human PSCs lines, offering a more efficient and quantifiable way to evaluate potential and quality of human PSCs (Bock *et al.*, 2011; Tsankov *et al.*, 2015).

One of the major advantages of an iPSC -derived disease model is the ability to represent a patient-specific genotype in the *in vitro* disease tissue, thereby allowing study of the exact genetic defects contributing to pathogenesis. However, there is an inherent risk of genomic mutation during reprogramming thus reducing translation of the patient somatic cell genotype over to the corresponding iPSCs. Acquisition of genetic mutation during reprogramming or long-term culture of iPSCs can alter its differentiation capacity. Thus, cytogenetic analysis of the iPSC cell lines prior to its differentiation is recommended in order to detect any likely chromosomal structural variations. Although it has been reported that cells with chromosomal aberrations are eliminated via p-53 dependent apoptosis in the early stages of reprogramming, it has been shown that aneuploid cells can be successfully reprogrammed, implying that this kind of chromosomal aberration is not a barrier to reprogramming (Park *et al.*, 2008; Marion *et al.*, 2009). Additionally, as seen previously in **Chapter 1** (**section 1.1.5.2**), cytogenetic analysis might be particularly interesting for cells from SAA patients since they may present mutations that can affect genes encoding proteins involved in DNA repair pathways and telomere length homeostasis leading to accumulation of DNA damage, chromosome instability and premature cell senescence. Whereas no clinical cytogenetic imbalance was observed in WT1, WT2 and WT3 control-iPSC lines, SAA1-IPSC line revealed two small regions of CN-LOH in locus 3q11.2 and 7q22.1. CN-LOHs are stretches of homozygosity that might be particularly detrimental if the homozygous region includes recessive mutations. Presence of mutations in both alleles would lead to a defective/loss of gene function. However, regions were CN-LOH

are located in SAA1-iPSC have a relative small size with no significant clinical relevance since these regions do not include previously described disease-causing OMIM genes according to the UCSC Genome Browser database (Kent *et al.*, 2002). Both SAA2 and SAA3-iPSC lines presented cytogenetic abnormalities that were also present in the parental HDF indicating that these alterations did not arise during the reprogramming process or long-term iPSC culture. Finally, SAA4-iPSC presented a small deletion of 0.14 Mb not observed in the parental HDF. This deletion is located in locus 16p12.2 that includes the disease-causing OMIM gene *OTOA*. Deafness is the main disease phenotype associated with this gene with no association to haematological malignancies (Kent *et al.*, 2002). Cytogenetic alterations observed in SAA1 and SAA4-iPSC might be due to the reprogramming process since it has been previously reported that reprogrammed cells encounter a high replicative stress during reprogramming (Hussein *et al.*, 2011). However, it is worth mentioning that several authors reported that most of the CNV observed only in iPSC using SNP arrays were also detected in the starting populations of fibroblasts when using deep sequencing approach (Abyzov *et al.*, 2012; Kwon *et al.*, 2017). This study might suggest that low-frequency CNV present in parental fibroblasts might be beyond the detection limit of the SNP array but they are detected by more sensitive methods such as NGS. Thus, we cannot exclude the possibility that alterations detected specifically in SAA1 and SAA4-iPSC could have been present in the original fibroblast population in undetectable levels by the SNP analysis but, due to later clonal expansion of the specific clones of these SAA-iPSC lines were then detected by the SNP array.

Taken together these results provide enticing evidence of the successful generation of fully-reprogrammed iPSC suitable for further differentiation into haematopoietic progenitors as disease model for SAA. The generated control and SAA-iPSC lines presented characteristic features associated to PSC with no signs of reprogramming-associated alterations that could affect their differentiation potential therefore introducing bias to the study and distorting results.

Chapter 4. Differentiation of iPSC into Haematopoietic Progenitor Cells

4.1 Introduction

HSC from SAA patients would provide the best disease model to study the pathogenesis of SAA. However, the SAA disorder is characterized by low numbers of HSCs in the bone marrow of SAA patients and *ex vivo* expansion or pooling of bone marrow haematopoietic progenitor cells from SAA patients would be necessary in order to generate sufficient samples for disease studies (Zeng *et al.*, 2004). Likewise, *ex vivo* expansion of HSCs remains challenging due to the complexity controlling the regulatory networks involved in HSC differentiation (Walasek *et al.*, 2012). Remarkably, iPSC technology provides an excellent opportunity to investigate the pathogenesis of SAA. Due to the unique features of iPSC, widely discussed in **Chapter 1** (**section 1.2.3**), these cells can be used to provide a supply of unlimited numbers of patient-specific haematopoietic progenitor cells.

To date, several elegant methods have reported the generation of haematopoietic progenitor cells from human PSCs using different approaches such as directed differentiation, with cytokine stimulation and EB formation or co-culture on stromal cells, directed conversion or by way of teratomae formation *in vivo* as described in **Chapter 1** (**section 1.3.2.2**). However, the generation of functional HSCs with multi-lineage reconstitution ability and robust engraftment potential from PSC remains challenging (Ackermann *et al.*, 2015; Wahlster and Daley, 2016). Recently, Olivier *et al.* described the development of a robust, simple and highly efficient protocol, reporting the generation of 150 haematopoietic progenitor cells generated from a single PSC, primarily designed for the generation of erythroid cells from human PSCs but that can be also used in the first stages for the differentiation for the generation of HSPCs (Olivier *et al.*, 2016).

The aim of this chapter is to validate the use of the haematopoietic differentiation protocol by analysing:

- The capacity of the control-iPSC lines to generate the different populations of mesodermal, haemato-endothelial precursors and haematopoietic progenitor cells by flow cytometric analysis
- The potential of control-iPSC-derived haematopoietic progenitor cells to differentiate into haematopoietic colonies using CFU assays
- The robustness of the haematopoietic differentiation protocol in order to identify sources of variation present during the differentiation of iPSC lines.

4.2 Results

4.2.1 Directed differentiation of control-iPSC lines into haematopoietic progenitor cells

The method previously described by Olivier *et al.* (Olivier *et al.*, 2016) was used to differentiate control-iPSC lines into haematopoietic progenitor cells. This method promotes the induction of PSCs into mesoderm lineage and later specification of the mesodermal precursor into haematopoietic progenitors by combining the use of different cytokines and small molecules at specific time points (**Figure 27**).

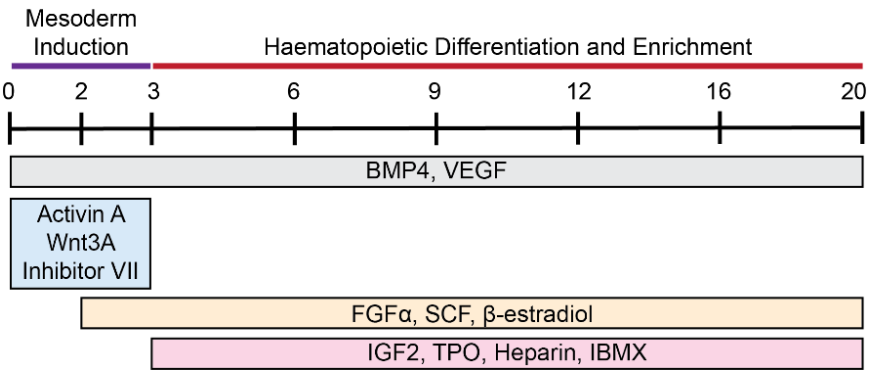


Figure 27. Differentiation scheme used for the generation of haematopoietic progenitors from iPSC

Protocol designed by Oliver *et al* (Olivier *et al.*, 2016).

As described in **Chapter 1** (**section 1.3.2.1**), different mesodermal and endothelial populations emerge during the differentiation of PSCs into haematopoietic progenitors. **Figure 28** describes visually the process of haematopoietic differentiation from PSCs including time points and key stages defining markers I chose to identify the different populations of mesodermal, haemato-endothelial precursors and haematopoietic progenitor cells, including MkP, Ery/MkP, EryP and myeloid progenitors (MyeP).

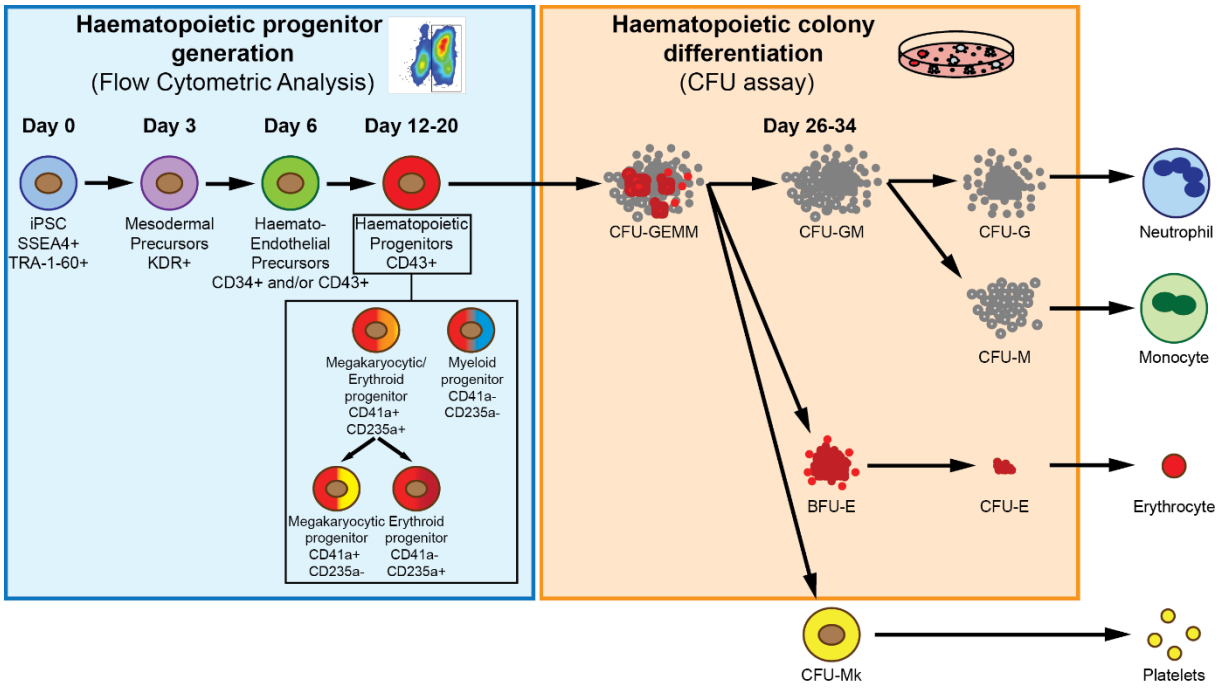


Figure 28. Schematic representation of the experimental design used to analyze control-iPSC haematopoietic differentiation capacity.

Identification of populations emerging during the process of generation of iPSC-derived haematopoietic progenitors was monitored by flow cytometric analysis. On day 3, mesodermal precursors were identified by KDR expression. On day 6, haemato-endothelial precursors were identified by CD34 and/or CD43 expression. Haematopoietic progenitors were identified at day 12, 16 and 20 by expression of CD43 marker including mekaryocytic/erythroid, megakaryocytic, erythroid and myeloid progenitors using CD41a and CD235a markers. Colony-forming potential of iPSC-derived haematopoietic progenitors was assessed by CFU assay on day 26-34. Myeloid and erythroid colonies were enumerated and identified according to size, morphology and cellular composition on day 26-34. CFU, colony-forming unit; CFU-GEMM, colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte; CFU-GM, colony forming unit-granulocyte, macrophage; BFU-E, burst forming unit-erythroid; CFU-G, colony forming unit-granulocyte; CFU-M, colony forming unit-macrophage; CFU-E, colony forming unit-erythroid.

WT2 and WT3 control-iPSC lines were used to validate the haematopoietic differentiation method published by Olivier *et al.* By day 3 of differentiation, formation of mesodermal cells was identified by expression of KDR marker also known as CD309 and vascular endothelial growth factor receptor 2 (**Figure 29A**). Presence of both CD34+CD43- endothelial progenitors, including HE cells, and emergence of cells expressing the haematopoietic marker CD43+ from the CD34+CD43- endothelial population was observed at day 6 of differentiation (**Figure 29B**).

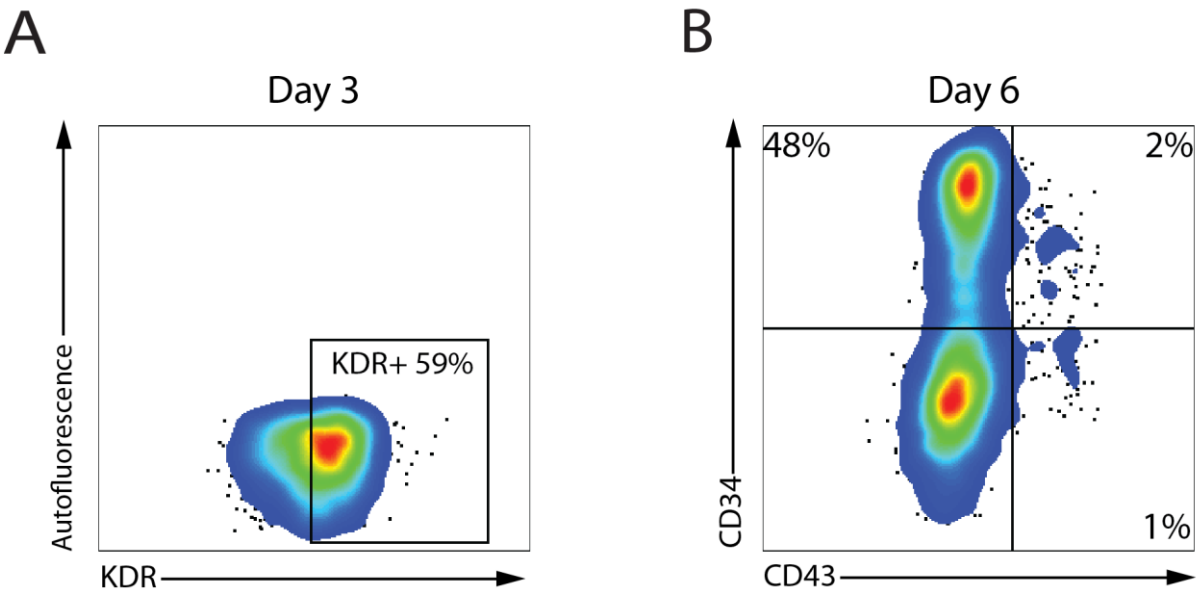


Figure 29. iPSC differentiation into mesodermal and haemato-endothelial progenitors.

(A) Flow cytometric analysis of KDR expression in control cell lines on day 3. (B) Flow cytometric analysis of CD34 and CD43 expression in control cell lines on day 6.

Expression of CD43 has been previously described to confer antiadhesive properties interfering with cell-cell adhesion (Ardman *et al.*, 1992). This would explain the presence of floating cells budding from loosely attached haemato-endothelial clusters firstly observed at day 6 (**Figure 30A**). An increase in the numbers of these budding/floating cells was observed at day 9 (**Figure 30B**) becoming especially prominent at day 12 (**Figure 30C**).

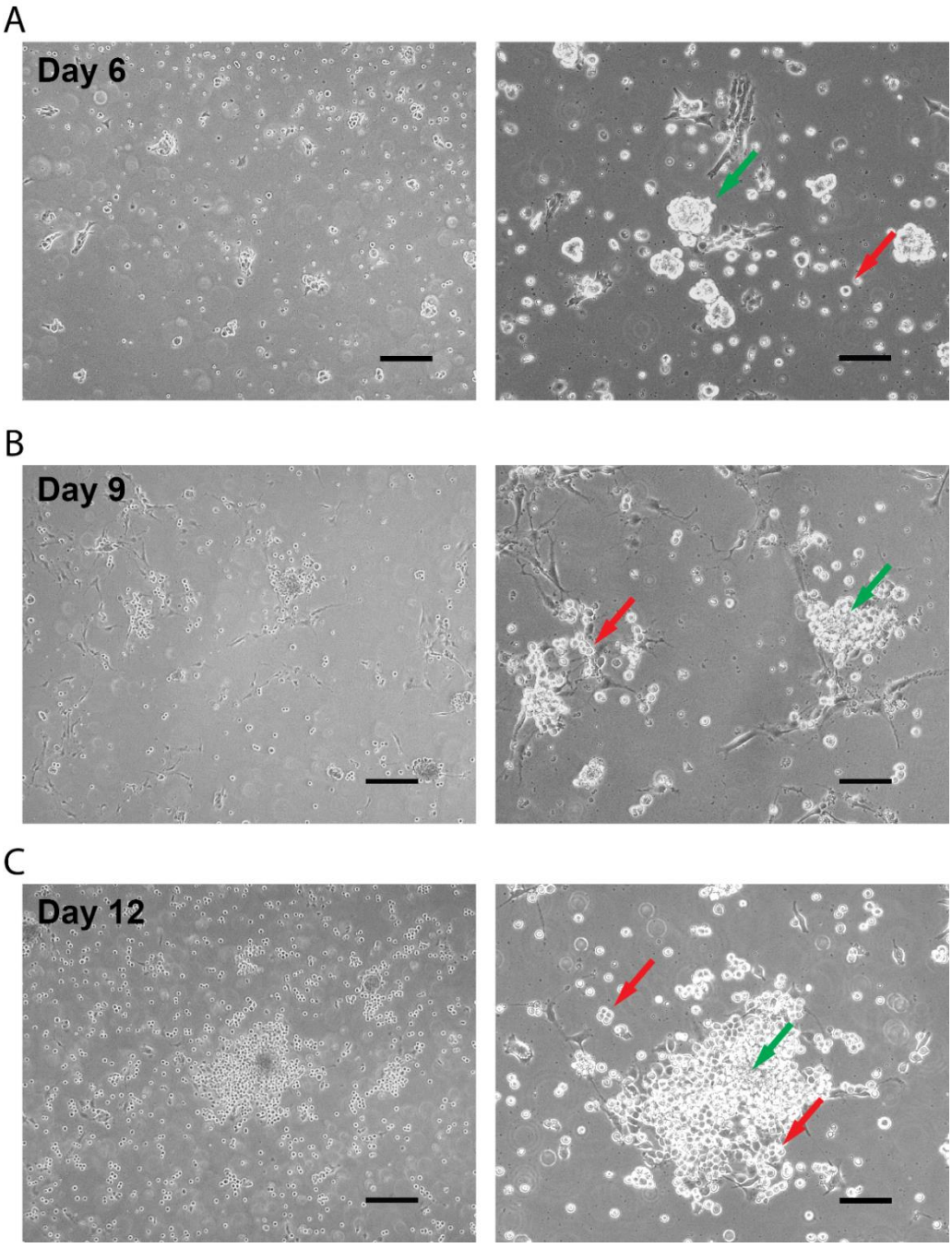


Figure 30. Appearance of haematopoietic progenitor cell like morphology.

Cell morphology of non-adherent cells which may be haematopoietic progenitors (red arrow) budding from haemato-endothelial clusters (green arrow). (A) Day 6. (B) Day 9. (C) Day 12. Scale bars, left column 200µm, right column 100µm.

A large population of CD43+ cells was identified by flow cytometric analysis of the whole population of cells between days 12 and 20 (**Figure 31A**). A gradual down-regulation in the expression of CD34 marker was observed within the CD43+ population from day 12, with 47% of CD34+CD43+ cells on day 12, 33% on day 16 and 27% on day 20. Likewise, analysis of the CD43+ subpopulations of progenitors revealed the generation of MkP (CD41a+CD235a-), Ery/MkP (CD41a+CD235a+), EryP (CD41a-CD235a+) and MyeP (CD41a-CD235a-) at day 12 (**Figure 31B**). A decrease in the Ery/MkP and EryP percentages was also observed in day 16 and day 20 most likely due to a maturation of the progenitors over time.

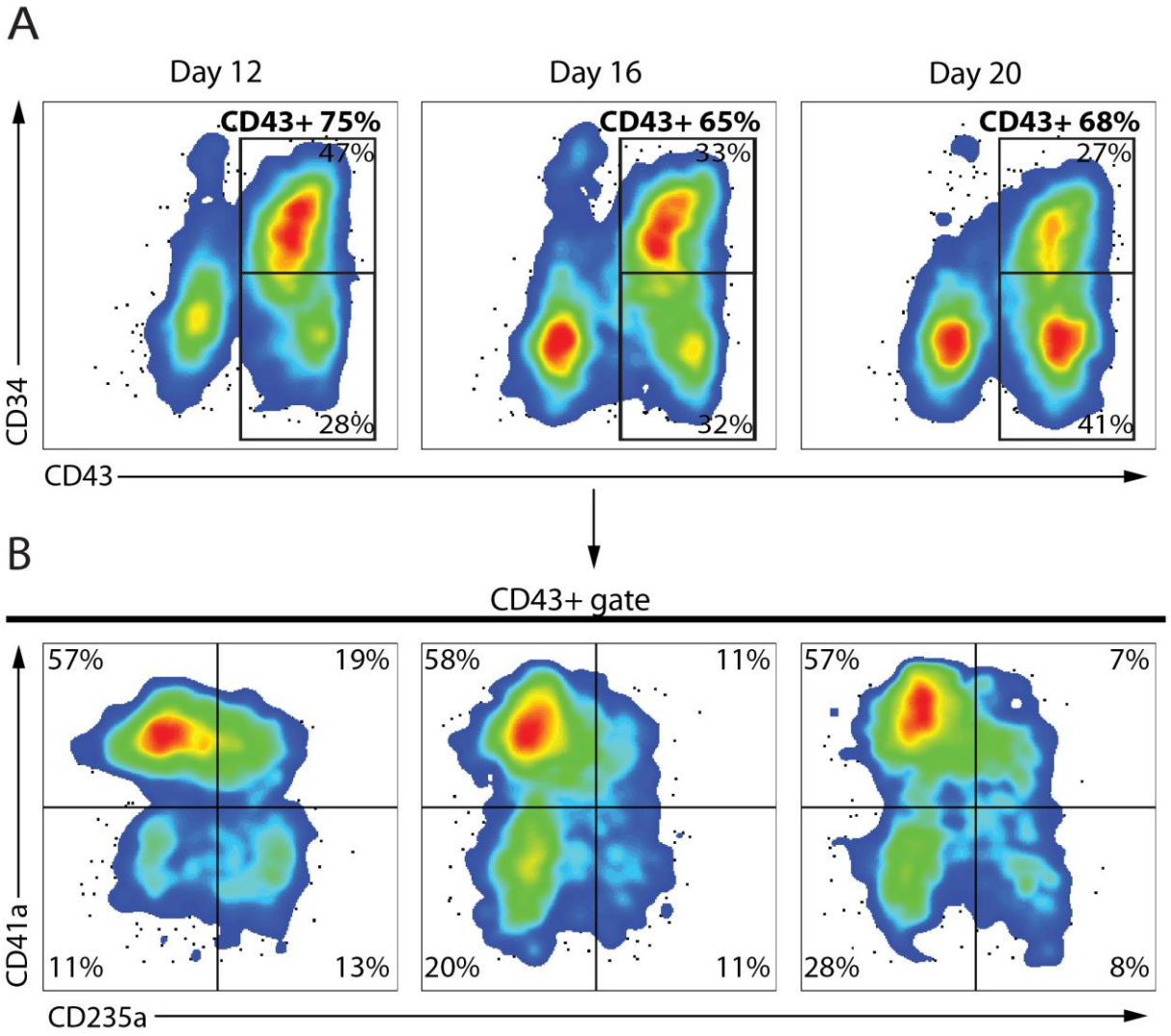


Figure 31. Identification of CD43+ haematopoietic progenitors and CD41a+/CD235a+ subpopulations.

(A) Flow cytometric analysis of CD34 and CD43 expression in control cell lines on day 12, 16 and 20. (B) Flow cytometric analysis of CD41a and CD235a expression in gated CD43+ cells in control cell lines on day 12, 16 and 20.

Analysis of the colony-forming capacity of the control-iPSC-derived haematopoietic progenitors generated at day 12, 16 and 20 was evaluated by CFU assays using methylcellulose–based medium enriched with haematopoietic-specific cytokines. This assay allows the enumeration of CFU colonies from both erythroid lineage (CFU-E and BFU-E), myeloid lineage (CFU-GEMM, CFU-GM, CFU-G and CFU-M) after 14-16 days of culture according to size, morphology and cellular composition following standard criteria (Coutinho, 1993; Eaves, 1995) (**Figure 32**). Maturation capacity of progenitors from megakaryocyte lineages was not evaluated due to the difficulties on enumerating CFU-Mk based on morphology and cellular composition using methylcellulose-based media.

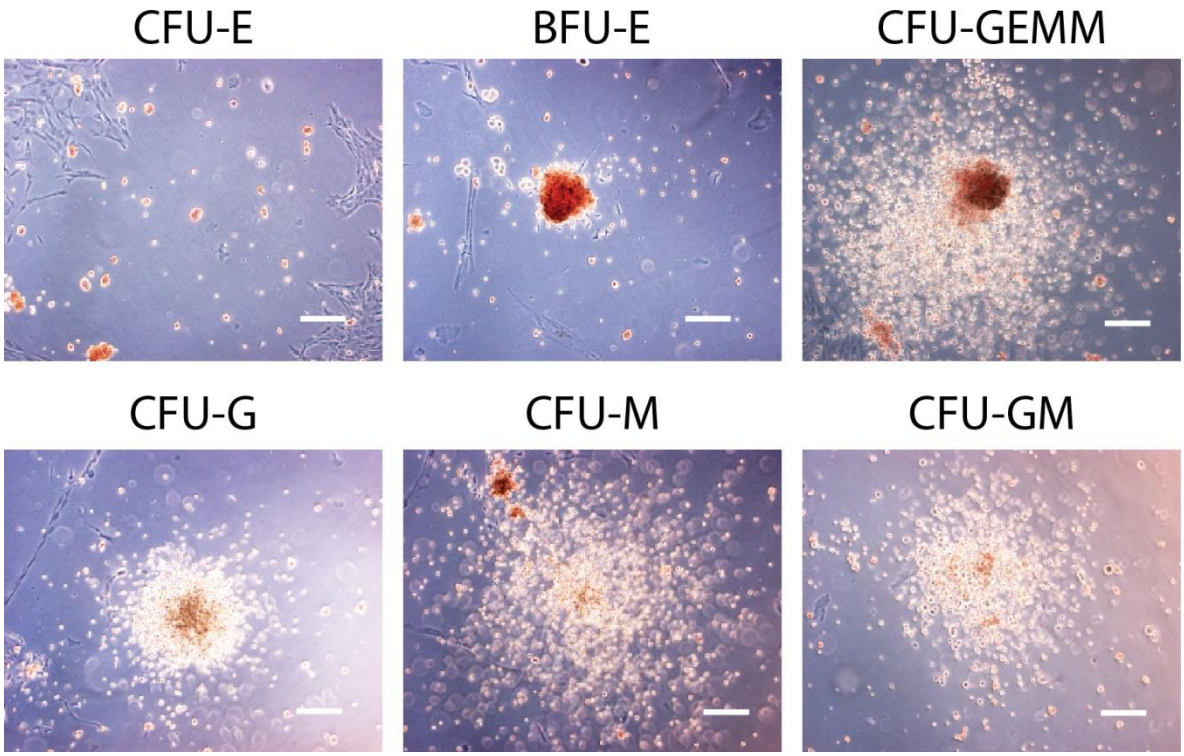


Figure 32. Morphological appearance of haematopoietic colonies from iPSC-derived haematopoietic progenitors formed in CFU-assay.

Representative colony types of CFU-E, BFU-E, CFU-GEMM, CFU-M, CFU-G and CFU-GM haematopoietic colonies obtained from differentiation of control-iPSC-derived haematopoietic progenitors. Scale bars, 200µm.

CD43+ haematopoietic progenitors obtained from whole population of cells at day 12 showed a higher colony-forming capacity with increased numbers of CFUs in cytokine-enriched medium compared to day 16 and day 20 (**Figure 33**).

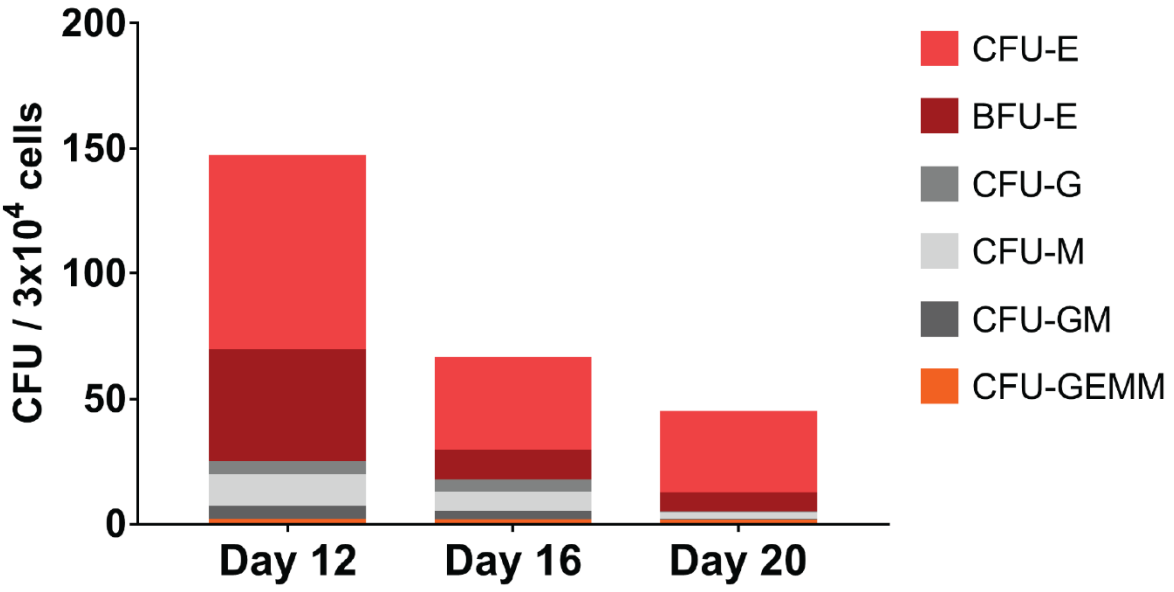


Figure 33. Colony-forming potential of iPSC-derived haematopoietic progenitor cells.

CFU assay showing number of haematopoietic colonies and colony type generated by control-iPSC-derived haematopoietic progenitor cells on day 12, 16 and 20**.**

4.2.2 Haematopoietic potential of CD43+ subset

To identify the population of cells at day 12 with haematopoietic colony-forming potential, subsets of CD34/CD43 cells obtained from WT3-iPSC line were enriched by fluorescence-activated cell sorting (FACS) and assayed for colony-forming potential by CFU assay (**Figure 34A**). As expected, only CD43+ cells showed ability to form haematopoietic colonies whereas CD43- populations showed complete absence of haematopoietic colonies (**Figure 34B**). This indicates that only CD43+ fraction displays haematopoietic colony-forming potential. Both CD43+ subpopulations, CD34+ and CD34-, demonstrated colony-forming potential although it is noteworthy that CD34+CD43+ showed a higher number of CFU compared to the CD34-CD43+ population although the CD34+CD43+ cells on day 12 showed a lower percentage of cells, 16%, compared to the 68% of CD34-CD43+ cells.

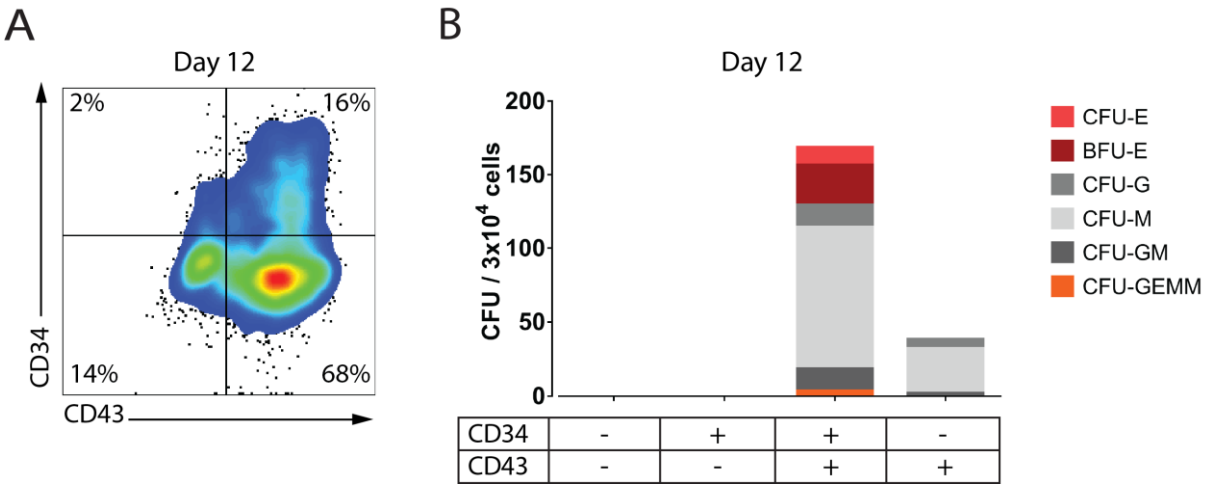


Figure 34. Colony-forming potential of FACS-sorted CD34/CD43 subsets.

(A) Flow cytometric analysis of CD34 and CD43 expression in WT3 cell line on day 12. (B) CFU assay showing number of haematopoietic colonies and colony type according to the different subpopulations sorted with indicated phenotype (+/- chart) generated by WT3-iPSC-derived haematopoietic progenitor cells on day 12.

4.2.3 Analysis of sources of variation in the haematopoietic differentiation of iPSC

To identify potential contributors to variability in the differentiation process of the iPSC lines into haematopoietic progenitors, I analysed the variation introduced by different experimental variables when using the previously described haematopoietic differentiation method such as differentiation experiment, passage number of the iPSC line (passage), iPSC clone (clone) and iPSC line (genetic background) (**Figure 35**).

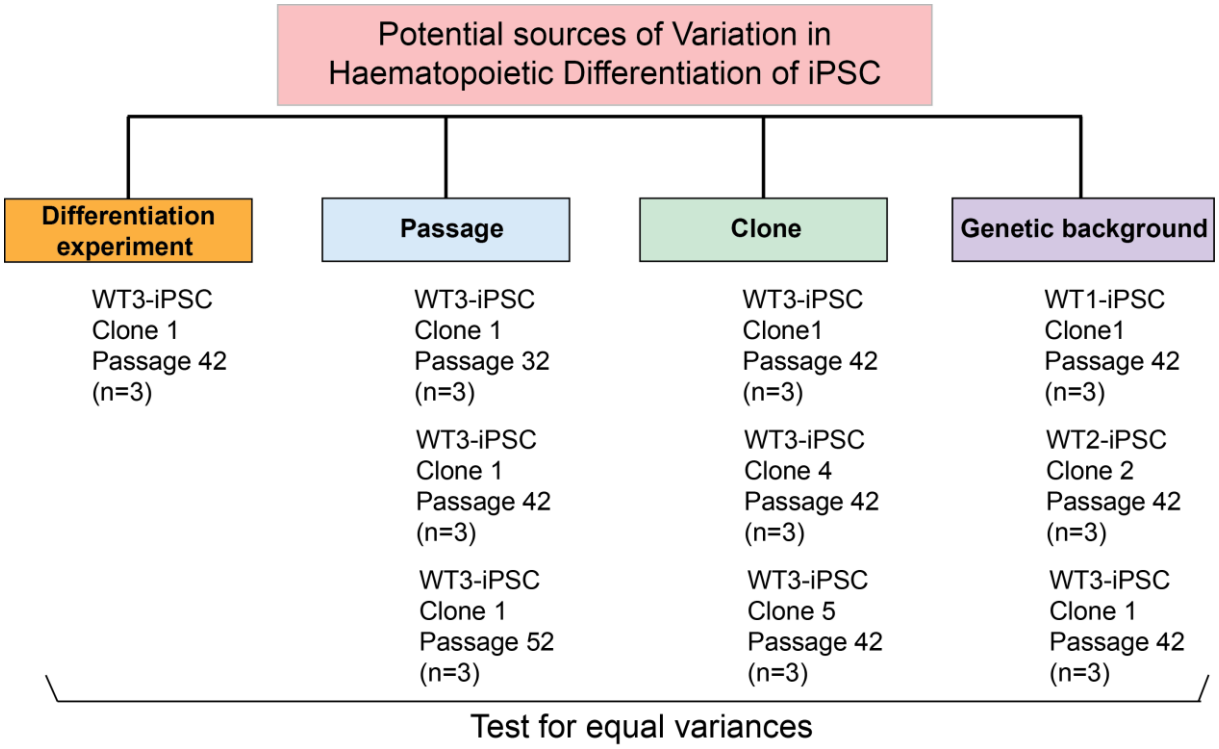
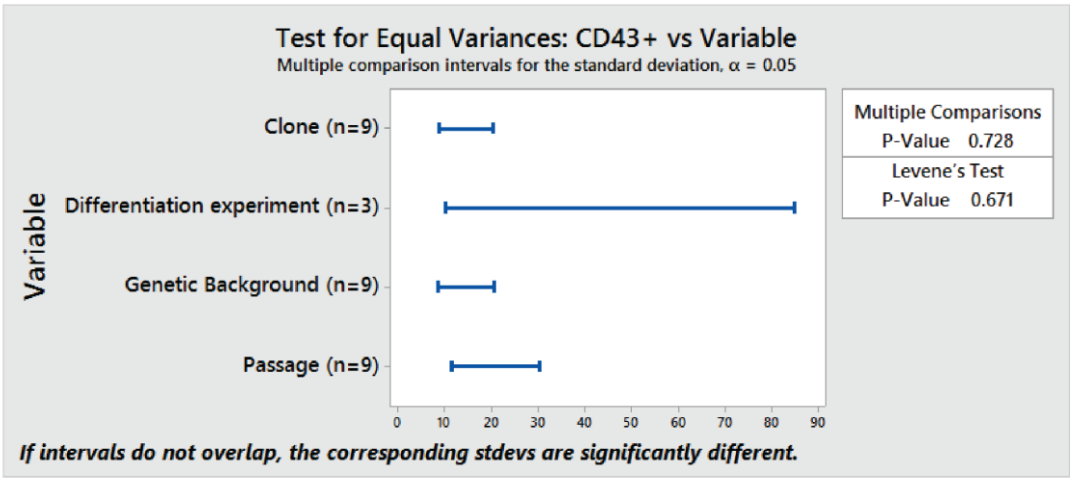


Figure 35. Schematic representation of the experimental design to analyse variation in generation of haematopoietic progenitor cells from iPSC on day 12.

Differentiation experiment refers to the variation observed in sample biological repeats (intra-experimental) (variable is differentiation experiment). Passage refers to the variation observed in experiments using an individual clone from the same iPSC line with different passage number (variable is passage number). Clone refers to the variation observed in experiments using different clones from the same iPSC line with same passage number (variable is clone). Genetic background refers to the variation observed in experiments using different iPSC lines with same passage number (variable is genetic background between iPSC lines)

Percentages of positive cells for the different populations analysed in this experiment are shown in **Appendix B**. By using a test for equal variances for multiple comparisons, the different variances obtained for each variable were compared when differentiating the iPSC into CD43+ haematopoietic progenitors and the different subpopulations of haematopoietic progenitors (Ery/MkP, MkP, EryP and MyeP). No statistical differences were observed in the variances obtained from the variables of interest and the differentiation experiment variable for any of the haematopoietic progenitor populations (**Figure 36A-E**). However, variance observed in the EryP population was close to significance (*p* value=0.051) (**Figure 36D**). As shown in **Figure 36D**, interval of data represented for genetic background in EryP barely overlaps with the interval of data for differentiation experiment, indicating that differences of both standard deviations are close to significance (red rectangle, **Figure 36D**). This suggests that genetic background is the main driver of variation when generating EryP from iPSC lines in our study, although the differences observed were not significant.

A



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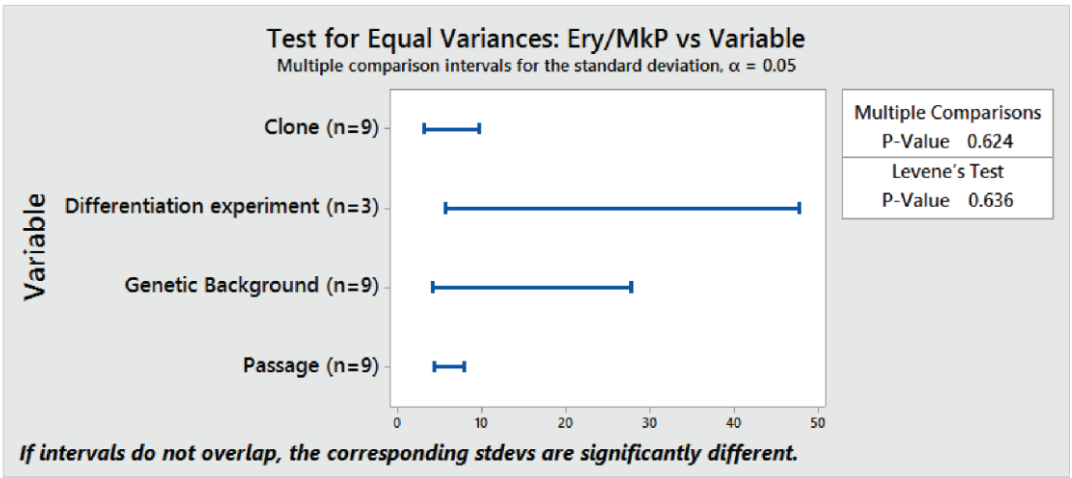

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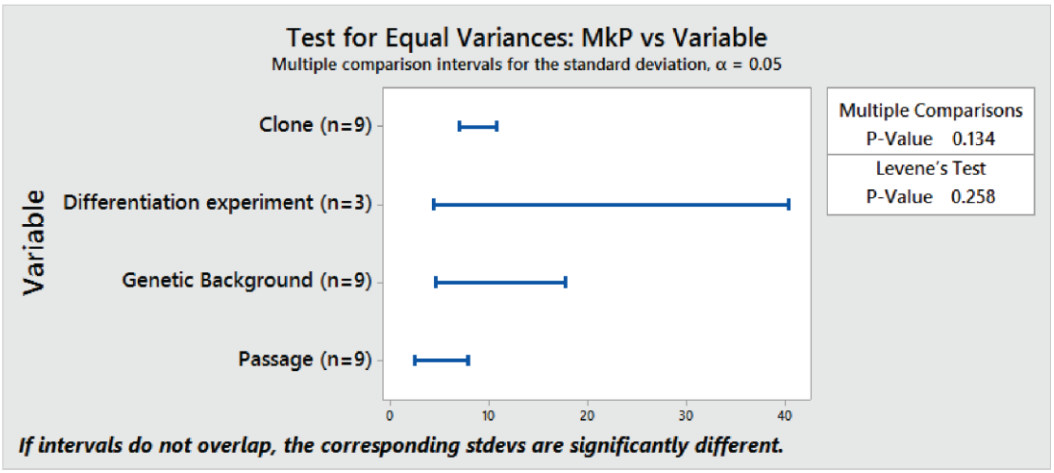

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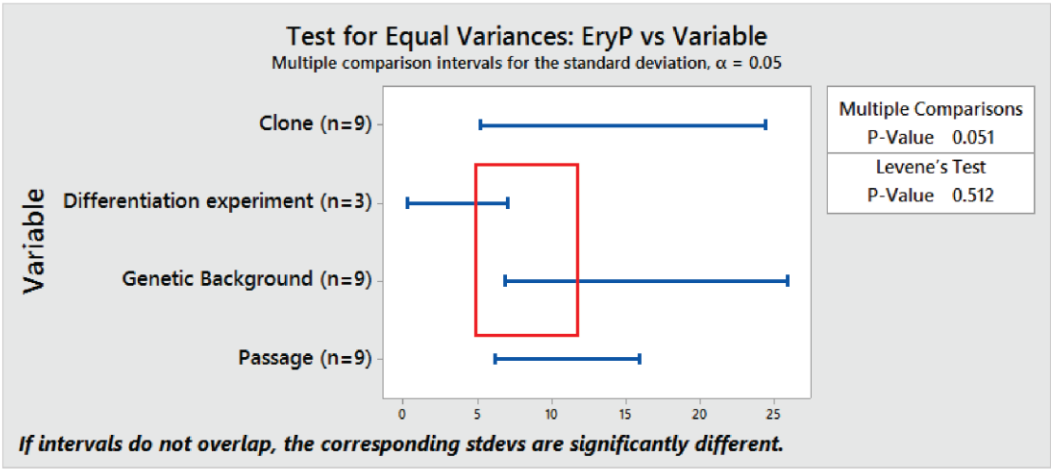

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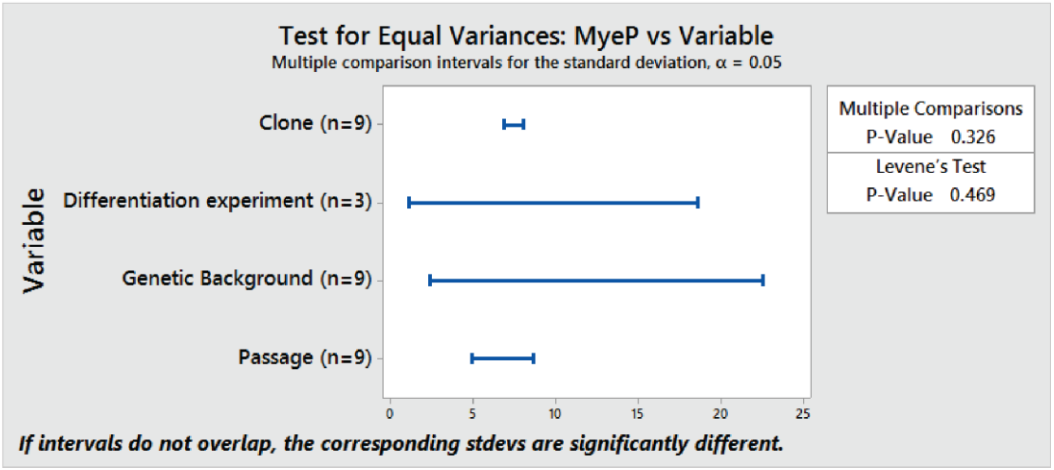

Figure 36. Analysis of variation in the differentiation of WT3-iPSC into haematopoietic progenitor cells and subpopulation of progenitors on day 12.

(A) Haematopoietic progenitors (CD43+). (B) Ery/MkP. (C) MkP. (D) EryP. Red rectangle indicates minimum overlap between differentiation experiment and genetic background variables. (E) MyeP. Test for equal variances for multiple comparisons was used for statistical comparison between the different variables. Data is presented as mean of at least 3 independent experiments.

4.3 Discussion

The generation of patient-specific SAA haematopoietic progenitor cells represents a "disease-in-a-dish" approach that we consider extremely valuable to investigate the unknown pathogenesis of SAA. In this chapter, the process of generation and identification of haematopoietic progenitors from iPSC is described and the robustness of the method used to obtain these progenitors in order to identify potential sources of experimental variation is evaluated. By using the method developed by Olivier *et al.*, mesodermal KDR+ cells were successfully induced on day 3 from control-iPSCs lines. The formation of primitive mesodermal KDR+ cells at this stage is induced by the signalling activators BMP4, Activin A and WNT3A used in the differentiation media previously described by different studies as essential in establishing posterior streak mesoderm progenitors, with haematopoietic and endothelial potential (Sumi *et al.*, 2008; Zhang *et al.*, 2008; Wang and Nakayama, 2009). Additionally, stabilization of β-catenin by GSK3β inhibitor (Inhibitor VII) used in the differentiation media activates WNT signalling effect in the formation of mesoderm with haemato-vascular potential (Sumi *et al.*, 2008; Olivier *et al.*, 2016). Likewise, addition of VEGF, FGFα and SCF to the differentiation media promoted the formation of haematopoietic progenitors via haemato-vascular specification of the primitive mesoderm (Pick *et al.*, 2007). Thus, at day 6, the formation of endothelial cells was detected by expression of endothelial marker CD34 and absence of haematopoietic marker CD43. CD34+ population at this stage is heterogeneous including committed endothelial cells and haemogenic endothelium (HE) cells, defined as a specialized population of endothelial cells that lack of ability to form haematopoietic colonies in semi-solid media but have the capacity to form haematopoietic progenitors with erythroid, myeloid and T-lymphoid potential when cultured on OP9 mouse stromal cells (Choi *et al.*, 2012; Kennedy *et al.*, 2012). We observed that CD34+CD43- cells at day 6 showed very similar pattern and percentages to the ones reported by studies using similar differentiation method (Kennedy *et al.*, 2012). Likewise, the first early haematopoietic progenitors emerging form the CD34+CD43- cells were detected on day 6 although at low levels. This population could be designated as angiogenic haematopoietic progenitors, previously described as the first haematopoietic progenitors to emerge at this stage from the endothelial population with potential to form haematopoietic colonies (Choi *et al.*, 2012). However, CFU assays would be required to confirm the haematopoietic potential of this $CD43^{low}$ population.

From day 10-12 a burst in the proliferation of cells was observed with an elevated presence of floating cells. This could be explained by the use of cytokines and small molecules such as SCF, FGF2, TPO, IGF2 and IBMX at later stages in order to enforce and enrich the generation of haematopoietic progenitors by enhancing haematopoietic proliferation and increasing the transcription of key genes involved in haematopoietic specification (Perlingeiro *et al.*, 2003; Zhang *et al.*, 2006; Olivier *et al.*, 2016). As described in **section 4.2.1**, CD43 pan-haematopoietic marker allows the identification of the haematopoietic progenitor population generated from PSCs and it has been widely used in a high number of studies involving haematopoiesis from PSCs (Vodyanik *et al.*, 2006; Timmermans *et al.*, 2009; Choi *et al.*, 2012; Kennedy *et al.*, 2012; Elcheva *et al.*, 2014; Ronn *et al.*, 2015; Nishizawa *et al.*, 2016) and modelling of haematopoietic disorders using iPSCs (Garcon *et al.*, 2013; Mills *et al.*, 2013). Days 12, 16 and 20 showed similar percentages of CD43+ cells (75% at day 12, 65% at day 16 and 68 % at day 20) and CD43+ subpopulations including Ery/MkP, MkP, EryP and MyeP were also detected. However, CFU assays revealed a higher colony-forming capacity of day 12 haematopoietic progenitors and a decreased colony-forming potential of the day 16 and day 20 progenitor populations. This could be explained by a down-regulation in the expression of CD34 within the CD43+ population observed at days 16 and 20 previously described as a sign of commitment to more mature progenitors and decreased colony-forming potential (Vodyanik *et al.*, 2006; Kennedy *et al.*, 2012). Likewise, the decrease in Ery/MkP and EryP population observed on days 16 and 20 could be also attributable to a maturation of the progenitors with loss of CD43 expression associated to a commitment to erythroid lineages (Remold-O'Donnell *et al.*, 1987)

To confirm the colony-forming potential of the CD43+ population, CD34/CD43 fractions at day 12 were sorted according to surface marker expression and plated in cytokine-enriched media in order to assess the colony-forming potential of the different fractions. As expected, CFU-assays showed the presence of CFUs from both erythroid and myeloid lineages only in the CD43+ subsets, CD34+CD43+ and CD34-CD43+, confirming that CD43 marker can be used to reliably separate population of progenitors with haematopoietic colony-forming potential as previously described by other studies (Vodyanik *et al.*, 2006; Timmermans *et al.*, 2009; Kennedy *et al.*, 2012). Interestingly,

CD34+CD43+ fraction generated a higher number of CFU, 170 per 30.000 cells plated, compared to CD34-CD43+ fraction, 40 per 30.000 cells plated, with a lower percentage of cells at day 12 (16% for CD34+CD43+ vs 68% for CD34-CD43+). These results confirm that the loss of CD34 expression is associated to a reduced colony-forming potential probably due to a maturation of the progenitors but not a complete lack of capacity to form haematopoietic colonies.

As described earlier in **Chapter 1** (**section 1.2.5**) the use of iPSC in disease modelling presents important limitations. Analysis of potential sources of variation introduced during the generation of the iPSC lines was addressed in **Chapter 3**. The results in this **Chapter 3** demonstrated that the iPSC lines generated for this study presented no major complications with regards to reprogramming vector-related alterations, incomplete reprogramming or presence of genetic variations due to reprogramming or long-term passaging of the cell lines. In this chapter, we address complications related to potential heterogeneity induced by the differentiation of iPSC cells into haematopoietic progenitors that can diminished the value of iPSC in SAA disease modelling. Thus, I analysed the contribution of different sources of variation previously described in iPSC-based disease modelling such as passage number variability (Nishino *et al.*, 2011), clone-to-clone variability within same iPSC lines (Thatava *et al.*, 2013) and donor-to-donor variability between iPSC lines due to different genetic background (Mills *et al.*, 2013). Cell type of origin has been also previously described as potential source of variation suggesting retention of cell-type epigenetic memory that bias the iPSC differentiation potential towards the cell type of origin (Bar-Nur *et al.*, 2011). However, we did not consider this variable since all the different iPSC lines used for this study were generated by reprogramming of the same cell type, HDF. Thus, for each variable group, different iPSC lines that differ mainly in the variable of interest (passage, clone or genetic background) were differentiated and variances obtained for day 12 CD43+ haematopoietic progenitors, including EryP, Ery/MkP, MkP and MyeP, were compared to the differentiation experiment variable which is included in each variable group. Thus, any statistical significant differences observed when comparing variances of any of the variable groups to the differentiation experimentation group could be interpreted as variation introduced by the variable and not just experimental variability. Results obtained by using the test for equal variances by multiple comparisons method indicate that differences in variances between each of the

variables and the differentiation experiment variable were not statistically significant for any of the haematopoietic progenitor populations since the standard deviations calculated for each variable showed overlapping with the differentiation experiment variable. However, it is noteworthy that differences in variances for EryP were close to significance that might be attributable to the minimum overlap showed by the standard deviations of the genetic background variable and the differentiation experiment variable. So, it can be hypothesized that genetic background might be acting as a source of variation with regards to the generation of EryP from iPSC and a definitive answer to this question would require further experiments. These results are in agreement with previous studies highlighting the importance of genetic background as the main source of variation in iPSC at a transcriptomic and epigenetic level (Rouhani *et al.*, 2014; Choi *et al.*, 2015; Kyttala *et al.*, 2016; Carcamo-Orive *et al.*, 2017) and at haematopoietic differentiation potential of iPSCs and ESCs (Mills *et al.*, 2013; Tulpule *et al.*, 2013; Kotini *et al.*, 2015; Feraud *et al.*, 2016). Thus, as suggested by Cahan *et al.*, it is possible that genetic background variations observed in iPSC differentiation are amplified due to the fact that *in vitro* differentiation methods do not completely reproduce aspects of early embryonic development and signalling pathways involved in differentiation are less efficiently activated (Cahan and Daley, 2013). The use of normal isogenic iPSC lines from same patient or iPSC lines sharing partial genetic background, such as parental relatives, would reduce the variation introduced by the genetic background. Unfortunately, samples from parental relatives of the SAA patients were not available and generation of isogenic iPSC lines from SAA patients is not plausible since presence of a genetic defect in these patients remains unknown and, therefore, there is no possibility to correct it to generate isogenic iPSC lines. Therefore, in the light of these results, in order to include genetic background variation when analysing the haematopoietic potential of SAA-iPSC in **Chapter 5**, I decided to use multiple control-iPSC lines to distinguish between phenotypic effects caused by disease-causing alterations and normal variation.

In summary, here I provide evidence of the effective generation from PSCs of previously described mesodermal/endothelial progenitors and, most importantly, the generation of haematopoietic progenitors with colony-forming potential, including Ery-P, Ery/MkP, MkP and MyeP when using the method published by Olivier *et al.* These results show that haematopoietic progenitors on day 12 have a higher colony-forming

capacity compared to later stages of the differentiation. Likewise, we confirmed that CD43 expression can be used to identify haematopoietic progenitors with colony-forming potential. Thus, in accordance with these results, we will base the analysis of the haematopoietic potential of the SAA cell lines described in the **Chapter 5** in investigating the CD43+ population generated by the SAA-iPSC on day 12. Finally, when studying the variation introduced by different experimental variables we identified genetic background as the variable showing the highest contribution to the variance in EryP. This indicates that genetic background variation should be considered when investigating the haematopoietic potential of the SAA-iPSC lines by including multiple lines from unaffected individuals in order to reduce the risk of misinterpreting differences in phenotypes due to genetic variation as disease-related phenotypes.

Chapter 5. Haematopoietic potential and telomere dynamics of SAA cell lines

5.1 Introduction

SAA is characterised by peripheral blood pancytopenia and hypocellular bone marrow. Several studies have reported reduced numbers of haematopoietic progenitors in the bone marrow of SAA patients and impaired differentiation capacity of these progenitors by *in vitro* clonogenic assays (**Chapter 1**, **section 1.1.5.2**). Although traditionally considered as an immune disorder, the presence of an underlying defect in haematopoietic progenitors of SAA patients has been also hypothesised to be associated to SAA pathogenesis. However, this stem cell dysfunction has been difficult to investigate due to absence of *in vitro* disease models and unsuitability of current animal models (**see section 1.1.6**). By using iPSC technology, SAA haematopoietic progenitor cells could be generated at ease providing an *in vitro* disease model to investigate their properties and potential defects in the absence of immune system influence.

Different studies have reported the generation of iPSC from patients with BMFS such as FA (Raya *et al.*, 2009; Muller *et al.*, 2012; Yung *et al.*, 2013), DC (Agarwal *et al.*, 2010; Batista *et al.*, 2011; Wang *et al.*, 2012; Gu *et al.*, 2015), DBA (Garcon *et al.*, 2013; Ge *et al.*, 2015), SDS (Tulpule *et al.*, 2013) and SCN (Hiramoto *et al.*, 2013; Nayak *et al.*, 2015). These patient-specific iPSC models have successfully recapitulated the disease phenotype of patients and have been used as disease model to investigate the mechanisms associated with the pathogenesis of the disorders.

Excessive telomere attrition in highly proliferative cells such as HSPCs can lead to bone marrow failure (Calado and Young, 2008). Due to the up-regulation of telomerase function and telomere elongation occurring during the reprogramming process (Marion *et al.*, 2009; Zalzman *et al.*, 2010) , IPSC technology has been widely used in the past to investigate telomere dynamics in telomeropathies such as DC (Agarwal *et al.*, 2010; Batista *et al.*, 2011; Wang *et al.*, 2012; Gu *et al.*, 2015). I decided to investigate the telomere dynamics in our SAA-iPSC model since it has been reported that one third of

acquired AA patients present short telomeres in leukocytes with only 10% of these patients presenting mutations in known telomere-associated genes (Ball *et al.*, 1998; Brummendorf *et al.*, 2001; Young *et al.*, 2006).

The aim of this 5th chapter is to assess the feasibility of the iPSC approach to recapitulate the AA disease phenotype by analysing:

- The ability of SAA-iPSC to generate haematopoietic progenitor cells including mesodermal and haemato-endothelial precursors during haematopoietic differentiation
- The capacity of iPSC-derived haematopoietic progenitor cells to proliferate and differentiate into haematopoietic cells.
- The telomerase activity up-regulation and telomere length during reprogramming of SAA-fibroblasts and further SAA-iPSC haematopoietic differentiation.

5.2 Results

5.2.1 Generation of haematopoietic progenitor cells from SAA-iPSC lines

Control (WT1, WT2 and WT3) and SAA-iPSC lines (SAA1, SAA2, SAA3 and SAA4) were differentiated into haematopoietic progenitor cells by using the experimental design and differentiation protocol described in **Chapter 4** (**Figure 37**).

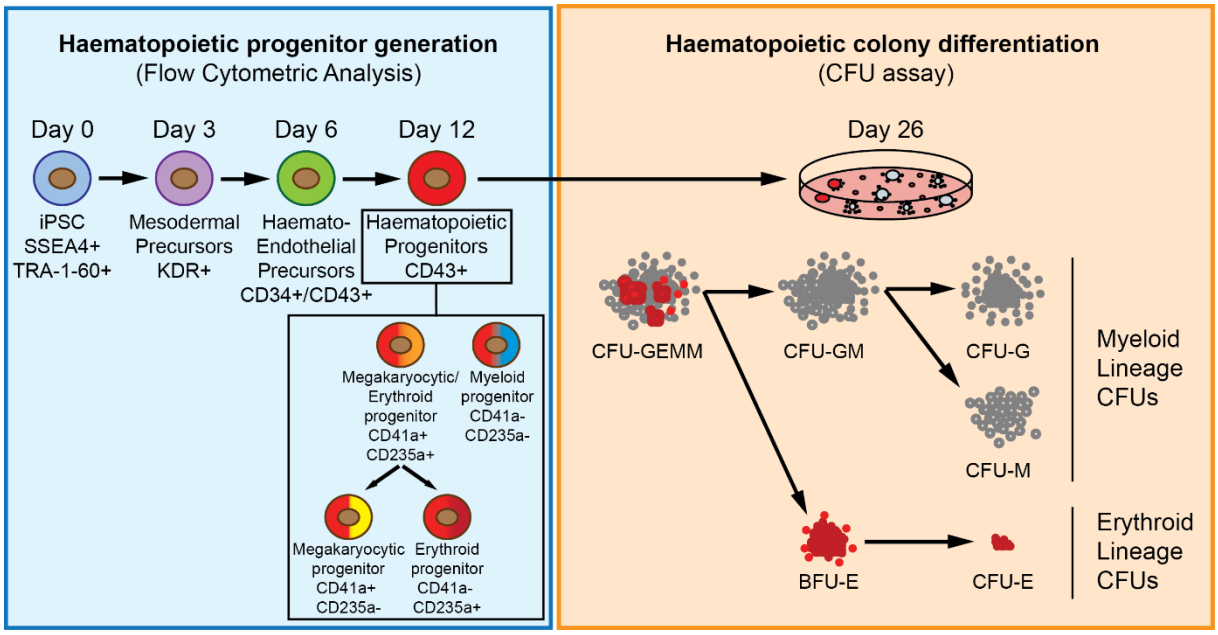

Figure 37. Schematic representation of the experimental design used to analyze the control and SAA-iPSC haematopoietic differentiation capacity

As described in **Chapter 1** (**section 1.2.5.3**), potential epigenetic memory retained in iPSC after reprogramming process can result in reduced differentiation capacity leading to biased results. Thus, to guarantee erasure of existing somatic epigenetic memory, both control and SAA-iPSC lines were cultured for at least 30 passages since it has been described that extended passaging of iPSC removes this epigenetic memory (Polo *et al.*, 2010). Early stages of mesoderm induction from iPSC cultures were monitored on day 3 of differentiation by expression of KDR (FLK1) (**Figure 38A**). No statistically significant differences were observed in the percentage of KDR+ cells between control and SAA cell lines indicating that patient-specific iPSC lines did not show impaired ability to differentiate into mesodermal lineages (**Figure 38B**). Formation of haemato-endothelial progenitors and the emergence of the first haematopoietic progenitors was detected at day 6 using CD34 and CD43, pan-haematopoietic marker (**Figure 38C**). SAA1-iPSC showed a significant reduction in the percentage of CD34+/CD43+ cells indicating a reduced potential of SAA1-iPSC to generate haemato-endothelial progenitors or a delay in the generation of these cells from iPSC (**Figure 38D**).

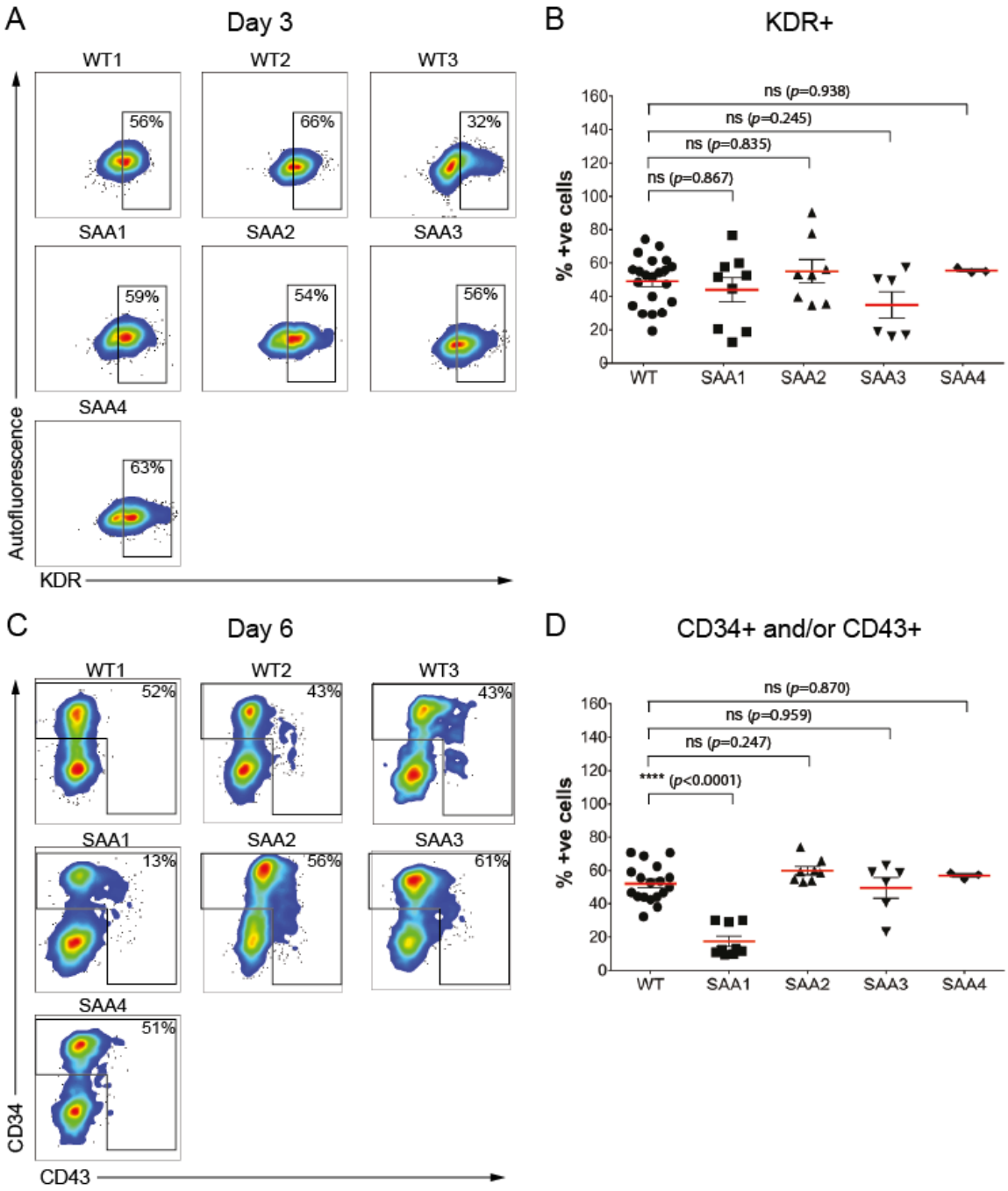

Figure 38. Generation of mesodermal and haemato-endothelial progenitors from control (WT) and SAA-iPSC lines.

(A) Representative images of flow cytometric analysis of KDR expression in differentiating control (WT) and SAA cell lines on day 3; (B) Scatter plot showing percentages of KDR+ cells in differentiating grouped control (WT) and SAA cell lines on day 3; (C) Representative images of flow cytometric analysis of CD34 and CD43 expression in differentiating control (WT) and SAA cell lines on day 6; (D) Scatter plot showing percentages of CD34+ and/or CD43+ cells in differentiating grouped control (WT) and SAA cell lines on day 6; B, D: One-way ANOVA with Dunnett's multiple comparison test was used for statistical comparison between grouped control (WT) and SAA cell lines. Data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

The presence of CD43+ haematopoietic progenitors including erythroid, megakaryocytic and myeloid subpopulations generated from control and SAA-iPSC lines was assessed at day 12 by flow cytometric analysis (**Figure 39**).

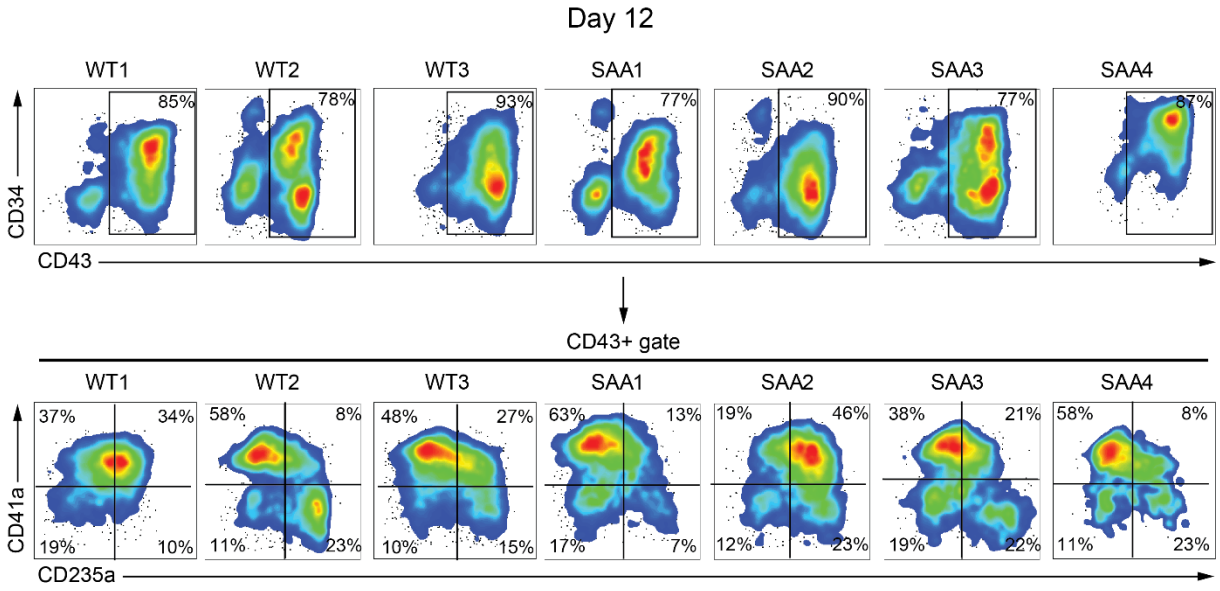

Figure 39. Generation of iPSC-derived CD43+ haematopoietic progenitors and Ery/MkP, MkP and MyeP subpopulations from control (WT) and SAA cell lines.

Representative images of flow cytometric analysis of CD34, CD43 expression and CD41 and CD235 expression for gated CD43+ population.

Statistical analysis did not reveal a significant reduction in the capacity to generate CD43+ haematopoietic progenitors in any of the SAA-iPSC cell lines compared to unaffected controls (**Figure 40A**). However, one of the SAA patient iPSC (SAA1) showed a statistically significant reduction in the potential to generate EryP (CD43+CD41a-CD235a+) (**Figure 40B**); whereas no impaired potential to generate the Ery/MkP, MkP and MyeP was observed in the other SAA-iPSC lines (**Figure 40C-E**).

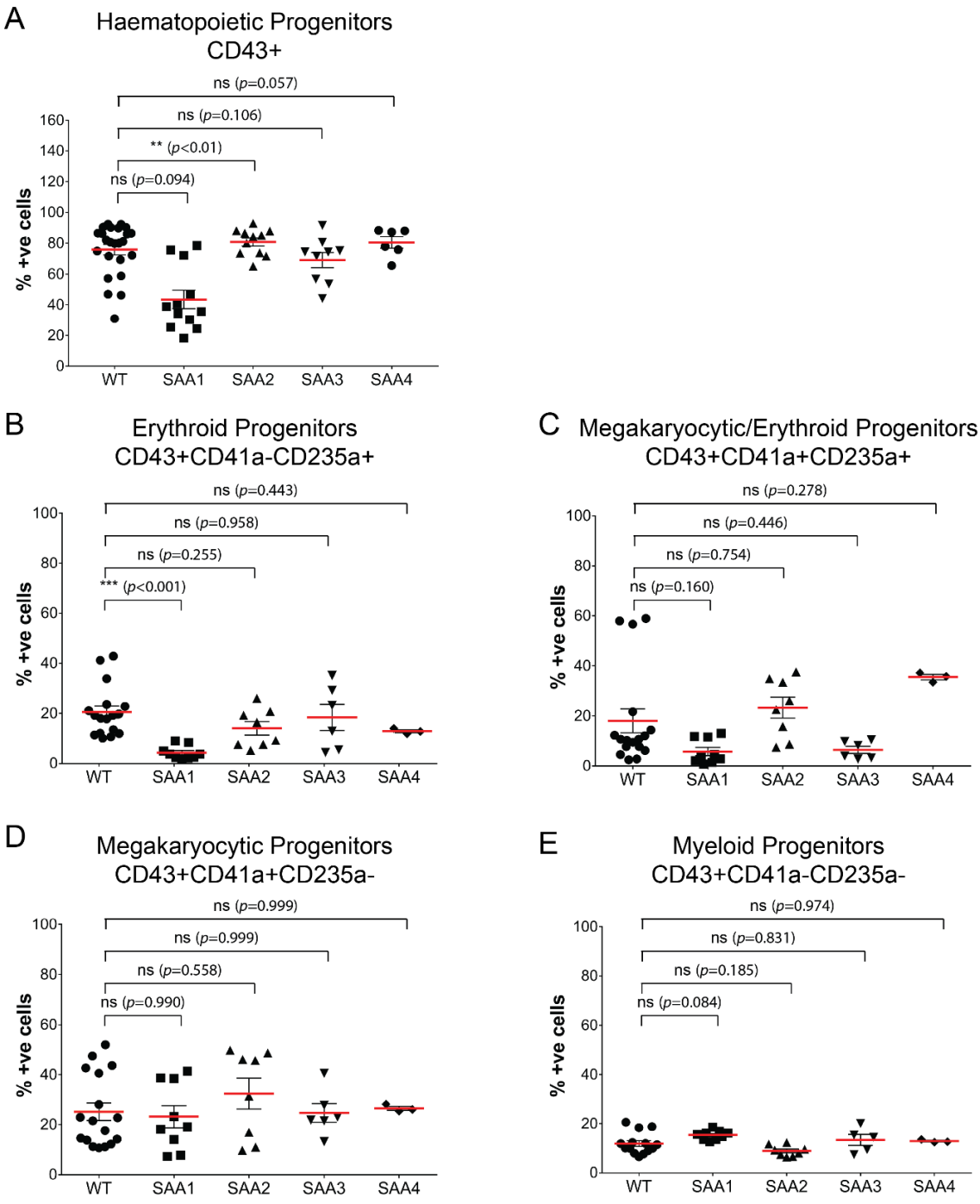

Figure 40. Comparison of haematopoietic differentiation potential of control (WT) and SAA-iPSC lines.

(A) Haematopoietic progenitors (CD43+); (B) Erythroid progenitors (CD43+CD41a-CD235a+); (C) Megakaryocytic/erythroid progenitors (CD43+CD41a+CD235a+); (D) Megakaryocytic progenitors (CD43+CD41a+CD235a-); (E) Myeloid progenitors (CD43+CD41a-CD235a-); A, C: Kruskal-Wallis test with Dunn's multiple comparison test was used for statistical comparison between grouped control (WT) and SAA cell lines; B, D, E: One-way ANOVA with Dunnett's multiple comparison test was used for statistical comparison between grouped control (WT) and SAA cell lines; A-E: Data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

5.2.2 Colony-forming potential of SAA-iPSC-derived haematopoietic progenitor cells

To assess the colony-forming potential of the SAA-iPSC cell lines, we performed CFU assays at day 12 by culturing iPSC-derived-haematopoietic progenitors for 14-16 days in methylcellulose-based media enriched with recombinant cytokines which promote the differentiation into committed erythroid progenitors (CFU-E and BFU-E), and myeloid lineage progenitors (CFU-G, CFU-M, CFU-GM and CFU-GEMM) (**Figure 37**). Enumeration of CFU colonies was carried out according to size, morphology and cellular composition as indicated in **Chapter 4** (**section 4.2.1**). Two of the patient cell lines (SAA2 and SAA3) failed to produce all the different types of CFU colonies usually obtained from haematopoietic progenitors (**Figure 41**). CFU colonies from SAA patients displayed similar morphology to CFUs from control cell lines with no apparent changes in size or cellular composition (**Figure 41**).

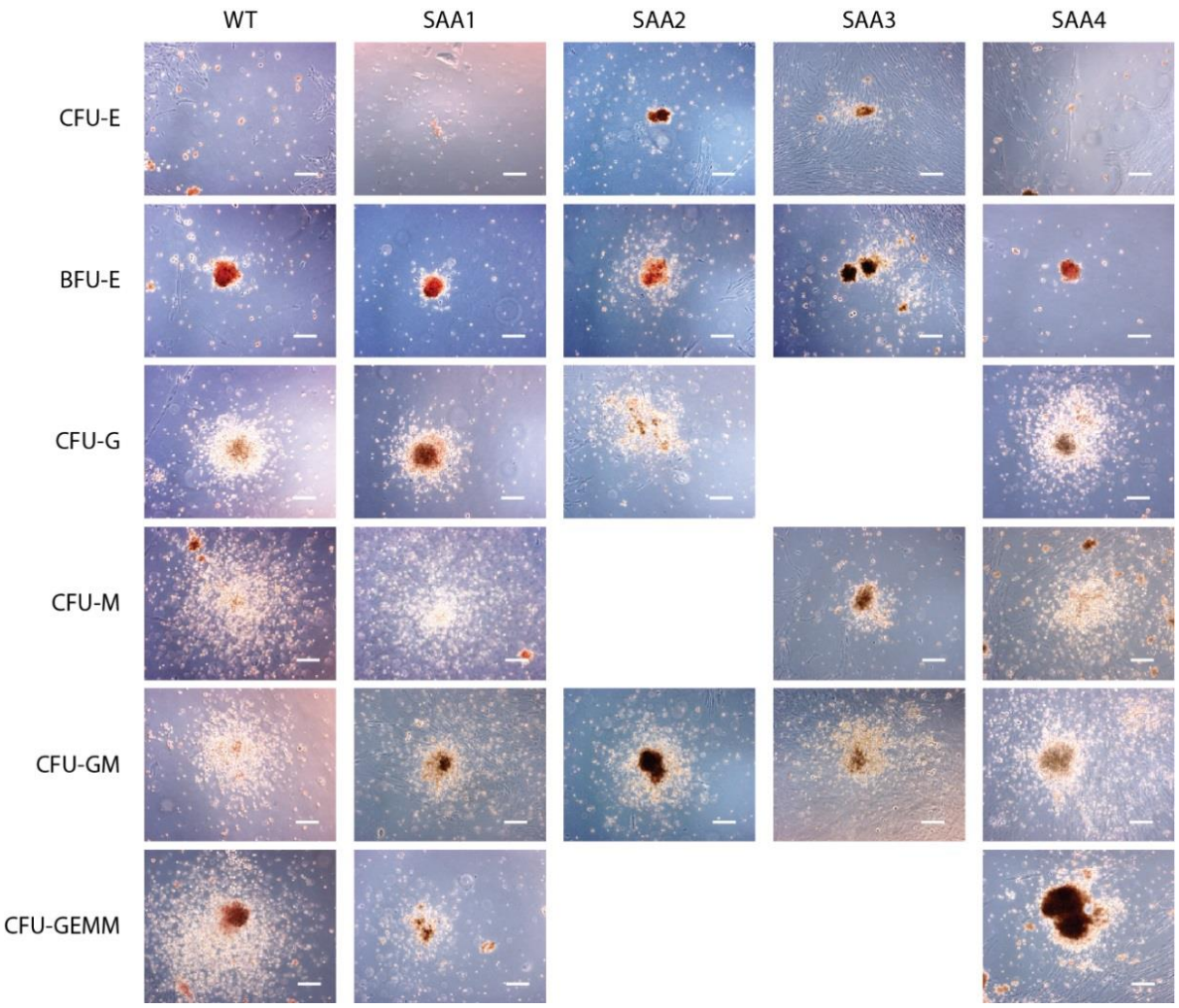

Figure 41. Representative pictures of CFU-GEMM, CFU-E, BFU-E, CFU-GM, CFU-G and CFU-M haematopoietic colonies in control (WT) and SAA cell lines.

No representative pictures for CFU-M/CFU-GEMM and CFU-G/CFU-GEMM were taken for SAA2 and SAA3 cell lines respectively due the scant number of colonies. Scale bars, 200 µm.

Determination of the number of CFUs revealed that three SAA-iPSC-derived haematopoietic progenitors (SAA1, SAA2 and SAA3) showed a significant reduction in the total number of CFUs (**Figure 42A**) indicating an impaired haematopoietic colony-forming capacity, including both erythroid and myeloid colony potential (**Figure 42B-C**). Of note, the main and common deficiency between the three affected SAA cell lines appeared at the stage of erythroid and myeloid colony formation from respective progenitor cells, a process which requires extensive cellular replication and differentiation.

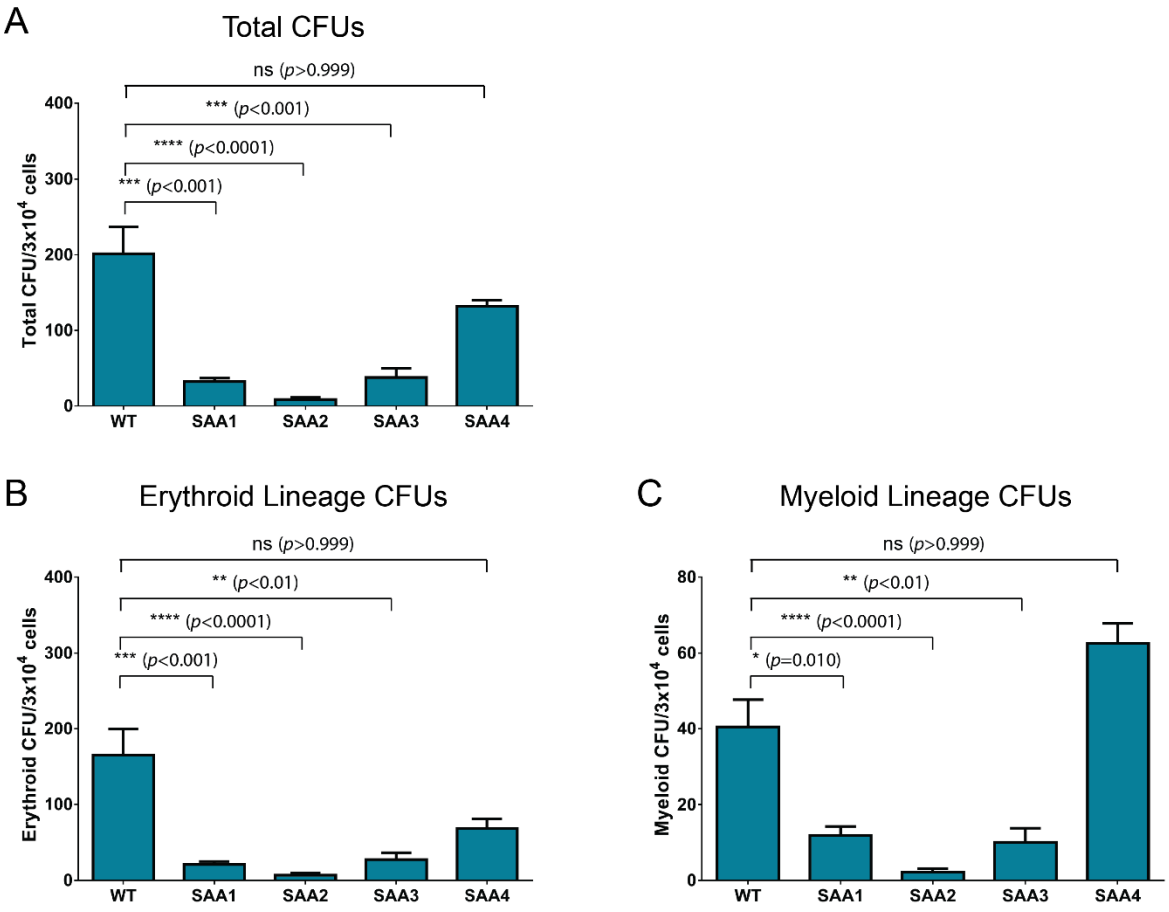

Figure 42. Comparison of the colony-forming potential of control (WT) and SAA-iPSC-derived haematopoietic progenitors.

(A) Total number of CFUs; (B) Erythroid-lineage CFUs; (C) Myeloid-lineage CFUs; A-C: Kruskal-Wallis test with Dunn's multiple comparison test was used for statistical comparison between grouped control (WT) and SAA cell lines. Data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

Analysis of the different haematopoietic colony types generated in CFU assay showed a complete absence of CFU-M and CFU-GEMM colony type in SAA2 cell line (**Figure 43**). However, these results are difficult to interpret due to the reduced total haematopoietic colony number observed in the affected SAA cell lines (**Figure 43**).

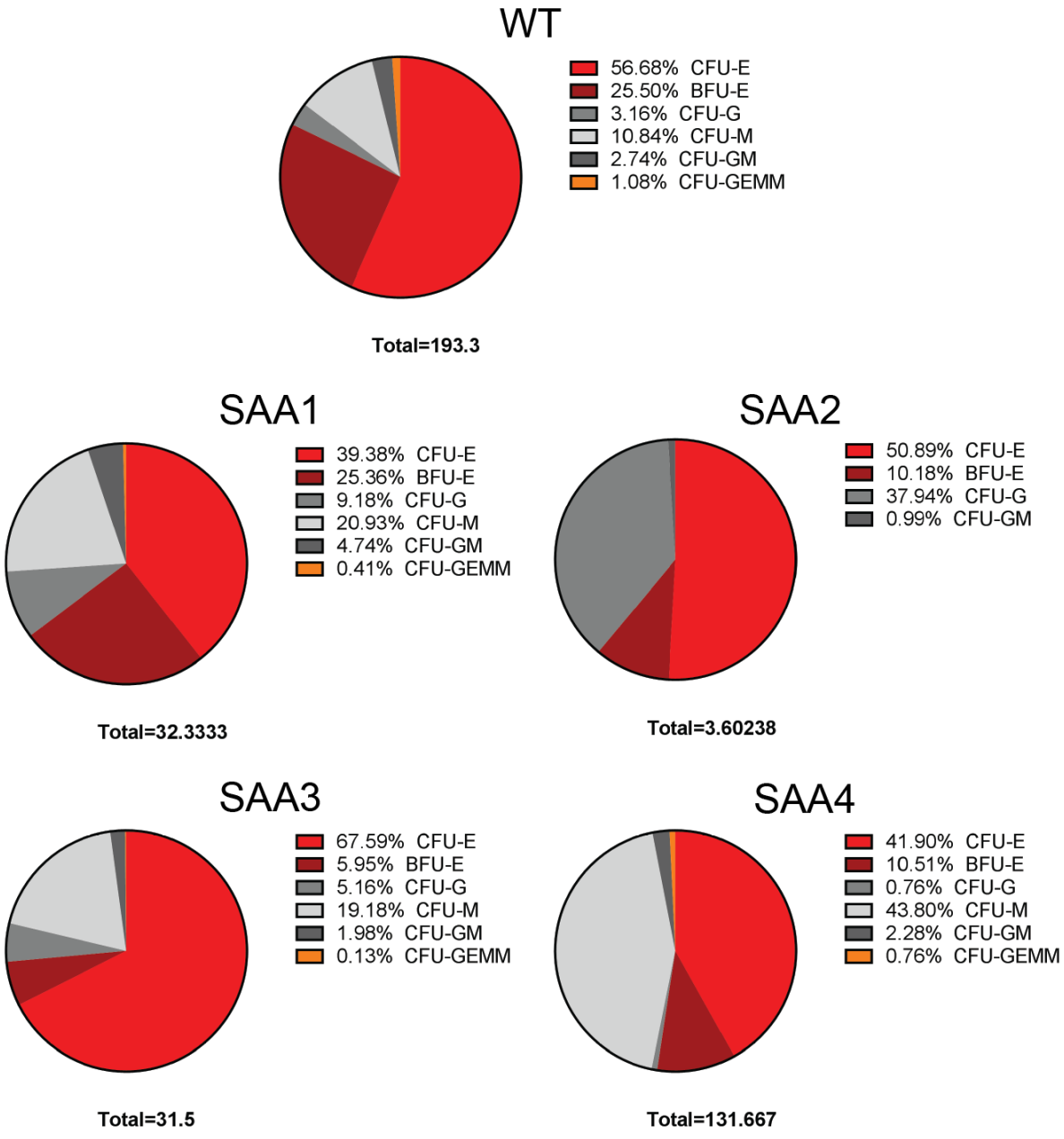

Figure 43. Distribution of haematopoietic colonies types in control (WT) and SAA cell lines represented as proportional percentage.

Data is presented as mean of at least 3 independent experiments. Data for all control cell lines is averaged in one group (WT).

5.2.3 Telomere dynamics in SAA cell lines

To assess the ability of the SAA cells to elongate telomeres during reprogramming, I measured telomere length in parental fibroblasts and iPSC at different passages by qPCR. Control-iPSC and one of the SAA patients (SAA4) showed longer telomeres in iPSC than in parental fibroblasts due to telomere elongation during reprogramming, corroborating previous reports (Marion *et al.*, 2009). Strikingly, no significant increases in telomere length were observed during the reprogramming of three of the SAA cell lines (SAA1, SAA2 and SAA3) (**Figure 44A**). Furthermore, one of the patients (SAA3) showed continued telomere shortening during the reprogramming process (**Figure 44A**), resulting in iPSC with telomeres which were significantly shorter than parental fibroblasts. The control-iPSC lines did not show a significant telomere shortening during the 12 day differentiation time course to haematopoietic lineages compared to iPSC passage 50; however the three SAA-iPSC lines showing defective telomere elongation (SAA1, SAA2 and SAA3) displayed a significant telomere attrition during the differentiation process, resulting in iPSC-derived haematopoietic progenitors with significantly shorter telomeres than corresponding iPSC passage 50 (**Figure 44B**).

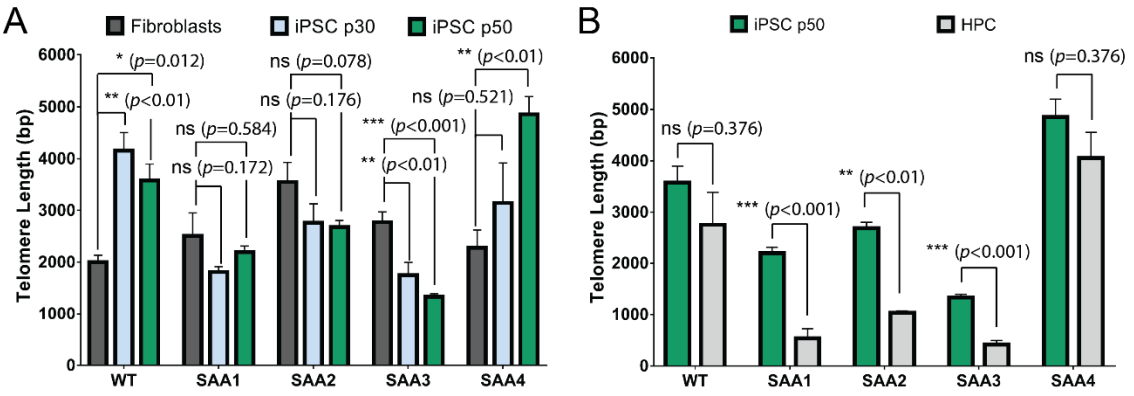

Figure 44. Telomere length analysis of control (WT) and SAA cell lines.

(A) Analysis of telomere length in parental fibroblasts (dark grey bars) and iPSC passage 30 (light blue bars) and 50 (green bars) in grouped control (WT) and SAA cell lines. One-way ANOVA with Tukey's multiple comparison test was used for statistical comparison between fibroblasts and iPSC passage 30 and passage 50; (B) Analysis of telomere length in iPSC passage 50 (green bars) and iPSC-derived haematopoietic progenitor cells (HPC) differentiated from iPSC passage 50 (light grey bars) in grouped control (WT) and SAA cell lines. Multiple t-test using Holm-Sidak method was used for statistical comparison between iPSC passage 50 and HPC; A-B: data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

We assessed up-regulation of telomerase activity in iPSC and iPSC-derived-haematopoietic progenitors by TRAP assay. Telomerase activity was significantly increased in iPSC when compared to parental fibroblasts indicating up-regulation of telomerase activity during reprogramming of both control and SAA-fibroblasts as reported by other studies (Takahashi *et al.*, 2007) (**Figure 45A**). Analysis of telomerase activity in iPSC-derived-haematopoietic progenitors revealed that only one SAA patient (SAA2) displayed a significantly reduced telomerase activity compared to iPSC passage 50 (**Figure 45B**). Together, these data indicate an impaired telomere elongation during the reprogramming of SAA-fibroblasts which was independent of telomerase activity assessed by *in vitro* TRAP assay. Furthermore, three SAA-iPSC lines (SAA1, SAA2 and SAA3) suffered significant telomere attrition during differentiation resulting in haematopoietic progenitors with shorter telomeres than controls corroborating data obtained with patient specific peripheral blood and bone marrow nucleated cells (Brummendorf *et al.*, 2001; Lee *et al.*, 2001; Sakaguchi *et al.*, 2014; Park *et al.*, 2016).

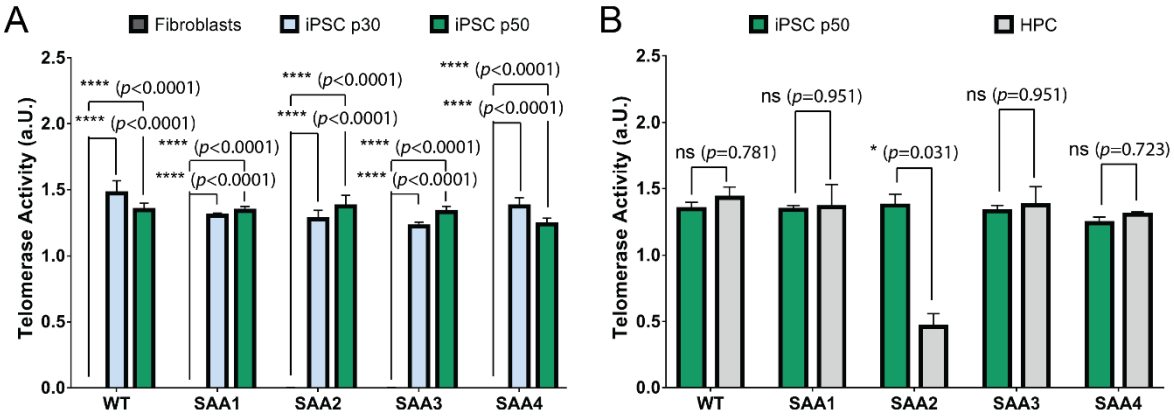

Figure 45. : Telomere activity analysis of control (WT) and SAA cell lines.

(A) Analysis of telomere activity in parental fibroblasts (dark grey bars) and iPSC passage 30 (light blue bars) and 50 (green bars) in grouped control (WT) and SAA cell lines. One-way ANOVA with Tukey's multiple comparison test was used for statistical comparison between fibroblasts and iPSC passage 30 and passage 50; (B) Analysis of telomere activity in iPSC passage 50 (green bars) and iPSC-based haematopoietic progenitor cells (HPC) differentiated from iPSC passage 50 (light grey bars) in grouped control (WT) and SAA cell lines. Multiple t-test using Holm-Sidak method was used for statistical comparison between iPSC passage 50 and HPC; A-B: data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

5.3 Discussion

Reduced number of haematopoietic progenitor cells in the bone marrow is the common clinical presentation in SAA patients. This reduced number of bone marrow progenitor cells has been traditionally associated to an immune-mediated disorder due to the fact that 60-70% of SAA patients show response to IST (Young *et al.*, 2006). However, there is an increasing recognition that a significant number of young adults may have instead a constitutional form of BMFS which affects the haematopoietic stem and/or progenitor compartments. In 2003, a study reported that five percent of SAA patients cases were undiagnosed cases of constitutional BMFS without the apparent abnormal phenotype commonly associated to these disorders (Fogarty *et al.*, 2003). Misdiagnosis of SAA in patients actually presenting constitutional defects in haematopoietic progenitors may have severe consequences in terms of both diagnosis and treatment. Firstly, unnecessary exposure of patients to the toxicity associated to IST and inadequacy of the use of these treatments in patients that might not respond due to the non-immune-associated nature of the disorder are elements that would benefit from the identification of potential genetic defects in SAA patients. Secondly, identification of potential genetic defects in SAA patients could also help to identify affected 'silent' siblings that should not be considered as donors in case of HSCT. Lastly, in the longer term, patients with inherited BMFS are predisposed to develop to haematopoietic malignancies (Zeng and Katsanis, 2015), necessitating more rigorous monitoring for later development and long term follow up of second malignancies. Thus, identification of constitutional forms of BMFS in SAA cases would have profound clinical implications in SAA patients that would benefit from more accurate diagnosis and tailored therapies.

SAA-iPSC lines generated in this study, as described in **Chapter 3**, were successfully differentiated into haematopoietic progenitors. In the process of haematopoietic differentiation, SAA-iPSC lines successfully generated KDR+ mesodermal precursors at levels similar to those in control cell lines indicating no impaired capacity to differentiate into mesodermal lineages. These results were expected since SAA patients do not show defects in cells from mesodermal lineage, other than haematopoietic cells, such as endothelial, vascular, bone, cardiac muscle and skeletal muscle cells. Mesodermal progenitors generated from SAA cell lines progressed into

differentiation and generated CD34+/CD43+ haemato-endothelial progenitors. One of the SAA cell lines (SAA1) generated a significantly reduced number of haemato-endothelial progenitors compared to control cells lines that could be interpreted as an impaired capacity to generate haemato-endothelial progenitors. Flow cytometric analysis of CD43+ haematopoietic progenitors on day 12 revealed that SAA cell lines did not show a significantly reduced percentage of CD43+ haematopoietic progenitors providing evidence of the potential of the SAA-iPSC lines to generate haematopoietic progenitors. Thus, generation of CD43+ by SAA1 in similar levels to those in control cell lines on day 12 suggest that the formation of CD34+/CD43+ haemato-endothelial progenitors occurred later than day 6 and the reduced percentages of these progenitors observed on day 6 in SAA1 might be explained by a delay in the generation of haemato-endothelial progenitors. However, SAA1 was the only patient cell line showing significantly reduced numbers of CD43+CD41a-CD235a+ EryP. These results could indicate a reduced capacity of SAA1 to generate progenitors from erythroid lineages. However, it is noteworthy that generation of EryP is highly influenced by genetic background variability as shown in **Chapter 4**. Thus, it would be important not to rule out the idea that the significant differences observed between control and SAA1 cell lines could be attributable to genetic background variation. A definite answer to this question would require an increased number of control cell lines to include a higher genetic background variation in the control group when comparing generation of EryP.

Strikingly, SAA-iPSC-derived haematopoietic progenitors of three of the patient cell lines (SAA1, SAA2 and SAA3) exhibited a significantly reduced ability to generate haematopoietic colonies, with CFUs from both erythroid and myeloid lineages affected equally, accurately recapitulating the phenotype that defines SAA. One of the remaining SAA patient cell line (SAA4) did not exhibit a significantly reduced colony-forming capacity compared to control cell lines suggesting normal functional ability. The presence of reduced number and colony-forming capacity of bone marrow haematopoietic progenitors in SAA patients has been reported by different studies (Marsh *et al.*, 1990; Maciejewski *et al.*, 1994; Scopes *et al.*, 1994; Maciejewski *et al.*, 1996; Rizzo *et al.*, 2002; Rizzo *et al.*, 2004). These quantitative and qualitative defects observed in bone marrow haematopoietic progenitors from SAA patients have been traditionally considered to be secondary to immune-mediated stem cell destruction

(Young *et al.*, 2006). The *in vitro* SAA-iPSC model used in this study provides evidence of an impaired differentiation capacity in SAA-iPSC-derived haematopoietic progenitors in the absence of the immune system in three of the patient cell lines (SAA1, SAA2 and SAA3), which is suggestive of a constitutional progenitor cell dysfunction in these patient cell lines. Thus, use of this SAA-iPSC model would potentially offer the opportunity of identifying SAA patients showing an underlying haematopoietic progenitor cell dysfunction away from the influence of immune system that could be treated accordingly. Likewise, by recapitulating the SAA disease phenotype, the SAA-iPSC model presented in this study could be used to identify pathogenic mechanisms associated to stem cell dysfunction in SAA disease modelling studies.

Functional reconstruction of telomeres and upregulation of telomerase activity during reprogramming represent a hallmark of induction to pluripotency (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007; Marion *et al.*, 2009). iPSC-based modelling of telomeropathies associated with BMFS caused by mutations in telomerase-associated genes such as *TERC, TERT* and *DKC* have shown defective telomere elongation in iPSC due to reduced telomerase function which impacts on the maintenance of the pluripotent phenotype and haematopoietic differentiation capacity (Batista *et al.*, 2011; Winkler *et al.*, 2013; Gu *et al.*, 2015). In AA, only 10% of patients with short telomeres display known mutations in telomere pathway components suggesting that mutations in uncharacterised genes might have a role in the disease phenotype observed in these patients (Young *et al.*, 2006). For this reason, we set out to investigate telomere dynamics in the SAA-iPSC lines and haematopoietic progenitors derived therefrom. Variability in telomere length among iPSC lines due to passage number and parent cell type has been previously described (Rohani *et al.*, 2014). Likewise, it has been reported that telomeres in iPSC are elongated gradually with increasing passages and stabilize after prolonged passaging at passage 15-30 (Marion *et al.*, 2009; Liu, 2017). Both control and SAA-iPSC lines were generated from fibroblast cells, minimizing the variability introduced by parental cell type. Additionally, in order to reduce the variability in telomere length due to passage number and ensure that telomere elongation was completed by the time of measurement, analysis of telomere dynamics in control and SAA-iPSC lines was done in iPSC passage 30 and 50. Our results revealed that three of the SAA-iPSC lines (SAA1, SAA2 and SAA3) failed to elongate telomeres during the reprogramming process despite up-regulation

of telomerase activity, pointing to an impaired telomere elongation during the reprogramming process that cannot be attributed to an absent/reduced telomerase activity. *In vivo* HSC exhibit detectable levels of telomerase activity although this activity is down-regulated during the process of *in vivo* generation of haematopoietic cells leading to shortening of telomere as the cells differentiate (Zimmermann and Martens, 2008; Hills and Lansdorp, 2009). Telomeres in control and SAA4 cell lines were not significantly shorter compared to the corresponding iPSC passage 50 used for differentiation. In contrast, iPSC-derived haematopoietic progenitors in the telomere elongation deficient SAA cell lines (SAA1, SAA2 and SAA3) showed excessive telomere shortening during haematopoietic differentiation. Thus, I hypothesized that the presence of short telomeres due to accelerated telomere attrition may be responsible for the impaired haematopoietic differentiation capacity we observed in SAA1, SAA2 and SAA3 cell lines as previously described (Winkler *et al.*, 2013). Interestingly, SAA4 cell line showed normal colony-forming capacity and telomere elongation in iPSC with no accelerated telomere attrition upon iPSC haematopoietic differentiation, supporting the hypothesis that impaired haematopoietic differentiation capacity observed in SAA1, SAA2 and SAA3 might be attributed to excessive telomere shortening. Alternatively, the short telomeres can be a consequence of reiterative rounds of divisions carried out by a smaller number of proliferating haematopoietic progenitor cells in SAA patients which was highlighted by our study as a compensatory mechanism to maintain homeostasis (Beier *et al.*, 2012). It is difficult to distinguish between these possibilities without knowing the genetic defect that may underline the pathogenesis of SAA in these patients.

In summary, iPSC-derived haematopoietic progenitors of three of the SAA cell lines (SAA1, SAA2 and SAA3) exhibited a reduced capacity to generate haematopoietic cells despite an equal ability to generate haematopoietic progenitor cells, thus corroborating data obtained from the study of SAA patients. Secondly, SAA cell lines with reduced haematopoietic differentiation potential (SAA1, SAA2 and SAA3) showed a deficient telomere elongation in iPSC during reprogramming and accelerated telomere shortening upon differentiation into haematopoietic progenitors. These results suggest an intrinsic link between telomere shortening and haematopoietic differentiation; however more work at the gene analysis is needed to establish whether

these patients harbour mutations in any of the genes that maintain telomere length and telomerase activity.

Chapter 6. Insights into SAA pathophysiology using molecular studies

6.1 Introduction

iPSC-derived haematopoietic progenitors generated from three SAA cell lines (SAA1, SAA2 and SAA3) exhibited a reduced capacity to generate both erythroid and myeloid cells in CFU assays as described in **Chapter 5**. Likewise, these three SAA cell lines showed defective telomere elongation during reprogramming and, most importantly, accelerated telomere attrition upon haematopoietic differentiation of the iPSC lines. Progressive telomere shortening leads eventually to cell cycle arrest and apoptotic cell death (Harley *et al.*, 1990; Blasco, 2005). Extensive replication associated with haematopoietic differentiation may lead to accumulation of DNA damage, which, if unrepaired, can induce replicative senescence or apoptosis of HSPCs (Zeman and Cimprich, 2014; Moehrle and Geiger, 2016). Impaired DNA damage repair capacity in HSPCs has been associated with inherited BMFS such as FA (Dokal and Vulliamy, 2010).

In this chapter I set out to investigate whether the observed impaired haematopoietic differentiation potential of SAA-iPSC derived haematopoietic progenitors of the three affected SAA cell lines could be associated to a haematopoietic progenitor cell dysfunction such as abnormal proliferation, apoptosis rate and DNA repair capacity. To this end, SAA-iPSC-derived haematopoietic progenitors were cultured under replicative-stress conditions in order to synchronize cell cycle for subsequent proliferation analysis. Ability to repair DNA damage and apoptosis rate in normal and replicative-stress conditions in SAA-iPSC-derived haematopoietic progenitor cells were determined using flow cytometric analysis and CFU assays.

As discussed in **Chapter 1** (**section 1.2.4.1**), one of the main advantages that iPSC technology offers is the possibility of testing the efficacy of drugs and provide insights into the drug mechanism of action in patient specific iPSC-derived cells. Several studies have demonstrated the use of iPSC technology as disease model to identify drugs that can rescue the disease phenotype in patient-derived iPSC cells (Huang *et al.*, 2011; Cooper *et al.*, 2012). Recently, it has been reported that EP, a TPO receptor

agonist, stimulates bi- and tri-lineage haematopoiesis by increasing platelet and neutrophil counts and haemoglobin levels with overall response in 40% of SAA patients at 3-4 months (Desmond *et al.*, 2014) (**Chapter 1, section 1.1.4.2**). Thus, in view of these findings, I investigated whether the observed reduced colony-forming potential and proliferation of the affected SAA-iPSC-derived haematopoietic progenitors could be rescued by the addition of EP to the differentiation media.

The aim of this 6th chapter is to investigate the following:

- The proliferation of SAA-iPSC-derived haematopoietic progenitors
- The ability of SAA-iPSC-derived haematopoietic progenitors to repair DNA damage under normal and replicative-stress conditions
- The apoptosis of SAA-iPSC haematopoietic progenitors under normal and replicative-stress conditions
- The effect of EP in colony-forming potential, proliferation and DNA repair capacity of SAA-iPSC-derived haematopoietic progenitors under normal and replicative-stress conditions

6.2 Results

6.2.1 Proliferative capacity of SAA-iPSC-derived haematopoietic progenitors

To analyse proliferation capacity, I synchronized the iPSC-derived haematopoietic progenitors from control cell lines in G1/S phase by treatment with ribonucleotide reductase inhibitor hydroxyurea (HU) for 24 hours (**Figure 46A**). This was followed by culture in HU-free media and pulsing with 5-bromo-2-deoxyuridine (BrdU) for 1hour. Flow cytometric analysis of DNA content and incorporation of BrdU in control-iPSC-derived-haematopoietic progenitors indicated a high percentage of cells arrested in G1/S phase at 1 and 3 hours post-release from HU due to depletion of deoxyribonucleotide pools (**Figure 46B**). These arrested cells re-entered the cell cycle, progressed through S-phase and showed a similar cell cycle profile to that of untreated cells at 24 hours post treatment (**Figure 46B-C**), indicating that iPSC-derived haematopoietic progenitors require at least 24 hours to restore normal cell-cycle profile.

Next, I analysed the proliferation rate of the synchronized control- and SAA-iPSC-derived haematopoietic progenitors (marked by CD43 expression) by comparing the percentage of BrdU-incorporating cells (S-phase) at 24 hours post-release from HU. Interestingly, SAA-iPSC-derived-haematopoietic progenitors from the three affected SAA cell lines (SAA1, SAA2 and SAA3) showed a significant reduction in the number of BrdU-incorporating cells (proliferative cells) compared with control counterparts, indicating a reduced proliferative capacity (**Figure 46D**). IPSC-derived haematopoietic progenitors from SAA4 showed no significant differences in the percentages of proliferative cells compared to control cell lines, indicating normal proliferative capacity (**Figure 46D**).

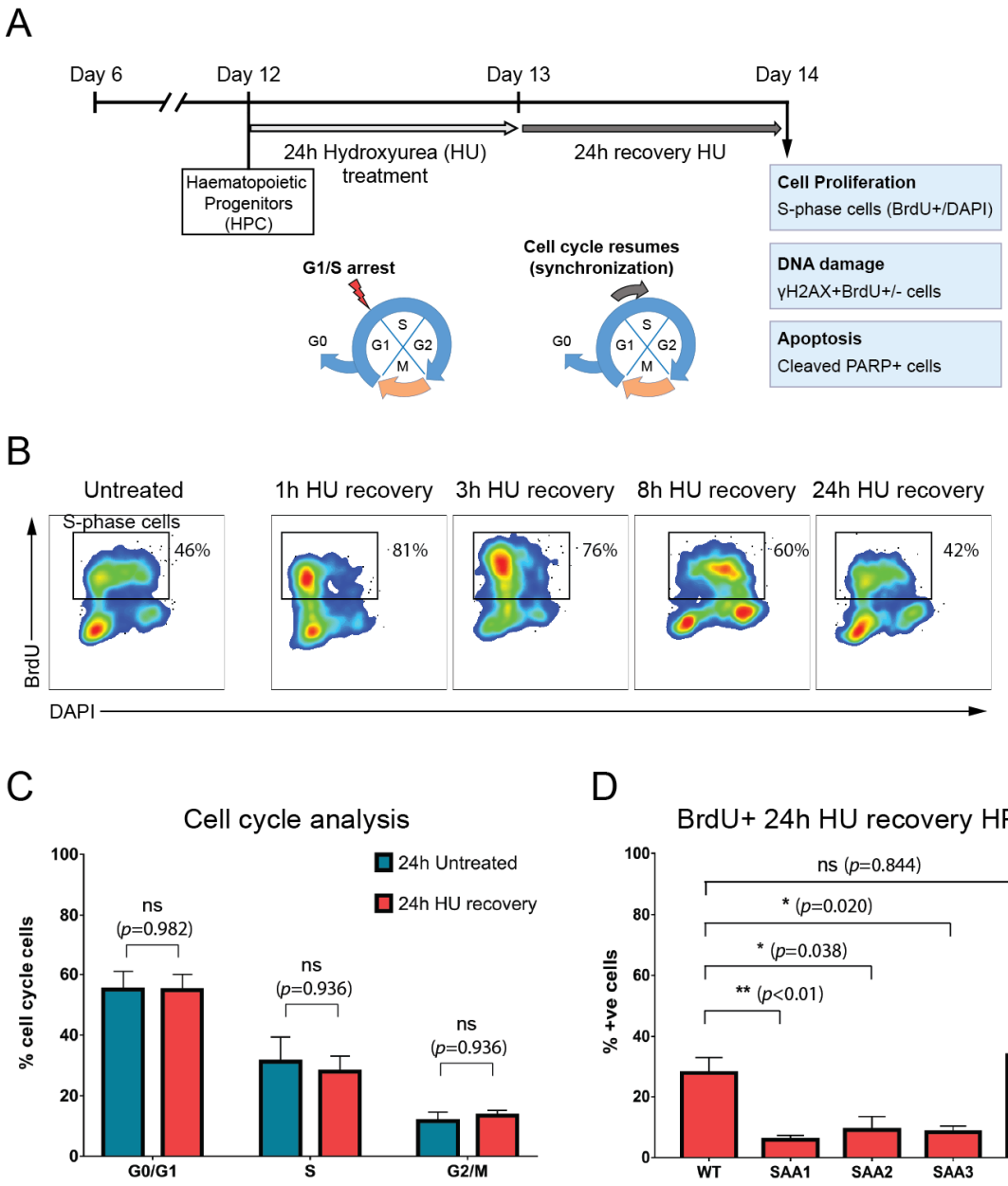

Figure 46. Analysis of proliferative capacity of control and SAA-iPSC-derived haematopoietic progenitors.

(A) Schematic of the experimental design used to analyse the proliferation, DNA repair capacity and apoptosis in iPSC-derived-haematopoietic progenitor cells (HPC); (B) Flow cytometric analysis of BrdU and DAPI incorporation in untreated, 1 hour HU recovery, 3 hour HU recovery, 8 hour HU recovery and 24 hour HU recovery control-iPSC-derived haematopoietic progenitor cells; (C) Analysis of cell cyle in untreated (dark blue bars) and 24 hour HU recovery (red bars) control-iPSC-derived haematopoietic progenitor cells. Multiple t-test using Holm-Sidak method was used for statistical comparison between untreated and 24 hour HU recovery WT cells; (D) Analysis of BrdU-incorporating cells in control (WT) and SAA-iPSC-derived-haematopoietic progenitors. One-way ANOVA with Dunnett's multiple comparison test was used for statistical comparison between WT and SAA cell lines. B-D: data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

6.2.2 Ability to repair DNA damage associated to replicative stress in SAA-iPSC-derived haematopoietic progenitors

Induction of replicative stress by depletion of deoxyribonucleotide pools with long exposure to HU leads to stalled replication forks and accumulation of DNA damage (Petermann *et al.*, 2010). To address whether the reduced haematopoietic potential of SAA-iPSC-derived-haematopoietic progenitors might be attributed to an impaired ability to repair DNA damage associated with replicative stress, I analysed the percentage of DNA damage induced by HU treatment by flow cytometric analysis in proliferating (BrdU+) and non-proliferating cells (BrdU-). First, to assess the reversibility of the DNA damage induced by HU exposure, I measured the formation of the phosphorylated histone variant H2AX at serine 139 (γH2AX) at 1, 3, 8 and 24 hours after HU release in control-iPSC-derived-haematopoietic progenitors. This analysis indicated accumulation of γH2AX+ in BrdU+ cells at 1 and 3 hours after HU release indicating accumulation of DNA damage in replication forks after HU replication arrest (**Figure 47A**). The γH2AX foci progressively disappeared showing similar levels of γH2AX levels to those of untreated cells at 24 hours (**Figure 47A**). Thus, analysis of accumulation of DNA damage in proliferating (γH2AX+BrdU+) and non-proliferating (γH2AX+BrdU-) iPSC-derived-haematopoietic progenitors showed that control cell lines were able to repair the induced DNA damage after 24 hours release from HU (**Figure 47B-C**).

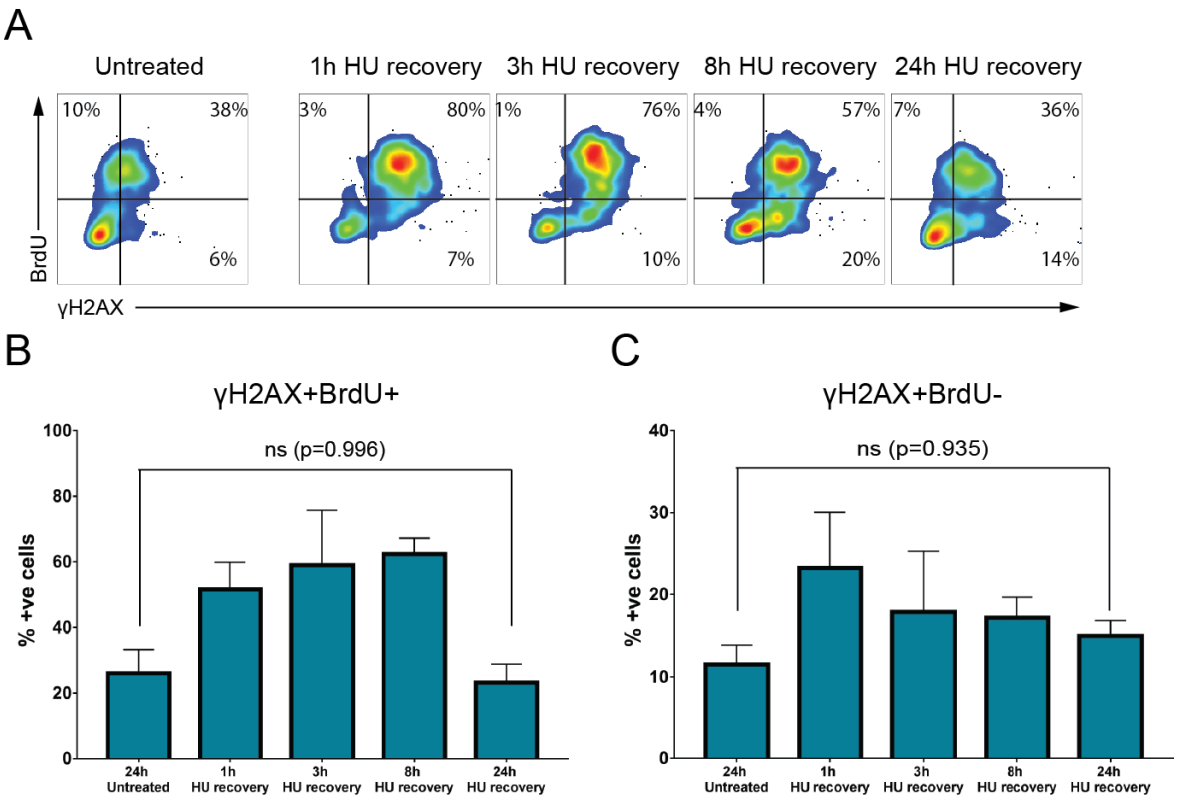

Figure 47. DNA damage induced by replicative stress (HU) in control-iPSC-derived haematopoietic progenitors.

(A) Flow cytometric analysis of BrdU incorporation and γH2AX detection in untreated, 1 hour, 3 hours, 8 hours and 24 hours post-HU recovery; (B) Analysis of γH2AX in BrdU+ untreated, 1 hour, 3 hours, 8 hours and 24 hours post-HU recovery. Student's *t*-test was used for statistical comparison between 24h untreated and 24 HU recovery groups; (C) Analysis of γH2AX in BrdU- untreated, 1 hour, 3 hours, 8 hours and 24 hours post-HU recovery. Student's *t*-test was used for statistical comparison between 24h untreated and 24 post-HU recovery groups. A-C: data is presented as mean of at least 3 independent experiments +/- S.E.M.

To investigate the ability of SAA cell lines to repair DNA damage associated to replicative stress, I then analysed the accumulation of γH2AX in proliferating (BrdU+) and non-proliferating (BrdU-) in SAA-iPSC-derived-haematopoietic progenitors at 1 and 24 hours after HU release. No significant increase in the accumulation of γH2AX+BrdU+ was observed at 1 hour post HU release in SAA-iPSC-derived haematopoietic progenitors (**Figure 48A**), unlike the control counterparts which showed a significant accumulation of DNA damage at 1 hour post HU release. Of note, low levels of DNA damage observed in BrdU+ 1h after replicative stress in SAA1, SAA2 and SAA3 iPSC-derived haematopoietic progenitors points to a reduced proliferation capacity likely due to a reduced formation of replication forks, in agreement with our

previous results showing a significantly reduced number of BrdU-incorporating cells in SAA1, SAA2 and SAA3 cell lines (**Figure 46D**). Thus, higher levels of γH2AX+BrdU+ cells were observed in control-iPSC-derived haematopoietic progenitors compared to those in SAA cell lines, likely due to a higher number of replication forks, although the differences were not statistically significant (**Figure 48B**).

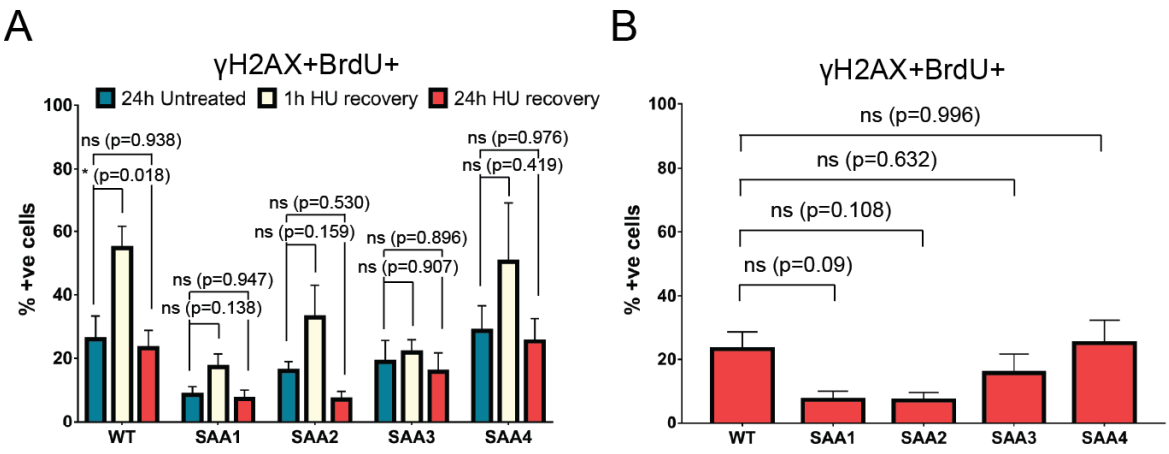

Figure 48. DNA damage induced by replicative stress (HU) in proliferating iPSC-derived haematopoietic progenitors.

(A) Analysis of γH2AX in BrdU+ cells in untreated (dark blue bars), 1 hour after HU recovery (beige bars) and 24 hours after HU recovery (red bars) iPSC-derived-haematopoietic progenitors. One-way ANOVA with Tukey's multiple comparison test was used for statistical comparison between untreated cells and 1 hour after HU recovery and 24 hours after recovery; (B) Analysis of γH2AX in BrdU+ cells in control (WT) and SAA-iPSC-derived-haematopoietic progenitors. One-way ANOVA with Dunnett's multiple comparison test was used for statistical comparison between control (WT) and SAA cell lines. A-B: data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

Interestingly, analysis of DNA damage in non-proliferative (BrdU-) progenitors revealed a significant increase in the level of γH2AX at 24 hours post HU release in two of the patient-derived-haematopoietic progenitors (SAA2 and SAA4) compared to equivalent cells generated from the controls (**Figure 49A**), suggesting an impaired ability to restore normal levels of DNA damage after HU treatment in non-proliferating iPSC-derived haematopoietic progenitors. Notwithstanding this, the percentage of γH2AX+BrdU-cells in the haematopoietic progenitors derived from these two patient iPSC lines was not significantly higher when compared to equivalent cells generated from the unaffected controls (**Figure 49B**). Together these data indicate that proliferating SAA-iPSC-derived-haematopoietic progenitors from the three affected

patient cell lines (SAA1, SAA2 and SAA3) tend to accumulate less DNA damage soon after HU treatment most likely as a result of their reduced proliferation. Furthermore, a subset of SAA-iPSC-derived-haematopoietic progenitors may be slower or have an impaired ability to restore DNA damage in the non-proliferative compartment. However, the overall level of DNA damage induced in response to replicative stress is not significantly different compared to control-derived-haematopoietic progenitors, excluding DNA damage accumulation as a key factor underlying the impaired haematopoietic differentiation of SAA-iPSC lines.

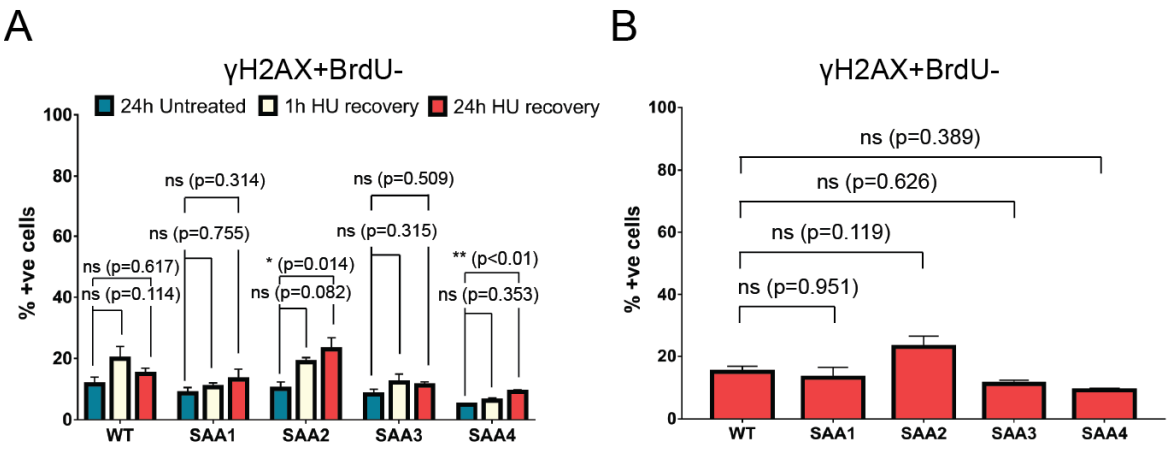

Figure 49. DNA damage induced by replicative stress (HU) in non-proliferating iPSC-derived haematopoietic progenitors.

(A) Analysis of γH2AX in BrdU- cells in untreated (dark blue bars), 1 hour post-HU recovery (beige bars) and 24 hours post-HU recovery (red bars) iPSC-derived-haematopoietic progenitors. One-way ANOVA with Tukey's multiple comparison test was used for statistical comparison between untreated cells and 1 hour post-HU recovery and 24 hours post-recovery; (B) Analysis of γH2AX in BrdU- cells in control (WT) and SAA-iPSC-derived-haematopoietic progenitors. One-way ANOVA with Dunnett's multiple comparison test was used for statistical comparison between control (WT) and SAA cell lines. A-B: data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

6.2.3 Apoptosis rate in SAA-iPSC-derived haematopoietic progenitors under normal and replicative-stress conditions

To investigate whether apoptosis was increased in SAA-iPSC-derived-haematopoietic progenitors, I measured the presence of cleaved Poly (ADP-ribose) polymerase-1 (PARP) under normal and replicative-stress conditions by flow cytometric analysis. Reduced presence of apoptotic cells in both control and SAA cell lines was observed

in the analysis of cleaved-PARP in iPSC-derived haematopoietic progenitor cells cultured under normal conditions (**Figure 50A**). However, a significant increase in the percentage of apoptotic cells was observed in SAA-iPSC-derived haematopoietic progenitors derived from one of the patients (SAA2) when compared to control cells (**Figure 50B**), which suggests an increased apoptosis rate in this patient. However, these results are difficult to interpret due to the extremely low number of apoptotic cells detected.

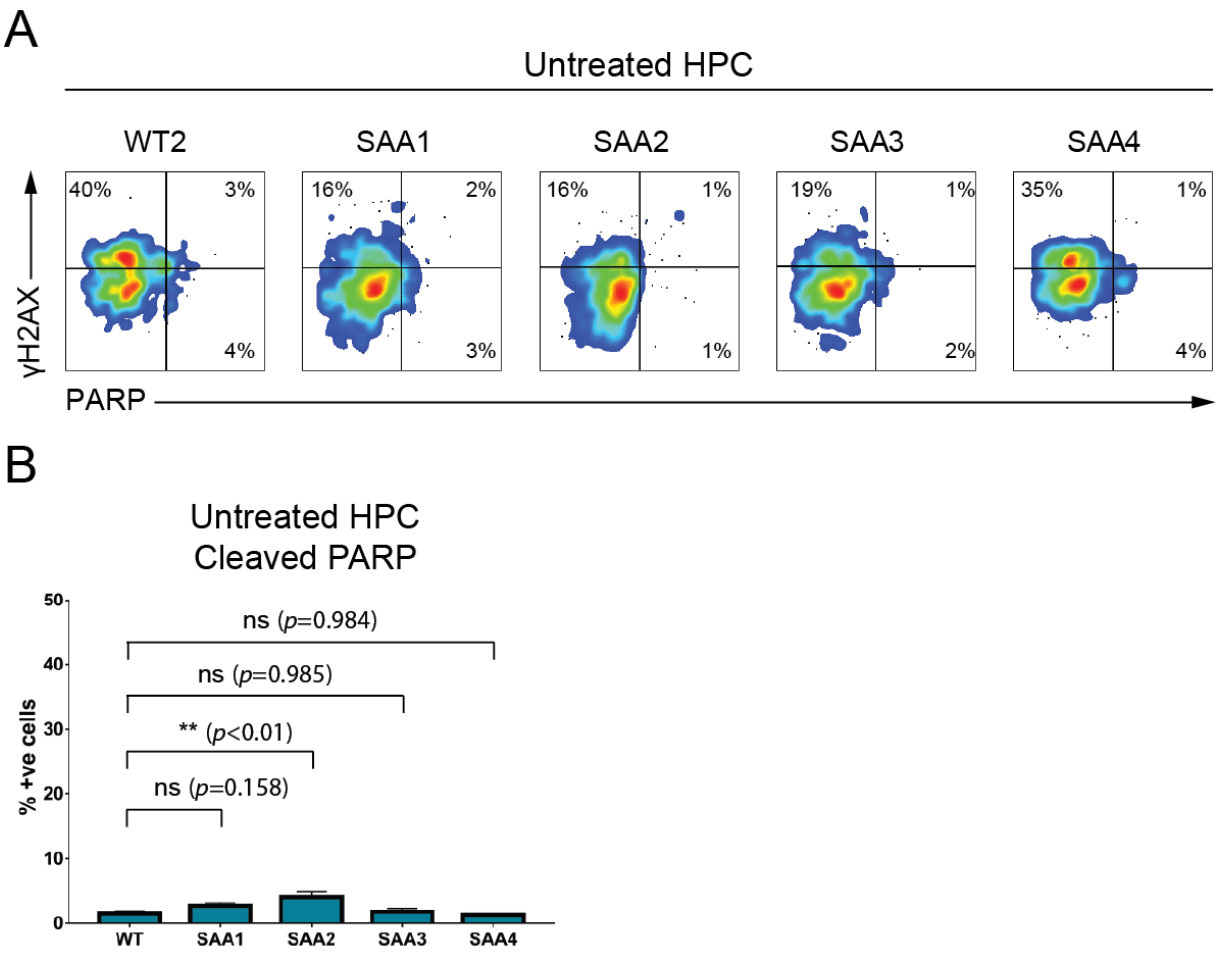

Figure 50. Analysis of apoptotic cells in SAA-iPSC-derived haematopoietic progenitors in normal conditions.

(A) Flow cytometric analysis of γH2AX and cleaved-PARP (PARP) detection in control (WT) and SAA-iPSC-derived haematopoietic progenitor cells (HPC) under normal conditions; (B) Analysis of cleaved PARP in control (WT) and SAA-iPSC-derived haematopoietic progenitor cells in non-replicative-stress conditions. One-way ANOVA with Dunnett's multiple comparison test was used for statistical comparison between control (WT) and SAA cell lines. A-B: data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

Levels of apoptotic cells remained low in haematopoietic progenitor cells from control and SAA cell lines when subjected to replicative-stress conditions (**Figure 51A**). Thus, no significant increase was observed in the apoptosis rate of SAA-iPSC-derived haematopoietic under replicative stress when compared to control cells (**Figure 51B**). In summary, these findings provide no convincing evidence of increased apoptosis in SAA cell lines under normal or after replicative stress excluding this mechanism as potential haematopoietic progenitor cell dysfunction associated to impaired haematopoietic differentiation potential in SAA cell lines.

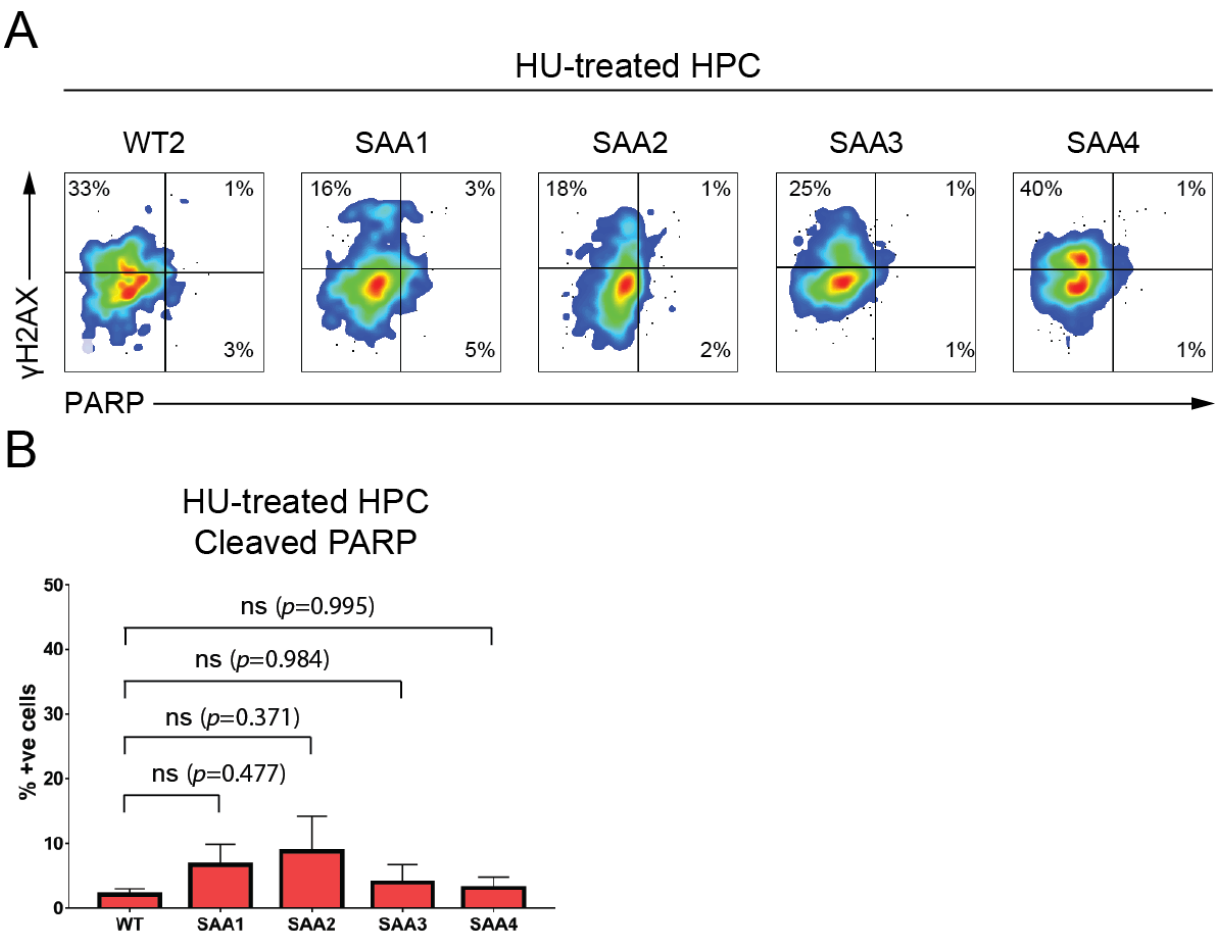

Figure 51. Analysis of apoptotic cells in control and SAA-iPSC-derived haematopoietic progenitors under replicative stress conditions.

(A) Flow cytometric analysis of γH2AX and cleaved-PARP (PARP) detection in control (WT) and SAA-iPSC-derived haematopoietic progenitor cells (HPC) under replicative-stress conditions (HU-treated); (B) Analysis of cleaved PARP in control (WT) and SAA-iPSC-derived haematopoietic progenitor cells under replicative-stress conditions (HU-treated). One-way ANOVA with Dunnett's multiple comparison test was used for statistical comparison between control (WT) and SAA cell lines. A-B: data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

6.2.4 Effect of EP in SAA-iPSC-derived haematopoietic progenitors

To assess the impacts of EP on iPSC-derived haematopoietic progenitors, SAA cell lines showing impaired haematopoietic potential (SAA1, SAA2 and SAA3) were cultured under normal and replicative-stress conditions in the presence and absence of EP from day 6 of differentiation and tested for colony-forming potential by CFU assay at day 14 of differentiation (**Figure 52**).

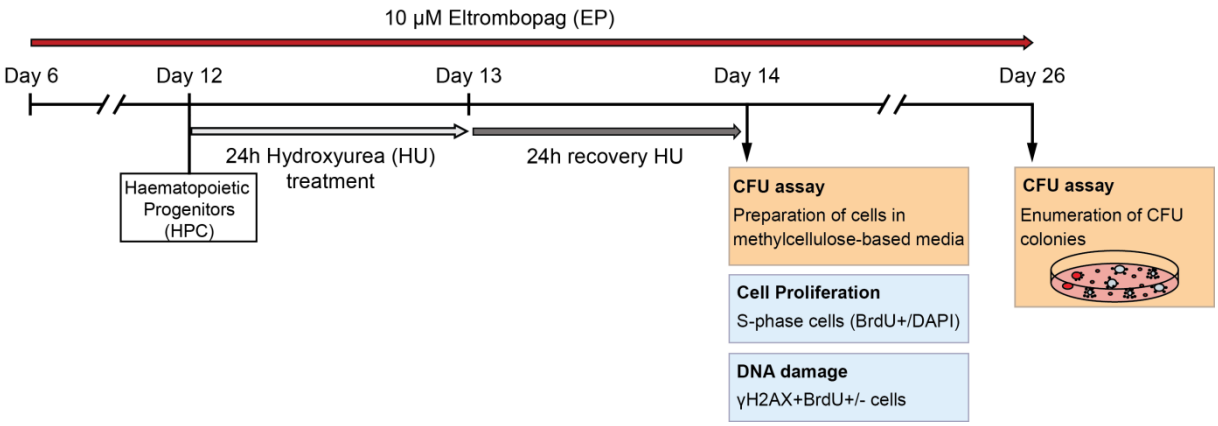

Figure 52. Schematic of the experimental design used to analyse the effect of EP on the colony-forming potential, proliferation and DNA repair capacity in SAA-iPSC-derived haematopoietic progenitor cells.

First, I performed pilot experiments in control-iPSC-derived haematopoietic progenitors to validate the effect of EP. Generation of CD43+ cells with a high presence of erythroid progenitors (CD43+CD41a-CD235a+) was observed in the control-iPSC-derived haematopoietic progenitors cultured in the presence of EP (**Figure 53A**). Thus, addition of EP to TPO-containing differentiation media induced a significant increase in the percentage of erythroid progenitors (CD43+CD235a+CD41a-) at the expense of megakaryocytic progenitors (CD43+CD235a-CD41+) compared to the control group containing only TPO in differentiation media (**Figure 53B**). These results are very similar to what has been reported in CD34+ bone marrow cells (Jeong *et al.*, 2015) and indicate that EP is biologically active in our iPSC differentiation system.

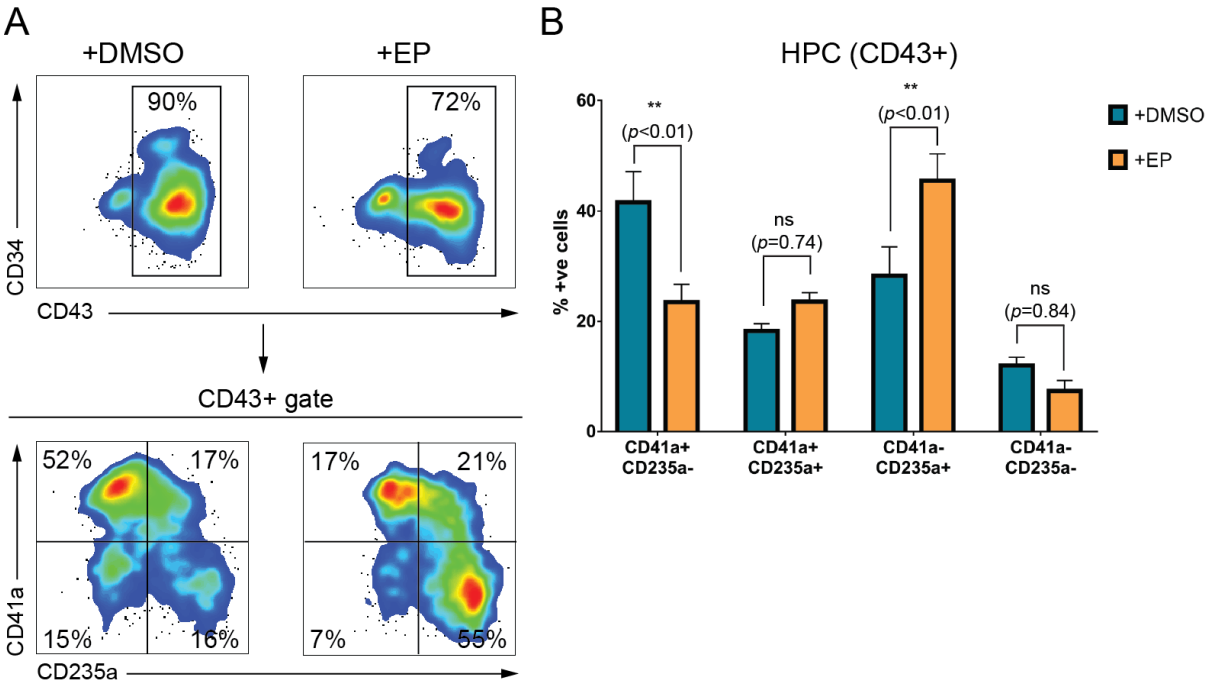

Figure 53. Validation of the effect of EP in control-iPSC-derived haematopoietic progenitors.

(A) Representative images of flow cytometric analysis of CD34 and CD43 expression and CD41 and CD235 expression for gated CD43+ population in control-iPSC-derived haematopoietic progenitor cells; (B) Analysis of the different haematopoietic progenitor cell (HPC) subpopulations, megakaryocitic (CD41a+CD235a-), megakaryocytic/ertythroid (CD41a+CD235a+), erythroid (CD41a-CD235a+) and myeloid (CD41a-CD235a-), in DMSO-treated and EP-treated groups. Multiple t-test using Holm-Sidak method was used for statistical comparison between DMSO-treated and EP-treated. A-B: data is presented as mean of at least 3 independent experiments +/- S.E.M.

CFU assay results did not show significant differences between DMSO- and EP-treated groups in the total number of CFU colonies (**Figure 54A**) or in the number of erythroid lineage (**Figure 54B**) and myeloid lineage colonies (**Figure 54C**) generated from SAA-iPSC-derived-haematopoietic progenitors cultured in absence of HU.

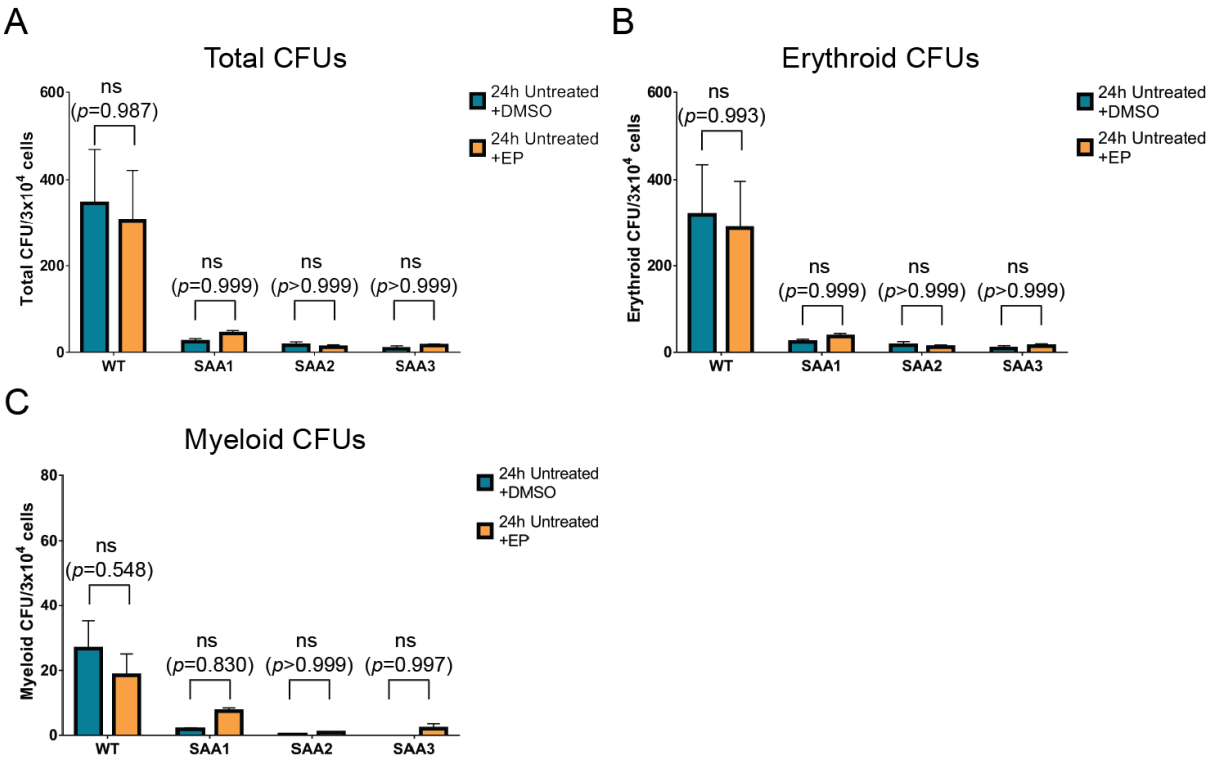

Figure 54. Colony-forming capacity of SAA-iPSC-derived haematopoietic progenitors in the presence and absence of EP in normal conditions.

Analysis of CFUs generated in DMSO (dark blue bars) and EP-treated (orange bars) iPSC-derived-haematopoietic progenitors in control (WT) and SAA cell lines in non-replicative-stress conditions; (A) Total CFUs, (B) erythroid-lineage CFUs, (C) myeloid-lineage CFUs. A-C: Multiple t-test using Holm-Sidak method was used for statistical comparison between DMSO and EP groups. Data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

Similarly, no differences in the total number of CFU colonies (**Figure 55A**) or in the number of erythroid lineage (**Figure 55B**) and myeloid lineage colonies (**Figure 55C**) were observed when EP was added in parallel to replicative stress inducing agent, HU.

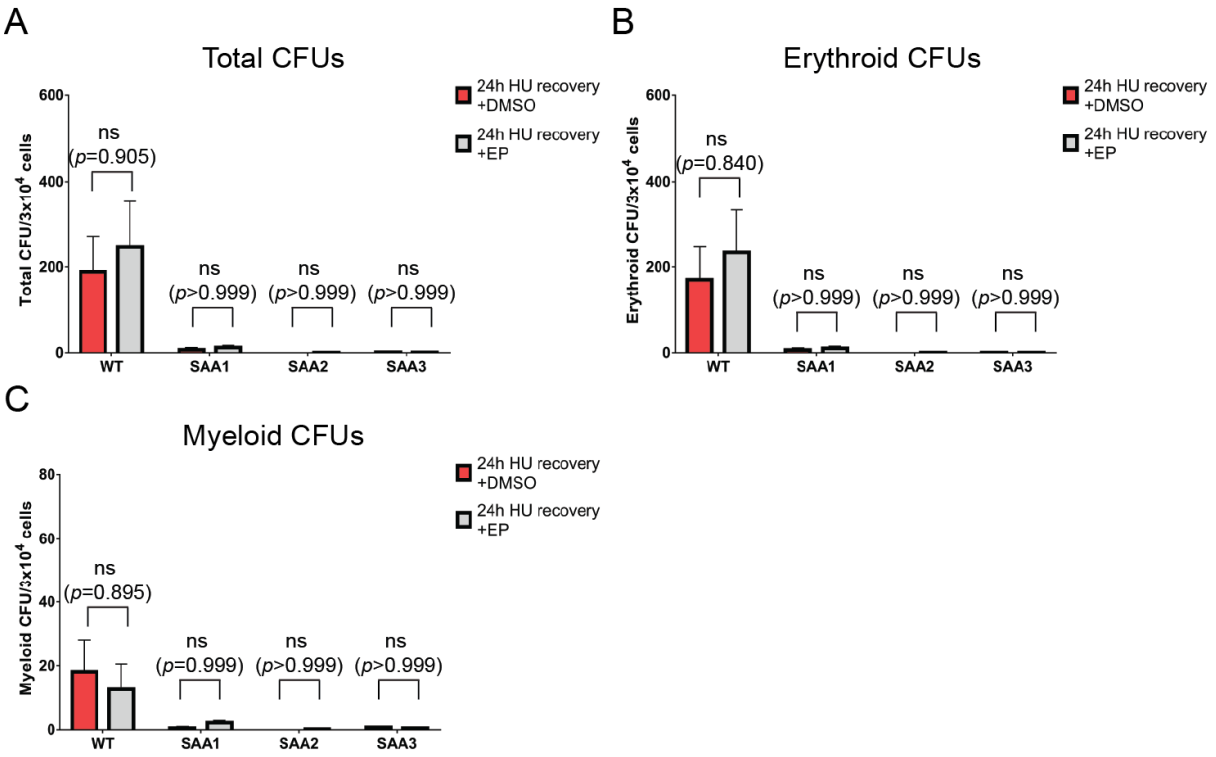

Figure 55. Colony-forming capacity of SAA-iPSC-derived haematopoietic progenitors in the presence and absence of EP in replicative-stress conditions (HU).

Analysis of CFUs generated in DMSO (red bars) and EP-treated (grey bars) iPSC-derived-haematopoietic progenitors in control (WT) and SAA cell lines in replicative-stress conditions; (A) Total CFUs, (B) erythroid-lineage CFUs, (C) myeloid-lineage CFUs. A-C: Multiple t-test using Holm-Sidak method was used for statistical comparison between DMSO and EP groups. Data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

EP favours the proliferation and DNA double-strand break (DSB) repair after γ-irradiation in human HSPCs (Sun *et al.*, 2012; Cheruku PS, 2015). To investigate whether EP is affecting the proliferative and DNA damage repair capacity of the SAA-iPSC-derived-haematopoietic progenitors under conditions of replicative stress induced by HU, flow cytometric analysis for BrdU incorporation and accumulation of γH2AX was carried out after EP treatment. No significant differences were observed in the percentage of BrdU+ cells, indicating that EP does not affect the proliferation of control or SAA-iPSC-derived haematopoietic progenitors (**Figure 56A**). Similarly, no significant changes were observed in the percentage of proliferating and non-proliferating control-and SAA-iPSC-derived-haematopoietic progenitors with γH2AX foci (**Figure 56B-C**). Together these data indicate that EP does not affect the proliferative capacity or DNA repair ability of SAA-iPSC-derived-haematopoietic progenitor cells. In summary, these findings demonstrate that SAA-iPSC-derived haematopoietic progenitors generated in this study do not respond to EP treatment suggesting that the potential underlying haematopoietic progenitor defect observed in the SAA patients analysed herein might not be associated with TPO signalling or other effects of EP independent of TPO receptor stimulation.

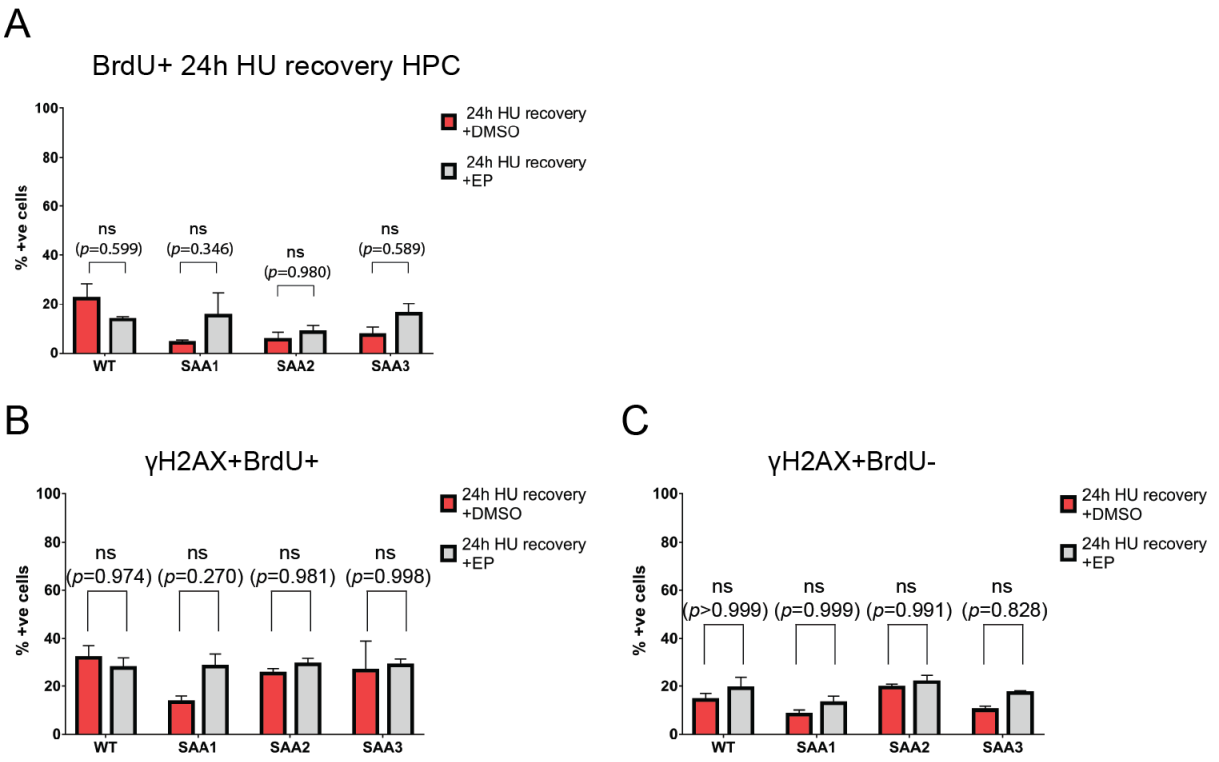

Figure 56. Analysis of proliferation and DNA repair capacity of control and SAA-iPSC-derived haematopoietic progenitors in the presence and absence of EP under replicative-stress conditions.

(A) Analysis of BrdU-incorporating cells in DMSO (red bars) and EP-treated (grey bars) iPSC-derived-haematopoietic progenitors in control (WT) and SAA cell lines; (B) Analysis of γH2AX in BrdU+ cells in DMSO (red bars) and EP-treated (grey bars) iPSC-derived-haematopoietic progenitors in control (WT) and SAA cell lines; (C) Analysis of γH2AX in BrdU- cells in DMSO (red bars) and EP-treated (grey bars) in iPSC-derived-haematopoietic progenitors in control (WT) and SAA cell lines. A-C: Multiple t-test using Holm-Sidak method was used for statistical comparison between DMSO and EP groups. Data is presented as mean of at least 3 independent experiments +/- S.E.M. Data for all control cell lines is averaged in one group (WT).

6.3 Discussion

Qualitative and quantitative defects such as reduced colony-forming capacity (Marsh *et al.*, 1990; Rizzo *et al.*, 2002; Rizzo *et al.*, 2004) and presence of very low numbers of haematopoietic progenitors (Maciejewski *et al.*, 1994; Scopes *et al.*, 1994; Maciejewski *et al.*, 1996) as measured by colony assays have been described in SAA patients. Presence of low numbers of progenitors in SAA patients and potential influence of the patient's immune system makes it difficult to investigate whether these defects are associated to a dysfunctional immune response, as described in many SAA patients, or to presence of an underlying haematopoietic progenitor cell defect. iPSC approach offers two main advantages: 1) SAA patient-specific iPSC-derived haematopoietic progenitors can be generated at ease enabling the use of these progenitors for disease modelling studies and 2) enables the study of SAA haematopoietic progenitors in an *in vitro* setting, therefore in the absence of patient's immune system influence, consequently removing one of the variables potentially associated to SAA pathophysiology.

Previous studies have shown the potential use of iPSC approach in disease modelling to provide insights into pathological mechanisms of BMFS (**Chapter 1, section 1.3.4.2**). One of the most recent examples of the use of iPSC technology in DC disease modelling was reported by Gu *et al.* showing that dysregulation of WNT signalling in DC-iPSC may contribute to the DC pathogenesis (Gu *et al.*, 2015). Thus, in view of the impaired haematopoietic differentiation capacity of the SAA-iPSC-derived haematopoietic progenitors and defective telomere maintenance observed in three of the SAA cell lines in **Chapter 5**, I decided to use the SAA-iPSC model to investigate potential mechanisms in order to provide evidence of an underlying haematopoietic progenitor cell dysfunction.

Defective telomere maintenance can lead to presence of short telomeres resulting in reduced numbers of haematopoietic cells in DC patients (Calado and Young, 2009). It has been previously described that the presence of critically short telomeres can result in the activation of DNA damage checkpoint pathway leading to senescence and blocking entry of haematopoietic progenitors into the cell cycle (Allsopp *et al.*, 2003; d'Adda di Fagagna *et al.*, 2003; Zimmermann and Martens, 2008). For this reason, I decided to investigate the proliferation rate of SAA-iPSC-derived-haematopoietic

progenitors. Our results revealed a significantly reduced proliferative capacity in the iPSC-derived haematopoietic progenitors of the three SAA cell lines displaying excessive telomere shortening (SAA1, SAA2 and SAA3). This reduced proliferation might be secondary to the presence of short telomeres as reported previously (Allsopp *et al.*, 2003; Raval *et al.*, 2015). Interestingly, SAA4-iPSC-derived haematopoietic progenitors showed levels of proliferative cells similar to those in control cell lines. As shown in **Chapter 5**, SAA4 displayed normal haematopoietic colony-forming potential and no excessive telomere shortening in iPSC-derived haematopoietic progenitors. These results support the idea that reduced proliferation observed in SAA1, SAA2 and SAA3-iPSC-derived haematopoietic progenitors is associated to the presence of short telomeres in the progenitors of these cell lines. Whether this reduced proliferation is specific of the iPSC-derived haematopoietic progenitors is worth investigating. Analysis of the proliferation capacity of the affected SAA-iPSC lines would provide an answer to this question and would support the hypothesis that the reduced proliferation observed in the affected SAA-iPSC-derived haematopoietic progenitors is secondary to the presence of short telomeres. Replicative senescence induced by telomere shortening leads to an increased activity and expression of the lysosomal hydrolase β-galactosidase (Bernadotte *et al.*, 2016). Thus, it would be interesting to determine β-galactosidase activity in iPSC-derived haematopoietic progenitor cells to provide more evidence of the link between reduced proliferation and telomere shortening observed in the affected SAA cell lines. It has been described that the presence of short telomeres activates DNA damage responses leading to up-regulation of p21 and blocking cell cycle in G1 (Herbig *et al.*, 2004; Deng *et al.*, 2008). Hence, it is worth analysing the expression levels of p21 and G1 arrest of the affected SAA-iPSC-derived haematopoietic progenitors in order to provide more evidence of a potential telomere dysfunction in these SAA cell lines. Likewise, short telomeres are detected by the cell as double-stranded DNA breaks activating the recruitment of γH2AX in dysfunctional telomeres, the so-called telomere-induced foci (TIF), and DNA-damage checkpoint factors (d'Adda di Fagagna *et al.*, 2003; Takai *et al.*, 2003). In this setting, analysis of co-localization of γH2AX and telomeres would be useful to indicate an increased telomeric damage pointing to dysfunctional telomeres that could potentially explain the reduced proliferation and consequent impaired haematopoietic differentiation in the affected SAA cell lines. Another future line of investigation that remains interesting would be to analyse the expression of cell cycle regulators as described in **Chapter 1**

(**section 1.1.5.2**). Zeng *et al.* showed that proliferation and cell cycle control genes such as *CDK6*, which is mainly involved in G1/S transition (Malumbres and Barbacid, 2001), were down-regulated in haematopoietic progenitors from SAA patients (Zeng *et al.*, 2004). Of note, up-regulation of p21 expression in cells with short telomeres leads to the down-regulation of CDK cell cycle stimulator proteins (Harper *et al.*, 1993; Harper *et al.*, 1995), so it is likely that potential *CDK6* down-regulation observed in SAA haematopoietic progenitors might be secondary to presence of dysfunctional telomeres.

Next, to investigate the DNA damage associated to increased proliferation during haematopoietic differentiation, SAA-iPSC-derived haematopoietic progenitors were subjected to replicative-stress conditions by deoxynucleotide depletion with HU leading to arrested replication forks. HU is mainly active in the S-phase of the cell cycle, affecting mainly proliferative cells, and prolonged exposure at lower doses can lead to collapsed forks generating DNA strand breaks and oxidative stress (Singh and Xu, 2016). Thus, analysis of accumulation of DNA damage in stalled replication forks induced by HU revealed that proliferating (BrdU+) SAA-iPSC-derived haematopoietic progenitor cells did not show higher levels of H2AX phosphorylation under conditions of replicative stress, most likely indicating a reduced formation of replication forks (Gagou *et al.*, 2010; Zeman and Cimprich, 2014). These results corroborate the reduced proliferative capacity findings of SAA-iPSC-derived-haematopoietic progenitors and exclude the accumulation of replicative stress- induced DNA damage as a causative factor for impaired haematopoietic differentiation. However, these results do not provide information regarding the ability of SAA cell lines to repair DNA damage since no replicative stress-associated DNA damage was observed due to the reduced number of proliferative cells. Therefore, it would be interesting to determine the ability of the SAA cell lines to repair different types of DNA lesions such as DSB or DNA crosslinks with agents such as ionizing radiation and bifunctional alkylators (i.e. cisplatin and mitomycin C). Depending on the nature of the DNA lesion different DNA repair pathways are activated. Thus, double-strand breaks induced by ionizing radiation are mainly repaired by non-homologous end joining (NHEJ), whereas DNA crosslinks induced by cisplatin or mitomycin C are repaired by homologous recombination (HR) or FA pathway (Helleday *et al.*, 2008). Tilgner *et al.* reported the use of a ligase IV-iPSC model to highlight the role of NHEJ-mediated-DSB repair in

the survival and differentiation of ligase IV-iPSC-derived haematopoietic progenitors after induction of DNA damage with ionizing radiation (Tilgner *et al.*, 2013). Thus, it would be interesting to use the SAA-iPSC model to determine the DNA repair capacity of the SAA cell lines to specific DNA damage by performing a similar analysis.

EP is a non-peptide molecule mimetic to TPO that stimulates bi- and tri-lineage haematopoiesis with 40% of the SAA patients responding to EP treatment at 3-4 months (Olnes *et al.*, 2012; Desmond *et al.*, 2014). However, the mechanism by which EP is promoting the generation of blood cells and impacting HSPC in AA patients is not fully understood. I studied the ability of EP to rescue the impaired haematopoietic differentiation capacity in our SAA-iPSC model. Interestingly, SAA-iPSC-derived haematopoietic progenitors did not show a significant increase in the number of erythroid or myeloid-lineage CFUs, proliferative capacity or DNA damage repair capacity under conditions of replicative stress, upon adding EP during the differentiation process. Given the complete lack of any cellular improvements in the presence of EP, it is likely that the potential underlying stem cell dysfunction present in our SAA cell lines is not associated with the TPO signalling pathway. Interestingly, it has been reported that EP failed to improve severe thrombocytopenia in patients with inherited BMFS including DC and DBA (Trautmann *et al.*, 2012). Thus, this lack of response to EP by DC patients supports our hypothesis that constitutional defects in the telomere-associated genes may be at the root of the impaired haematopoietic differentiation observed in the SAA-iPSC-derived haematopoietic progenitors. Different authors have hypothesized with the idea that EP might be involved in immune cell function by modulating regulatory T cell function in SAA patients (Desmond *et al.*, 2014; Marsh and Mufti, 2014) as observed in chronic ITP patients treated with EP (Bao *et al.*, 2010). Although Desmond *et al.* did not find significant changes in regulatory T cell subsets in 25 SAA patients treated with EP (Desmond *et al.*, 2014), the lack of response to EP observed in the SAA cell lines using the iPSC model might suggest that EP could have an underlying immune-regulatory function and therefore showing no effect on SAA cases with haematopoietic progenitors displaying defects associated to telomere maintenance. Nonetheless, it would be also of interest to investigate if this group of patients will respond to other available therapies, for example danazol, although, to date, it is unclear whether danazol related improvements are due to upregulation of telomerase activity through an increase in *TERT* expression or to a

telomerase-independent elongation of telomeres (Calado *et al.*, 2009; Townsley *et al.*, 2016b).

Taken together, these findings indicate, first, a reduced proliferative capacity in the SAA-iPSC-derived haematopoietic progenitors, likely associated with excessive telomere shortening observed in these haematopoietic progenitors. Second, in line with these findings, affected SAA-iPSC-derived haematopoietic progenitors exhibited reduced replicative-stress-associated DNA damage suggesting a reduced formation of replication forks. Third, apoptosis was not increased in SAA-iPSC-derived haematopoietic progenitors cultured under normal conditions and under replicative-stress conditions, excluding apoptotic death as potential dysfunctional mechanisms causing the reduced differentiation potential observed in the affected SAA patient cell lines. Lastly, addition of EP did not improve the proliferative capacity or rescue the disease phenotype observed in the affected SAA-iPSC-derived haematopoietic progenitors implying that EP signalling is probably not associated to the potential haematopoietic progenitor dysfunction present in the affected SAA cell lines. Thus, these results suggest that the presence of dysfunctional telomeres observed in SAA1, SAA2 and SAA3 cell lines could cause the reduced proliferation of the SAA-iPSC-derived haematopoietic progenitors observed in this study leading to impaired haematopoietic colony-forming capacity.

Chapter 7. Summary and future work

7.1 Summary

IPSC technology offers an excellent opportunity to study the complex pathophysiology of SAA for various reasons. First, it provides an unlimited source of SAA patient-specific haematopoietic progenitor cells that can be used in disease modelling studies and assessment of drug effectiveness and toxicity. This is especially relevant in the context of SAA due to the difficulties of obtaining haematopoietic progenitors from SAA patients for study of disease biology due to their reduced number in the patient's bone marrow. Second, it provides a disease model that enables the study of SAA patient-specific haematopoietic progenitors in the absence of immune system and identification of underlying haematopoietic progenitor dysfunction. Despite the fact that SAA is considered immune in nature, there is an increasing recognition that a subgroup of patients diagnosed with SAA might actually have underlying genetic defects. Thus, use of SAA-iPSC-derived haematopoietic progenitor cells in disease modelling studies would allow the identification of underlying dysfunction in these SAA progenitor cells in an *in vitro* setting opening a door to the study of the cellular pathways involved in this dysfunction. Identification of constitutional HSPC defects may have profound clinical implications on systematic diagnosis and treatment of SAA patients. Correct diagnosis of constitutive cases of SAA displaying haematopoietic progenitor dysfunction would lead to appropriate treatment with either HSCT, androgen or other newly emerging therapies (Miano and Dufour, 2015), avoiding toxicities from inappropriate and costly therapies, such as ATG, to which most show no or only transient response (Song *et al.*, 2013). Likewise, patients presenting genetic defects associated with BMFS have a predisposition towards haematological malignancies (Zeng and Katsanis, 2015), therefore rigorous follow-up studies of these patients would be imperative. Finally, identification of constitutional haematopoietic progenitor cell defects would ultimately lead to family screenings including testing of siblings to avoid using affected 'silent' siblings that might carry the same genetic defect as donors. This body of work was designed firstly to generate a SAA-iPSC model that could recapitulate the disease phenotype observed in SAA patients and, secondly, to investigate the existence of a potential haematopoietic progenitor cell dysfunction in cells from SAA patients by using this model.

The main conclusions inferred from this study are summarised as follows:

- iPSC from the four SAA patients (three paediatric and 1 young adult) and three unaffected volunteers generated for this study met all the standard criteria of pluripotency, including expression of pluripotency markers and formation of trilineage teratomae. Moreover, presence of reprogramming transgenes or chromosomal abnormalities was not detected, providing additional evidence of the fully-reprogramming of the generated iPSC.

- Haematopoietic progenitor cells, marked by expression of CD43 and capacity to generate haematopoietic colonies in CFU assays, and subsequent progenitor populations (erythroid, megakaryocytic, erythroid/megakaryocytic and myeloid) were successfully generated from iPSC lines by using a haematopoietic differentiation protocol previously described (Olivier *et al.*, 2016).

- SAA-iPSC showed similar potential to generate haematopoietic progenitor cells to control cell lines. However, iPSC-derived haematopoietic progenitor cells from three of the SAA cell lines (SAA1, SAA2 and SAA3) failed to generate mature haematopoietic colonies in CFU assays in similar levels than those in control cell lines pointing to a reduced haematopoietic colony-forming potential in these SAA cell lines. This reduced haematopoietic differentiation potential observed in the affected SAA-iPSC-derived haematopoietic progenitor cells successfully mirrors characteristic SAA-associated cytopenia observed in patients, therefore, confirming the capacity of iPSC technology to generate a *bona fide* SAA disease model.

- Telomere length measurement revealed a reduced reprogramming-induced telomere elongation in iPSC of three SAA cell lines (SAA1, SAA2 and SAA3) despite up-regulation of telomerase activity. Moreover, these affected SAA-iPSC cell lines displayed an excessive telomere shortening upon differentiation towards haematopoietic progenitor cells.

- SAA1, SAA2 and SAA3 iPSC-derived haematopoietic progenitor cells showed a reduced number of BrdU-incorporating cells indicating reduced proliferation whereas SAA4 showed proliferation capacity similar to that in control cell lines. Additionally, a reduced number of replication-induced DNA damage was observed in the affected SAA cell lines suggesting a reduced formation of replication forks and, therefore, confirming previous observation of reduced proliferation in these SAA cell lines.

- Addition of EP to cell culture media failed to rescue the affected SAA cell lines (SAA1, SAA2 and SAA3) from impaired haematopoietic differentiation capacity or increase the proliferative capacity of these cell lines. These findings suggest that potential haematopoietic progenitor cell dysfunction present in these affected SAA cell lines is not associated with TPO signalling or alternative effects of EP independent of TPO stimulation.

Based on these findings, I hypothesized that the impaired haematopoietic colony-forming capacity observed in the iPSC-derived haematopoietic progenitors of SAA1, SAA2 and SAA3 cell lines is caused by a defective telomere maintenance leading to dysfunctional telomeres in these haematopoietic progenitor cells. This hypothesis is supported by the absence of reprogramming-induced telomere elongation in the affected SAA-iPSC lines and excessive telomere shortening upon differentiation into haematopoietic progenitors most likely due to extensive cellular replication required in this process. This accelerated telomere attrition would lead to dysfunctional telomeres and subsequent induction of replicative senescence as cellular response to avoid genome instability, ultimately resulting in reduced formation of haematopoietic colonies in CFU assays. The fact that the remaining SAA cell line, SAA4, did show telomere elongation in iPSC, colony-forming potential and proliferation at similar levels than control cell lines most likely indicates that the SAA-disease phenotype in this patient might not be caused by stem cell dysfunction. However, much more work will be required to test the validity of this hypothesis as it is discussed in the next section. A representation of the proposed model is presented in **Figure 57** in a visual format.

Accelerated telomere shortening in SAA patients has been associated to increased proliferative stress observed in the first years following allogeneic HSCT due to increased HSPC turnover to replenish the bone marrow stem cell compartment (Gadalla and Savage, 2011). It is also plausible that SAA-iPSC derived haematopoietic progenitors show a defective proliferation capacity as the primary cause of the impaired haematopoietic colony-forming potential. As described in **Chapter 1**, **section 1.1.5.2**, Zeng *et al.* reported the up-regulation of genes associated with inhibition of cell cycle entry and down-regulation of cell proliferation and cell-cycle progress-enhancing genes (Zeng *et al.*, 2004). Thus, telomeres shortening observed in the affected SAA cell lines would be a consequence of an increased compensatory proliferation in a smaller number of non-defective SAA-iPSC derived haematopoietic progenitors to compensate for the loss of progenitors as reported by others (Beier *et al.*, 2012). However, this possibility would imply the presence of a heterogeneous population containing defective and non-defective haematopoietic progenitors which seems unlikely if a constitutional defect is considered. Likewise, proliferation analysis did not show presence of proliferative SAA-iPSC derived haematopoietic progenitors in the affected SAA cell lines as it would be expected if the non-defective underwent

replicative stress as compensatory mechanism. Based on the data generated in this study is difficult to distinguish between these two possibilities and further experiments would be required to identify the primary cause associated to the impaired colony-forming potential of the affected SAA cell lines.

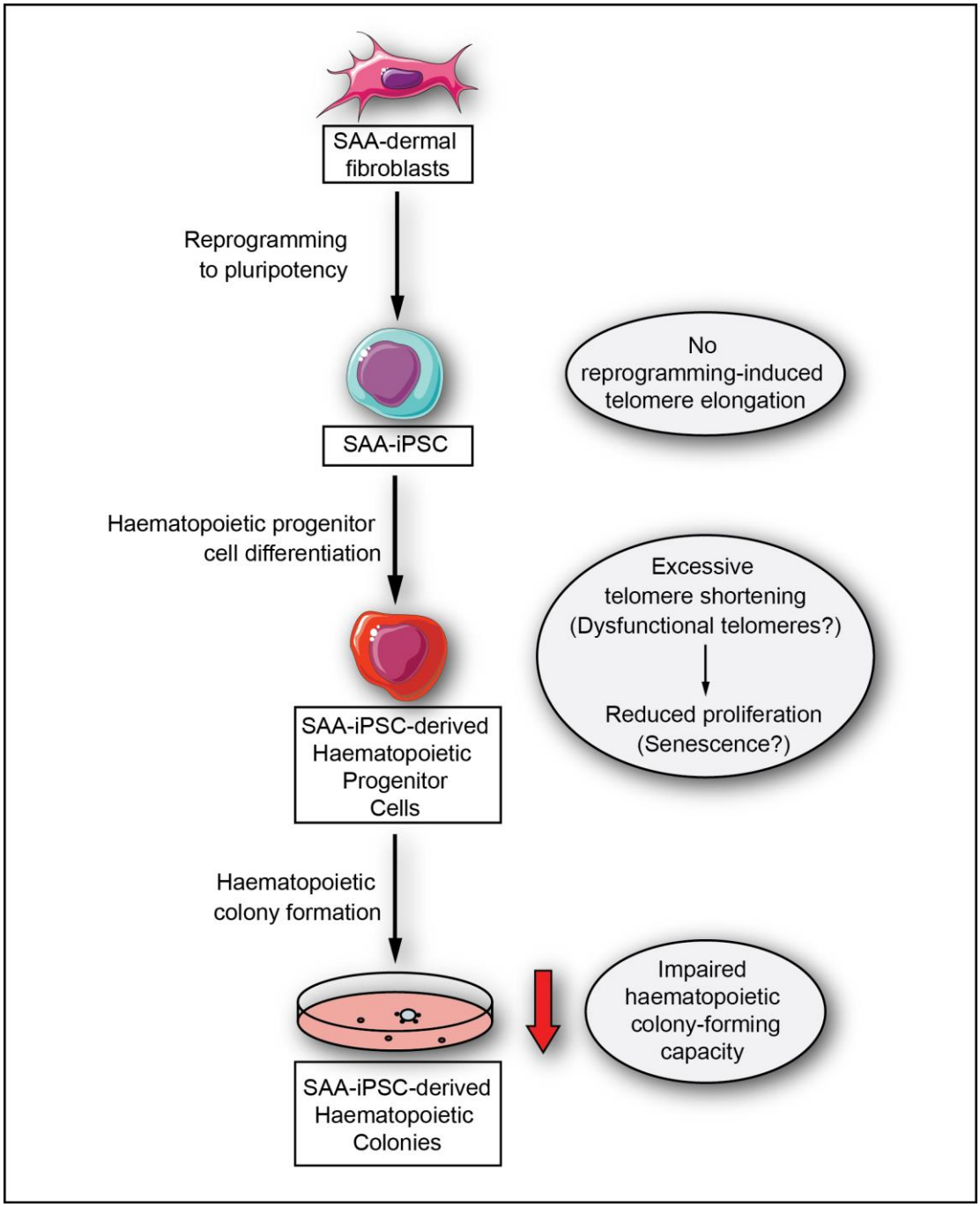

Figure 57. Proposed model for the role of telomere maintenance dysfunction found in three of the SAA-iPSC lines.

The potential defective telomere maintenance would imply the presence of genetic defects in telomere-associated genes in the affected SAA cell lines. SAA-iPSC lines were originally derived from HDF from SAA patients, underscoring the inherited nature of these genetic defects. Patients used for this study were diagnosed with SAA after exclusion of inherited BMFS suggesting absence of mutations in known genes associated with these syndromes. However, the majority of SAA patients with short telomeres lack mutations in previously characterized telomere-associated genes, suggesting the existence of undescribed mutations and genes that could contribute to defective telomere maintenance (Vulliamy *et al.*, 2005; Calado and Young, 2008; Walne and Dokal, 2009; Zeng and Katsanis, 2015; Allegra *et al.*, 2017). Novel mutations in genes associated with telomere pathway are rapidly emerging in syndromes characterized by short telomeres underscoring the complexity associated with telomeric function and regulation and blurring the historical clear distinction between SAA and inherited BMFS (Young, 2013; Gandhi *et al.*, 2015; Martinez and Blasco, 2015). Thus, assuming that the abnormal phenotype is caused defects by a single gene mutation, iPSC technology would provide a model to investigate as-yet-unknown constitutional defects in telomere-associated genes for further genetic studies. This would include correction of gene mutations in SAA-iPSC lines or introduction of specific mutations into non-affected control-iPSC lines with genome engineering to confirm causal relationship between genetic defect and disease phenotype (Musunuru, 2013)

In summary, these data provide evidence for the usefulness of iPSC-based disease modelling to replicate key phenotypes associated with SAA. Likewise, SAA-iPSC model provides a good platform to identify potential haematopoietic progenitor dysfunction, facilitating the identification of cryptic BMFS over acquired/immune-type AA. This in turn could prove extremely useful when applied in studies with a larger number of patients who are subsequently followed up with exome sequencing to uncover specific genetic variants associated with SAA. With current advances in genetic medicine, such information can easily be incorporated into diagnostic tests for patients and families with SAA, allowing the timely provision of the appropriate treatment modality. In conclusion, SAA-iPSC model stands out as an excellent tool for the identification of constitutional HSPC defects and uncovering novel constitutional

defects for further genetic studies. Further, this model can help SAA patients by predicting patient specific drug efficacy and safety *in vitro*.

I acknowledge that the use of iPSC technology in disease modelling of haematopoietic disorders presents some limitations that should be considered in order to avoid identification of phenotype due to reprogramming/differentiation artefacts as disease-relevant phenotype. Variability introduced during the reprogramming and differentiation process, either by clonal heterogeneity or genetic background, and the lack of protocols that enable the robust generation of *bona fide* HSCs are among the factors that could diminish the utility of iPSC for modelling haematopoietic diseases with a late onset. In the next section I discussed different approaches that could help overcome some of these hurdles.

7.2 Future work

The work described so far describes the feasibility of using iPSC technology to create a SAA disease model to study SAA pathogenesis. In this section, I suggest additional studies that would be necessary to improve this SAA-iPSC model to identify underlying SAA haematopoietic progenitor dysfunction and the cellular pathways involved in this abnormal behaviour:

- *Identification of fully-reprogrammed iPSC clones*.
  Successful identification of fully-reprogrammed iPSC clones is one of the main challenges associated with iPSC generation in order to avoid the so-called clonal heterogeneity. Conventional methods used in the characterization of iPSC are not sufficient in many aspects due to lack of standardization and ability to specifically identified *bona fide* iPSC clones (Asprer and Lakshmipathy, 2015). Interestingly, different studies have reported recently the successful identification of markers such as *IGF2/TRIM58* and *CHCHD2* to predict the differentiation potential of iPSC lines towards haematopoietic (Nishizawa *et al.*, 2016) or neuroectodermal lineages (Zhu *et al.*, 2016) respectively. Thus, Nishizawa *et al.* reported that downregulation of *IGF2* and/or aberrant DNA methylation of *TRIM58* during the reprogramming process would lead to a reduced haematopoietic differentiation potential in iPSC clones. Therefore, it would be interesting to determine the levels of expression of *IGF2* and methylation pattern of *TRIM58* to confirm that the impaired haematopoietic differentiation potential observed in affected SAA-iPSC lines is associated to a haematopoietic progenitor cell dysfunction and not as consequence of clonal heterogeneity due to aberrant DNA methylation acquired during the reprogramming process.

- *Genetic background variability*
  As discussed in **Chapter 4**, genetic background has been reported as the main driver of variation at a transcriptional, epigenetic and haematopoietic differentiation level. In this study, three different control-iPSC lines from unaffected individuals were used in order to include genetic background variation when comparing with SAA-iPSC lines. However, in order to identify

true biological phenotypes rather than just differences in phenotype due to genetic background, it would be more appropriate to use iPSC generated from unaffected parents/siblings, therefore minimizing variation introduced by genetic background. This might be especially relevant for sporadic disorders such as SAA in which differences in phenotype might be smaller than classical monogenic diseases with more apparent clinical manifestation (Bird, 2000).

Likewise, I discussed here further analysis that would be required to support the formulated hypothesis proposing defective telomere maintenance as underlying dysfunction in affected SAA-iPSC-derived haematopoietic progenitors leading to impaired colony-forming capacity of these progenitors.

- *Dysfunctional telomeres in SAA-iPSC-derived haematopoietic progenitor cells*

  Presence of dysfunctional telomeres in SAA-iPSC-derived haematopoietic progenitor cells would lead to induction of senescence and formation of TIF by accumulation of γH2AX in telomeres as DNA damage response to DSB. Detection of β-galactosidase activity would assess the induction of senescence (Bernadotte *et al.*, 2016) whereas formation of TIF would be detected by co-localization of γH2AX and telomeres by immunofluorescence staining (d'Adda di Fagagna *et al.*, 2003). Likewise, analysis of expression of p53 by Western blotting and p21 mRNA levels by quantitative RT-PCR would provide evidence of the activation of DNA damage response and induction of cellular senescence by dysfunctional telomeres (Herbig *et al.*, 2004; Deng *et al.*, 2008).

- G*enomic studies to identify pathogenic variants*

  With the advent of NGS technology, identification of pathogenic genetic variants in patients is progressing rapidly. NGS has enabled the identification of more than 100 causative genes in Mendelian disorders (Rabbani *et al.*, 2012), including *MPL* (Rabbani *et al.*, 2012; Keel *et al.*, 2016), *TP53* (Keel *et al.*, 2016) and *SRP72* (Kirwan *et al.*, 2012) as causative gene

in SAA. Our group performed a whole exome sequencing (WES) analysis of SAA patient's HDF. Unfortunately, no DNA samples from siblings or parents of these SAA patients were available which would provide critical information for the detection of *de novo* mutations. WES results revealed that SAA1, SAA2 and SAA3 patients did not show DC-associated mutations previously described in patients. However, presence of deleterious mutations in other telomere-associated genes such as *RPA2, NCL, POLD3, TEP1, YLMP1, PIF1 and ERCC4* in which no mutations in patients have been described to date were detected in SAA1, SAA2 and SAA3 but not in SAA4 (**Data not shown**). Interestingly, the replication Protein A (RPA) has been reported to be necessary for telomere maintenance due to its role in unfolding of telomeric G-quadruplexes and preventing replication-fork stalling (Kobayashi *et al.*, 2010) whereas *NCL* codifies for the RNA chaperone nucleolin that regulates the nuclear localization of telomerase (Khurts *et al.*, 2004). Likewise, overexpression of *Ylpm1* in mouse ESCs leads to down-regulation of telomerase activity and telomere shortening resulting in reduced proliferation and hematopoietic differentiation ability (Armstrong *et al.*, 2004). In line with this, Batista *et al.* reprogrammed DC fibroblasts harbouring mutations in *TCAB1* gene, a protein involved in the trafficking on the telomerase complex along the telomeric ends, resulting in iPSC with very short telomeres despite upregulation of TERT, TERC and dyskerin (Batista *et al.*, 2011). These results would support the hypothesis that underlying telomere-associated genetic defects observed in our SAA-iPSC model would result in primary defective telomere elongation and telomere shortening and subsequently impact the proliferation and clonogenic capacity of iPSC-derived-hematopoietic progenitor. Thus, it would be interesting to carry out WES analysis of a larger cohort of SAA patients in order to confirm the prevalence of these variants in other affected individuals and identify common pathogenic genes. This approach is especially relevant for cases in which the abnormal phenotype is caused by single gene defects. However, it is more limited for cases in which the disease is caused by interplay of genetic and environmental factors.

- G*ene-editing approach to correct potential pathogenic mutations.*

  The correlation observed between telomere shortening and reduced colony-forming capacity in the SAA-iPSC derived haematopoietic progenitor cells of SAA1, SAA2 and SAA3 cell lines points to a dysfunction in telomere maintenance as the underlying cause in the pathogenesis of SAA in these patients. However, correlation does not imply causation and the definitive experimental evidence of pathogenicity due to underlying telomere dysfunction in the affected SAA cell lines would imply functional assays correcting the genetic defect and rescue of the SAA-iPSC-derived haematopoietic progenitors from the impaired haematopoietic capacity by restoring functionality in telomeres. Different studies have reported the successful combination of iPSC platform with recently developed gene editing technologies to correct disease phenotypes in BMFS such as FA and DC (Rio *et al.*, 2014; Osborn *et al.*, 2015; Bluteau *et al.*, 2016; Woo *et al.*, 2016). Thus, given that the list of previously identified deleterious variants in candidate telomere-associated genes using NGS approach may involve more than 10 candidates, it would be necessary to perform a proof-of-principle screen experiment to introduce the relevant wild-type gene using genetic manipulation by using lentiviral systems in affected SAA-iPSC lines. Genetically-modified SAA-iPSC lines would be differentiated towards haematopoietic progenitors in small scale differentiation experiments and followed by measurements of telomeres and CFU assays to confirm the restoration of telomere functionality and, subsequently, haematopoietic colony-forming capacity. If introduction of wild-type gene of interest by lentivirus systems successfully reverses the *in vitro* SAA cellular phenotype, it would be interesting to proceed with the correction of the identified deleterious variants in the selected candidate genes of the affected SAA-iPSC cell lines using clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (Cas9) genome editing technology and CRISPR corrected SAA-iPSC lines would be subjected to haematopoietic differentiation to confirm the rescue of the disease phenotype as described previously. Likewise, in order to ensure the reliable identification of the SAA disease-causing mutations, SAA-disease-phenotype would be induced in non-disease affected wild-type iPSC lines

by introducing the identified mutations using CRIPS/Cas9 systems. These approaches would enable in turn the generation of isogenic iPSC lines with the correction/introduction of the mutation as only variable minimizing variability due to genetic background which can be particularly important in the study of sporadic diseases in which phenotypic differences between unaffected and affected cell lines are anticipated to be subtle (Shi *et al.*, 2017)

- *Follow-up studies*

  SAA patients displaying short telomeres are more predisposed to develop clonal disorders such as MDS and PNH after receiving IST (Scheinberg *et al.*, 2010). Patient SAA1 and SAA2 were treated with IST with different outcome (**Table 7**). SAA2 relapsed and developed PNH, associated with clonal evolution. This could be explained by the presence of dysfunctional telomeres as pointed out by different authors (Afable *et al.*, 2011; Calado *et al.*, 2012; Dumitriu *et al.*, 2012). On the other hand SAA1 responded to IST. This contrasts with the possibility that this patient might actually present a constitutional telomere-associated defect since it has been reported that patients with inherited BMFS show reduced/lack of response to IST (Song *et al.*, 2013; Allegra *et al.*, 2017). However, it has been also described that patients with defects in *TERT* can show some response to standard IST (Young, 2013; Townsley *et al.*, 2014). Thus, due to the potential defective telomere maintenance observed in this patient using the SAA-iPSC model, it would be interesting to investigate if this patient has developed a clonal disorder that could provide more evidence of the presence of dysfunctional telomeres.

- *Efficacy of danazol to correct telomere dysfunction*

  Danazol, a synthetic sex hormone, has been reported to improve blood counts and elongate telomeres in blood leukocytes from patients with telomere diseases (Townsley *et al.*, 2016a). It has been hypothesized that danazol induces TERT upregulation similarly to that using sex hormones in human primary haematopoietic cells (Calado *et al.*, 2009). However, this still remains speculative and danazol mechanism of action have not been fully elucidated (Grossmann, 2016). Therefore, even though SAA-iPSC lines

showed an upregulation of telomerase activity induced by reprogramming, it would be interesting to investigate other possible mechanisms of action of danazol and its effect on telomere maintenance in order to correct telomere dysfunctionality and potentially rescue affected SAA cell lines from the impaired haematopoietic differentiation capacity.

References

Abyzov, A., Mariani, J., Palejev, D., Zhang, Y., Haney, M.S., Tomasini, L., Ferrandino, A.F., Rosenberg Belmaker, L.A., Szekely, A., Wilson, M., Kocabas, A., Calixto, N.E., Grigorenko, E.L., Huttner, A., Chawarska, K., Weissman, S., Urban, A.E., Gerstein, M. and Vaccarino, F.M. (2012) 'Somatic copy number mosaicism in human skin revealed by induced pluripotent stem cells', *Nature*, 492(7429), pp. 438-42.

Ackermann, M., Liebhaber, S., Klusmann, J.H. and Lachmann, N. (2015) 'Lost in translation: pluripotent stem cell-derived hematopoiesis', *EMBO Mol Med*, 7(11), pp. 1388-402.

Adam, S., Melguizo Sanchis, D., El-Kamah, G., Samarasinghe, S., Alharthi, S., Armstrong, L. and Lako, M. (2017) 'Concise Review: Getting to the Core of Inherited Bone Marrow Failures', *Stem Cells*, 35(2), pp. 284-298.

Afable, M.G., 2nd, Tiu, R.V. and Maciejewski, J.P. (2011) 'Clonal evolution in aplastic anemia', *Hematology Am Soc Hematol Educ Program*, 2011, pp. 90-5.

Agarwal, S., Loh, Y.H., McLoughlin, E.M., Huang, J., Park, I.H., Miller, J.D., Huo, H., Okuka, M., Dos Reis, R.M., Loewer, S., Ng, H.H., Keefe, D.L., Goldman, F.D., Klingelhutz, A.J., Liu, L. and Daley, G.Q. (2010) 'Telomere elongation in induced pluripotent stem cells from dyskeratosis congenita patients', *Nature*, 464(7286), pp. 292-6.

Alexander, W.S., Roberts, A.W., Nicola, N.A., Li, R. and Metcalf, D. (1996) 'Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl', *Blood*, 87(6), pp. 2162-70.

Alharbi, R.A., Pettengell, R., Pandha, H.S. and Morgan, R. (2013) 'The role of HOX genes in normal hematopoiesis and acute leukemia', *Leukemia*, 27(5), pp. 1000-8.

Allegra, A., Innao, V., Penna, G., Gerace, D., Allegra, A.G. and Musolino, C. (2017) 'Telomerase and telomere biology in hematological diseases: A new therapeutic target', *Leuk Res*, 56, pp. 60-74.

Allsopp, R.C., Morin, G.B., DePinho, R., Harley, C.B. and Weissman, I.L. (2003) 'Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation', *Blood*, 102(2), pp. 517-20.

Alper, J. (2009) 'Geron gets green light for human trial of ES cell-derived product', *Nat Biotechnol*, 27(3), pp. 213-4.

Amabile, G., Welner, R.S., Nombela-Arrieta, C., D'Alise, A.M., Di Ruscio, A., Ebralidze, A.K., Kraytsberg, Y., Ye, M., Kocher, O., Neuberg, D.S., Khrapko, K., Silberstein, L.E. and Tenen, D.G. (2013) 'In vivo generation of transplantable human hematopoietic cells from induced pluripotent stem cells', *Blood*, 121(8), pp. 1255-64.

Ardman, B., Sikorski, M.A. and Staunton, D.E. (1992) 'CD43 interferes with T-lymphocyte adhesion', *Proc Natl Acad Sci U S A*, 89(11), pp. 5001-5.

Armstrong, L., Lako, M., van Herpe, I., Evans, J., Saretzki, G. and Hole, N. (2004) 'A role for nucleoprotein Zap3 in the reduction of telomerase activity during embryonic stem cell differentiation', *Mech Dev*, 121(12), pp. 1509-22.

Asprer, J.S. and Lakshmipathy, U. (2015) 'Current methods and challenges in the comprehensive characterization of human pluripotent stem cells', *Stem Cell Rev*, 11(2), pp. 357-72.

Bacigalupo, A., Figari, O., Tong, J., Piaggio, G., Miceli, S., Frassoni, F., Caciagli, P., Badolati, G. and Marmont, A.M. (1992) 'Long-term marrow culture in patients with aplastic anemia compared with marrow transplant recipients and normal controls', *Exp Hematol*, 20(4), pp. 425-30.

Bacigalupo, A., Hows, J., Gluckman, E., Nissen, C., Marsh, J., Van Lint, M.T., Congiu, M., De Planque, M.M., Ernst, P., McCann, S. and et al. (1988) 'Bone marrow transplantation (BMT) versus immunosuppression for the treatment of severe aplastic anaemia (SAA): a report of the EBMT SAA working party', *Br J Haematol*, 70(2), pp. 177-82.

Ball, S.E., Gibson, F.M., Rizzo, S., Tooze, J.A., Marsh, J.C. and Gordon-Smith, E.C. (1998) 'Progressive telomere shortening in aplastic anemia', *Blood*, 91(10), pp. 3582-92.

Bao, W., Bussel, J.B., Heck, S., He, W., Karpoff, M., Boulad, N. and Yazdanbakhsh, K. (2010) 'Improved regulatory T-cell activity in patients with chronic immune thrombocytopenia treated with thrombopoietic agents', *Blood*, 116(22), pp. 4639-45.

Bar-Nur, O., Russ, H.A., Efrat, S. and Benvenisty, N. (2011) 'Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells', *Cell stem cell*, 9(1), pp. 17-23.

Bar, C., Povedano, J.M., Serrano, R., Benitez-Buelga, C., Popkes, M., Formentini, I., Bobadilla, M., Bosch, F. and Blasco, M.A. (2016) 'Telomerase gene therapy rescues telomere length, bone marrow aplasia, and survival in mice with aplastic anemia', *Blood*, 127(14), pp. 1770-9.

Batista, L.F., Pech, M.F., Zhong, F.L., Nguyen, H.N., Xie, K.T., Zaug, A.J., Crary, S.M., Choi, J., Sebastiano, V., Cherry, A., Giri, N., Wernig, M., Alter, B.P., Cech, T.R., Savage, S.A., Reijo Pera, R.A. and Artandi, S.E. (2011) 'Telomere shortening and loss of self-renewal in dyskeratosis congenita induced pluripotent stem cells', *Nature*, 474(7351), pp. 399-402.

Bautch, V.L. (2011) 'Stem cells and the vasculature', *Nat Med*, 17(11), pp. 1437-43.

Beier, F., Foronda, M., Martinez, P. and Blasco, M.A. (2012) 'Conditional TRF1 knockout in the hematopoietic compartment leads to bone marrow failure and recapitulates clinical features of dyskeratosis congenita', *Blood*, 120(15), pp. 2990-3000.

Ben-David, U. and Benvenisty, N. (2011) 'The tumorigenicity of human embryonic and induced pluripotent stem cells', *Nat Rev Cancer*, 11(4), pp. 268-77.

Bennett, J.M. and Orazi, A. (2009) 'Diagnostic criteria to distinguish hypocellular acute myeloid leukemia from hypocellular myelodysplastic syndromes and aplastic anemia: recommendations for a standardized approach', *Haematologica*, 94(2), pp. 264-8.

Bernadotte, A., Mikhelson, V.M. and Spivak, I.M. (2016) 'Markers of cellular senescence. Telomere shortening as a marker of cellular senescence', *Aging (Albany NY)*, 8(1), pp. 3-11.

Bernardes de Jesus, B. and Blasco, M.A. (2013) 'Telomerase at the intersection of cancer and aging', *Trends Genet*, 29(9), pp. 513-20.

Bhutani, K., Nazor, K.L., Williams, R., Tran, H., Dai, H., Dzakula, Z., Cho, E.H., Pang, A.W., Rao, M., Cao, H., Schork, N.J. and Loring, J.F. (2016) 'Whole-genome mutational burden analysis of three pluripotency induction methods', *Nat Commun*, 7, p. 10536.

Bird, T.D. (2000) 'Sporadic cases of possible genetic diseases: to test or not to test?', *Archives of neurology*, 57(3), pp. 309-10.

Blasco, M.A. (2005) 'Telomeres and human disease: ageing, cancer and beyond', *Nat Rev Genet*, 6(8), pp. 611-22.

Bluteau, D., Masliah-Planchon, J., Clairmont, C., Rousseau, A., Ceccaldi, R., Dubois d'Enghien, C., Bluteau, O., Cuccuini, W., Gachet, S., Peffault de Latour, R., Leblanc, T., Socie, G., Baruchel, A., Stoppa-Lyonnet, D., D'Andrea, A.D. and Soulier, J. (2016) 'Biallelic inactivation of REV7 is associated with Fanconi anemia', *J Clin Invest*, 126(9), pp. 3580-4.

Bock, C., Kiskinis, E., Verstappen, G., Gu, H., Boulting, G., Smith, Z.D., Ziller, M., Croft, G.F., Amoroso, M.W., Oakley, D.H., Gnirke, A., Eggan, K. and Meissner, A. (2011) 'Reference Maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines', *Cell*, 144(3), pp. 439-52.

Boiers, C., Carrelha, J., Lutteropp, M., Luc, S., Green, J.C., Azzoni, E., Woll, P.S., Mead, A.J., Hultquist, A., Swiers, G., Perdiguero, E.G., Macaulay, I.C., Melchiori, L., Luis, T.C., Kharazi, S., Bouriez-Jones, T., Deng, Q., Ponten, A., Atkinson, D., Jensen, C.T., Sitnicka, E., Geissmann, F., Godin, I., Sandberg, R., de Bruijn, M.F. and Jacobsen, S.E. (2013) 'Lymphomyeloid contribution of an immune-restricted progenitor emerging prior to definitive hematopoietic stem cells', *Cell Stem Cell*, 13(5), pp. 535-48.

Boisset, J.C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E. and Robin, C. (2010) 'In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium', *Nature*, 464(7285), pp. 116-20.

Brehm, M.A., Cuthbert, A., Yang, C., Miller, D.M., DiIorio, P., Laning, J., Burzenski, L., Gott, B., Foreman, O., Kavirayani, A., Herlihy, M., Rossini, A.A., Shultz, L.D. and Greiner, D.L. (2010) 'Parameters for establishing humanized mouse models to study human immunity: analysis of human hematopoietic stem cell engraftment in three immunodeficient strains of mice bearing the IL2rgamma(null) mutation', *Clin Immunol*, 135(1), pp. 84-98.

Brummendorf, T.H., Maciejewski, J.P., Mak, J., Young, N.S. and Lansdorp, P.M. (2001) 'Telomere length in leukocyte subpopulations of patients with aplastic anemia', *Blood*, 97(4), pp. 895-900.

Bueno, C., Roldan, M., Anguita, E., Romero-Moya, D., Martin-Antonio, B., Rosu-Myles, M., del Canizo, C., Campos, F., Garcia, R., Gomez-Casares, M., Fuster, J.L., Jurado, M., Delgado, M. and Menendez, P. (2014) 'Bone marrow mesenchymal stem cells from patients with aplastic anemia maintain functional and immune properties and do not contribute to the pathogenesis of the disease', *Haematologica*, 99(7), pp. 1168-75.

Cahan, P. and Daley, G.Q. (2013) 'Origins and implications of pluripotent stem cell variability and heterogeneity', *Nat Rev Mol Cell Biol*, 14(6), pp. 357-68.

Calado, R.T., Cooper, J.N., Padilla-Nash, H.M., Sloand, E.M., Wu, C.O., Scheinberg, P., Ried, T. and Young, N.S. (2012) 'Short telomeres result in chromosomal instability in hematopoietic cells and precede malignant evolution in human aplastic anemia', *Leukemia*, 26(4), pp. 700-7.

Calado, R.T., Yewdell, W.T., Wilkerson, K.L., Regal, J.A., Kajigaya, S., Stratakis, C.A. and Young, N.S. (2009) 'Sex hormones, acting on the TERT gene, increase telomerase activity in human primary hematopoietic cells', *Blood*, 114(11), pp. 2236-43.

Calado, R.T. and Young, N.S. (2008) 'Telomere maintenance and human bone marrow failure', *Blood*, 111(9), pp. 4446-55.

Calado, R.T. and Young, N.S. (2009) 'Telomere diseases', *N Engl J Med*, 361(24), pp. 2353-65.

Camitta, B.M., Rappeport, J.M., Parkman, R. and Nathan, D.G. (1975) 'Selection of patients for bone marrow transplantation in severe aplastic anemia', *Blood*, 45(3), pp. 355-63.

Carcamo-Orive, I., Hoffman, G.E., Cundiff, P., Beckmann, N.D., D'Souza, S.L., Knowles, J.W., Patel, A., Papatsenko, D., Abbasi, F., Reaven, G.M., Whalen, S., Lee, P., Shahbazi, M., Henrion, M.Y., Zhu, K., Wang, S., Roussos, P., Schadt, E.E., Pandey, G., Chang, R., Quertermous, T. and Lemischka, I. (2017) 'Analysis of Transcriptional Variability in a Large Human iPSC Library Reveals Genetic and Non-genetic Determinants of Heterogeneity', *Cell Stem Cell*, 20(4), pp. 518-532 e9.

Carpenter, L., Malladi, R., Yang, C.T., French, A., Pilkington, K.J., Forsey, R.W., Sloane-Stanley, J., Silk, K.M., Davies, T.J., Fairchild, P.J., Enver, T. and Watt, S.M. (2011) 'Human induced pluripotent stem cells are capable of B-cell lymphopoiesis', *Blood*, 117(15), pp. 4008-11.

Chadwick, K., Wang, L., Li, L., Menendez, P., Murdoch, B., Rouleau, A. and Bhatia, M. (2003) 'Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells', *Blood*, 102(3), pp. 906-15.

Chan, E.M., Ratanasirintrawoot, S., Park, I.H., Manos, P.D., Loh, Y.H., Huo, H., Miller, J.D., Hartung, O., Rho, J., Ince, T.A., Daley, G.Q. and Schlaeger, T.M. (2009) 'Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells', *Nat Biotechnol*, 27(11), pp. 1033-7.

Chang, K.H., Nelson, A.M., Cao, H., Wang, L., Nakamoto, B., Ware, C.B. and Papayannopoulou, T. (2006) 'Definitive-like erythroid cells derived from human embryonic stem cells coexpress high levels of embryonic and fetal globins with little or no adult globin', *Blood*, 108(5), pp. 1515-23.

Chao, Y.H., Peng, C.T., Harn, H.J., Chan, C.K. and Wu, K.H. (2010) 'Poor potential of proliferation and differentiation in bone marrow mesenchymal stem cells derived from children with severe aplastic anemia', *Ann Hematol*, 89(7), pp. 715-23.

Chen, J. (2005) 'Animal models for acquired bone marrow failure syndromes', *Clin Med Res*, 3(2), pp. 102-8.

Chen, J., Lipovsky, K., Ellison, F.M., Calado, R.T. and Young, N.S. (2004) 'Bystander destruction of hematopoietic progenitor and stem cells in a mouse model of infusion-induced bone marrow failure', *Blood*, 104(6), pp. 1671-8.

Chen, M.J., Yokomizo, T., Zeigler, B.M., Dzierzak, E. and Speck, N.A. (2009) 'Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter', *Nature*, 457(7231), pp. 887-91.

Cheruku PS, C.A., Dunbar CE, Young NS, Larochelle A (2015) 'The Thrombopoietin Receptor Agonist Eltrombopag Has DNA Repair Activity in Human Hematopoietic Stem and Progenitor Cells', *Blood*, 126, p. 2407.

Chin, M.H., Mason, M.J., Xie, W., Volinia, S., Singer, M., Peterson, C., Ambartsumyan, G., Aimiuwu, O., Richter, L., Zhang, J., Khvorostov, I., Ott, V., Grunstein, M., Lavon, N., Benvenisty, N., Croce, C.M., Clark, A.T., Baxter, T., Pyle, A.D., Teitell, M.A., Pelegrini, M., Plath, K. and Lowry, W.E. (2009) 'Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures', *Cell Stem Cell*, 5(1), pp. 111-23.

Choi, J., Lee, S., Mallard, W., Clement, K., Tagliazucchi, G.M., Lim, H., Choi, I.Y., Ferrari, F., Tsankov, A.M., Pop, R., Lee, G., Rinn, J.L., Meissner, A., Park, P.J. and Hochedlinger, K. (2015) 'A comparison of genetically matched cell lines reveals the equivalence of human iPSCs and ESCs', *Nature biotechnology*, 33(11), pp. 1173-81.

Choi, K.-D., Vodyanik, M.A., Togarrati, P.P., Suknuntha, K., Kumar, A., Samarjeet, F., Probasco, M.D., Tian, S., Stewart, R., Thomson, J.A. and Slukvin, I.I. (2012) 'Identification of the hemogenic endothelial progenitor and its direct precursor in human pluripotent stem cell differentiation cultures', *Cell reports*, 2(3), pp. 553-67.

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J.C. and Keller, G. (1998) 'A common precursor for hematopoietic and endothelial cells', *Development*, 125(4), pp. 725-32.

Choi, K.D., Vodyanik, M.A. and Slukvin, II (2009a) 'Generation of mature human myelomonocytic cells through expansion and differentiation of pluripotent stem cell-derived lin-CD34+CD43+CD45+ progenitors', *J Clin Invest*, 119(9), pp. 2818-29.

Choi, K.D., Yu, J., Smuga-Otto, K., Salvagiotto, G., Rehrauer, W., Vodyanik, M., Thomson, J. and Slukvin, I. (2009b) 'Hematopoietic and endothelial differentiation of human induced pluripotent stem cells', *Stem Cells*, 27(3), pp. 559-67.

Clarke, R.L., Yzaguirre, A.D., Yashiro-Ohtani, Y., Bondue, A., Blanpain, C., Pear, W.S., Speck, N.A. and Keller, G. (2013) 'The expression of Sox17 identifies and regulates haemogenic endothelium', *Nat Cell Biol*, 15(5), pp. 502-10.

Collins, K. and Mitchell, J.R. (2002) 'Telomerase in the human organism', *Oncogene*, 21(4), pp. 564-79.

Cooper, O., Seo, H., Andrabi, S., Guardia-Laguarta, C., Graziotto, J., Sundberg, M., McLean, J.R., Carrillo-Reid, L., Xie, Z., Osborn, T., Hargus, G., Deleidi, M., Lawson, T., Bogetofte, H., Perez-Torres, E., Clark, L., Moskowitz, C., Mazzulli, J., Chen, L., Volpicelli-Daley, L., Romero, N., Jiang, H., Uitti, R.J., Huang, Z., Opala, G., Scarffe, L.A., Dawson, V.L., Klein, C., Feng, J., Ross, O.A., Trojanowski, J.Q., Lee, V.M., Marder, K., Surmeier, D.J., Wszolek, Z.K., Przedborski, S., Krainc, D., Dawson, T.M. and Isacson, O. (2012) 'Pharmacological rescue of mitochondrial deficits in iPSC-derived neural cells from patients with familial Parkinson's disease', *Sci Transl Med*, 4(141), p. 141ra90.

Coutinho, L.M., Gillece, M.H., de Wynter, E.A., Will, A., Testa, N.G. (1993) *Haemopoiesis: a practical approach*. Oxford Univ. Press, New York.

Cowan, C.A., Atienza, J., Melton, D.A. and Eggan, K. (2005) 'Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells', *Science*, 309(5739), pp. 1369-73.

d'Adda di Fagagna, F., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P. and Jackson, S.P. (2003) 'A DNA damage checkpoint response in telomere-initiated senescence', *Nature*, 426(6963), pp. 194-8.

Daniel, M.G., Pereira, C.F., Lemischka, I.R. and Moore, K.A. (2016) 'Making a Hematopoietic Stem Cell', *Trends Cell Biol*, 26(3), pp. 202-14.

Davis, R.L., Weintraub, H. and Lassar, A.B. (1987) 'Expression of a single transfected cDNA converts fibroblasts to myoblasts', *Cell*, 51(6), pp. 987-1000.

Davis, R.P., Ng, E.S., Costa, M., Mossman, A.K., Sourris, K., Elefanty, A.G. and Stanley, E.G. (2008) 'Targeting a GFP reporter gene to the MIXL1 locus of human embryonic stem cells identifies human primitive streak-like cells and enables isolation of primitive hematopoietic precursors', *Blood*, 111(4), pp. 1876-84.

Dawson, L., Bateman-House, A.S., Mueller Agnew, D., Bok, H., Brock, D.W., Chakravarti, A., Greene, M., King, P.A., O'Brien, S.J., Sachs, D.H., Schill,

K.E., Siegel, A., Solter, D., Suter, S.M., Verfaillie, C.M., Walters, L.B., Gearhart, J.D. and Faden, R.R. (2003) 'Safety issues in cell-based intervention trials', *Fertil Steril*, 80(5), pp. 1077-85.

de Lange, T. (2005) 'Shelterin: the protein complex that shapes and safeguards human telomeres', *Genes Dev*, 19(18), pp. 2100-10.

De Los Angeles, A., Ferrari, F., Xi, R., Fujiwara, Y., Benvenisty, N., Deng, H., Hochedlinger, K., Jaenisch, R., Lee, S., Leitch, H.G., Lensch, M.W., Lujan, E., Pei, D., Rossant, J., Wernig, M., Park, P.J. and Daley, G.Q. (2015) 'Hallmarks of pluripotency', *Nature*, 525(7570), pp. 469-78.

Deng, J., Shoemaker, R., Xie, B., Gore, A., LeProust, E.M., Antosiewicz-Bourget, J., Egli, D., Maherali, N., Park, I.H., Yu, J., Daley, G.Q., Eggan, K., Hochedlinger, K., Thomson, J., Wang, W., Gao, Y. and Zhang, K. (2009) 'Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming', *Nat Biotechnol*, 27(4), pp. 353-60.

Deng, Y., Chan, S.S. and Chang, S. (2008) 'Telomere dysfunction and tumour suppression: the senescence connection', *Nature reviews Cancer*, 8(6), pp. 450-8.

Desmond, R., Townsley, D.M., Dumitriu, B., Olnes, M.J., Scheinberg, P., Bevans, M., Parikh, A.R., Broder, K., Calvo, K.R., Wu, C.O., Young, N.S. and Dunbar, C.E. (2014) 'Eltrombopag restores trilineage hematopoiesis in refractory severe aplastic anemia that can be sustained on discontinuation of drug', *Blood*, 123(12), pp. 1818-25.

Desmond, R., Townsley, D.M., Dunbar, C. and Young, N.S. (2015) 'Eltrombopag in aplastic anemia', *Semin Hematol*, 52(1), pp. 31-7.

Dezern, A.E. and Brodsky, R.A. (2011) 'Clinical management of aplastic anemia', *Expert Rev Hematol*, 4(2), pp. 221-30.

Dias, J., Gumenyuk, M., Kang, H., Vodyanik, M., Yu, J., Thomson, J.A. and Slukvin, II (2011) 'Generation of red blood cells from human induced pluripotent stem cells', *Stem Cells Dev*, 20(9), pp. 1639-47.

Dokal, I. and Vulliamy, T. (2010) 'Inherited bone marrow failure syndromes', *Haematologica*, 95(8), pp. 1236-40.

Donovan, P.J. and de Miguel, M.P. (2003) 'Turning germ cells into stem cells', *Curr Opin Genet Dev*, 13(5), pp. 463-71.

Donovan, P.J. and Gearhart, J. (2001) 'The end of the beginning for pluripotent stem cells', *Nature*, 414(6859), pp. 92-7.

Dorn, I., Klich, K., Arauzo-Bravo, M.J., Radstaak, M., Santourlidis, S., Ghanjati, F., Radke, T.F., Psathaki, O.E., Hargus, G., Kramer, J., Einhaus, M., Kim, J.B., Kogler, G., Wernet, P., Scholer, H.R., Schlenke, P. and Zaehres, H. (2015) 'Erythroid differentiation of human induced pluripotent stem cells is independent of donor cell type of origin', *Haematologica*, 100(1), pp. 32-41.

Doulatov, S., Vo, L.T., Chou, S.S., Kim, P.G., Arora, N., Li, H., Hadland, B.K., Bernstein, I.D., Collins, J.J., Zon, L.I. and Daley, G.Q. (2013) 'Induction of multipotential hematopoietic progenitors from human pluripotent stem cells via respecification of lineage-restricted precursors', *Cell Stem Cell*, 13(4), pp. 459-70.

Draper, J.S., Pigott, C., Thomson, J.A. and Andrews, P.W. (2002) 'Surface antigens of human embryonic stem cells: changes upon differentiation in culture', *J Anat*, 200(Pt 3), pp. 249-58.

Dravid, G.G. and Crooks, G.M. (2011) 'The challenges and promises of blood engineered from human pluripotent stem cells', *Adv Drug Deliv Rev*, 63(4-5), pp. 331-41.

Dumitriu, B., Feng, X., Townsley, D.M., Ueda, Y., Yoshizato, T., Calado, R.T., Yang, Y., Wakabayashi, Y., Kajigaya, S., Ogawa, S., Zhu, J. and Young, N.S. (2015) 'Telomere attrition and candidate gene mutations preceding monosomy 7 in aplastic anemia', *Blood*, 125(4), pp. 706-9.

Dumitriu, B., Ueda, Y., Kajigaya, S., Townsley, D.M. and Young, N.S. (2012) 'Very Short Telomeres of Peripheral Blood Leukocytes Precede Clinical Progression to Myelodysplasia with Monosomy 7 in Aplastic Anemia Patients', *Blood*, 120(21), pp. 1265-1265.

Dzierzak, E. and Speck, N.A. (2008) 'Of lineage and legacy: the development of mammalian hematopoietic stem cells', *Nat Immunol*, 9(2), pp. 129-36.

Easterbrook, J., Fidanza, A. and Forrester, L.M. (2016) 'Concise review: programming human pluripotent stem cells into blood', *Br J Haematol*, 173(5), pp. 671-9.

Eaves, C., Lambie, K. (1995) *Atlas of Human Hematopoietic Colonies*. StemCell Technologies, Vancouver.

Ebert, A.D., Yu, J., Rose, F.F., Jr., Mattis, V.B., Lorson, C.L., Thomson, J.A. and Svendsen, C.N. (2009) 'Induced pluripotent stem cells from a spinal muscular atrophy patient', *Nature*, 457(7227), pp. 277-80.

Eilken, H.M., Nishikawa, S. and Schroeder, T. (2009) 'Continuous single-cell imaging of blood generation from haemogenic endothelium', *Nature*, 457(7231), pp. 896-900.

Elbadry, M.I., Espinoza, J.L. and Nakao, S. (2017) 'Induced pluripotent stem cell technology: A window for studying the pathogenesis of acquired aplastic anemia and possible applications', *Exp Hematol*, 49, pp. 9-18.

Elcheva, I., Brok-Volchanskaya, V., Kumar, A., Liu, P., Lee, J.H., Tong, L., Vodyanik, M., Swanson, S., Stewart, R., Kyba, M., Yakubov, E., Cooke, J., Thomson, J.A. and Slukvin, I. (2014) 'Direct induction of haematoendothelial programs in human pluripotent stem cells by transcriptional regulators', *Nat Commun*, 5, p. 4372.

Elliott, A.M., Elliott, K.A. and Kammesheidt, A. (2010) 'High resolution array-CGH characterization of human stem cells using a stem cell focused microarray', *Mol Biotechnol*, 46(3), pp. 234-42.

Erickson-Miller, C.L., Delorme, E., Tian, S.S., Hopson, C.B., Landis, A.J., Valoret, E.I., Sellers, T.S., Rosen, J., Miller, S.G., Luengo, J.I., Duffy, K.J. and Jenkins, J.M. (2009) 'Preclinical activity of eltrombopag (SB-497115), an oral, nonpeptide thrombopoietin receptor agonist', *Stem Cells*, 27(2), pp. 424-30.

Evans, M.J. and Kaufman, M.H. (1981) 'Establishment in culture of pluripotential cells from mouse embryos', *Nature*, 292(5819), pp. 154-6.

Feng, Q., Lu, S.J., Klimanskaya, I., Gomes, I., Kim, D., Chung, Y., Honig, G.R., Kim, K.S. and Lanza, R. (2010) 'Hemangioblastic derivatives from human induced pluripotent stem cells exhibit limited expansion and early senescence', *Stem Cells*, 28(4), pp. 704-12.

Feng, Q., Shabrani, N., Thon, J.N., Huo, H., Thiel, A., Machlus, K.R., Kim, K., Brooks, J., Li, F., Luo, C., Kimbrel, E.A., Wang, J., Kim, K.S., Italiano, J., Cho, J., Lu, S.J. and Lanza, R. (2014) 'Scalable generation of universal platelets from human induced pluripotent stem cells', *Stem Cell Reports*, 3(5), pp. 817-31.

Feraud, O., Valogne, Y., Melkus, M.W., Zhang, Y., Oudrhiri, N., Haddad, R., Daury, A., Rocher, C., Larbi, A., Duquesnoy, P., Divers, D., Gobbo, E., Brunet de la Grange, P., Louache, F., Bennaceur-Griscelli, A. and Mitjavila-Garcia, M.T. (2016) 'Donor Dependent Variations in Hematopoietic Differentiation among Embryonic and Induced Pluripotent Stem Cell Lines', *PLoS One*, 11(3), p. e0149291.

Ferrell, P.I., Xi, J., Ma, C., Adlakha, M. and Kaufman, D.S. (2015) 'The RUNX1 +24 enhancer and P1 promoter identify a unique subpopulation of hematopoietic progenitor cells derived from human pluripotent stem cells', *Stem Cells*, 33(4), pp. 1130-41.

Fischbach, G.D. and Fischbach, R.L. (2004) 'Stem cells: science, policy, and ethics', *J Clin Invest*, 114(10), pp. 1364-70.

Fogarty, P.F., Yamaguchi, H., Wiestner, A., Baerlocher, G.M., Sloand, E., Zeng, W.S., Read, E.J., Lansdorp, P.M. and Young, N.S. (2003) 'Late presentation of dyskeratosis congenita as apparently acquired aplastic anaemia due to mutations in telomerase RNA', *Lancet*, 362(9396), pp. 1628-30.

Forrester, L.M. and Jackson, M. (2012) 'Mechanism of action of HOXB4 on the hematopoietic differentiation of embryonic stem cells', *Stem Cells*, 30(3), pp. 379-85.

Frelin, C., Herrington, R., Janmohamed, S., Barbara, M., Tran, G., Paige, C.J., Benveniste, P., Zuniga-Pflucker, J.C., Souabni, A., Busslinger, M. and Iscove, N.N. (2013) 'GATA-3 regulates the self-renewal of long-term hematopoietic stem cells', *Nat Immunol*, 14(10), pp. 1037-44.

French, A., Yang, C.T., Taylor, S., Watt, S.M. and Carpenter, L. (2015) 'Human induced pluripotent stem cell-derived B lymphocytes express sIgM and can be generated via a hemogenic endothelium intermediate', *Stem Cells Dev*, 24(9), pp. 1082-95.

Fusaki, N., Ban, H., Nishiyama, A., Saeki, K. and Hasegawa, M. (2009) 'Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome', *Proceedings of the Japan Academy Series B, Physical and biological sciences*, 85(8), pp. 348-62.

Gadalla, S.M. and Savage, S.A. (2011) 'Telomere biology in hematopoiesis and stem cell transplantation', *Blood Rev*, 25(6), pp. 261-9.

Gafni, O., Weinberger, L., Mansour, A.A., Manor, Y.S., Chomsky, E., Ben-Yosef, D., Kalma, Y., Viukov, S., Maza, I., Zviran, A., Rais, Y., Shipony, Z., Mukamel, Z., Krupalnik, V., Zerbib, M., Geula, S., Caspi, I., Schneir, D., Shwartz, T., Gilad, S., Amann-Zalcenstein, D., Benjamin, S., Amit, I., Tanay, A., Massarwa, R., Novershtern, N. and Hanna, J.H. (2013) 'Derivation of novel human ground state naive pluripotent stem cells', *Nature*, 504(7479), pp. 282-6.

Gagou, M.E., Zuazua-Villar, P. and Meuth, M. (2010) 'Enhanced H2AX phosphorylation, DNA replication fork arrest, and cell death in the absence of Chk1', *Mol Biol Cell*, 21(5), pp. 739-52.

Galic, Z., Kitchen, S.G., Subramanian, A., Bristol, G., Marsden, M.D., Balamurugan, A., Kacena, A., Yang, O. and Zack, J.A. (2009) 'Generation of T lineage cells from human embryonic stem cells in a feeder free system', *Stem Cells*, 27(1), pp. 100-7.

Gandhi, S., Abuarqoub, H., Kordasti, S., Jiang, J., Kulasekararaj, A., Mufti, G. and Marsh, J.C.W. (2015) 'Pathology of bone marrow failure syndromes', *Diagnostic Histopathology*, 21(5), pp. 174-180.

Garcon, L., Ge, J., Manjunath, S.H., Mills, J.A., Apicella, M., Parikh, S., Sullivan, L.M., Podsakoff, G.M., Gadue, P., French, D.L., Mason, P.J., Bessler, M. and Weiss, M.J. (2013) 'Ribosomal and hematopoietic defects in induced pluripotent stem cells derived from Diamond Blackfan anemia patients', *Blood*, 122(6), pp. 912-21.

Ge, J., Apicella, M., Mills, J.A., Garcon, L., French, D.L., Weiss, M.J., Bessler, M. and Mason, P.J. (2015) 'Dysregulation of the Transforming Growth Factor beta Pathway in Induced Pluripotent Stem Cells Generated from Patients with Diamond Blackfan Anemia', *PLoS One*, 10(8), p. e0134878.

Ghosh, Z., Wilson, K.D., Wu, Y., Hu, S., Quertermous, T. and Wu, J.C. (2010) 'Persistent donor cell gene expression among human induced pluripotent stem cells contributes to differences with human embryonic stem cells', *PLoS One*, 5(2), p. e8975.

Giannakoulas, N.C., Karakantza, M., Theodorou, G.L., Pagoni, M., Galanopoulos, A., Kakagianni, T., Kouraklis-Symeonidis, A., Matsouka, P., Maniatis, A. and Zoumbos, N.C. (2004) 'Clinical relevance of balance between type 1 and type 2 immune responses of lymphocyte subpopulations in aplastic anaemia patients', *Br J Haematol*, 124(1), pp. 97-105.

Gordon-Smith, E.C. (1991) 'Acquired aplastic anaemia. In: *Hematology, Basic Principles and Practice*'. Churchill Livingstone, New York.

Griffith, J.D., Comeau, L., Rosenfield, S., Stansel, R.M., Bianchi, A., Moss, H. and de Lange, T. (1999) 'Mammalian telomeres end in a large duplex loop', *Cell*, 97(4), pp. 503-14.

Grossmann, M. (2016) 'Danazol Treatment for Telomere Diseases', *N Engl J Med*, 375(11), p. 1095.

Gu, B.W., Apicella, M., Mills, J., Fan, J.M., Reeves, D.A., French, D., Podsakoff, G.M., Bessler, M. and Mason, P.J. (2015) 'Impaired Telomere Maintenance and Decreased Canonical WNT Signaling but Normal Ribosome Biogenesis in Induced Pluripotent Stem Cells from X-Linked Dyskeratosis Congenita Patients', *PLoS One*, 10(5), p. e0127414.

Guinan, E.C. (2011) 'Diagnosis and management of aplastic anemia', *Hematology Am Soc Hematol Educ Program*, 2011, pp. 76-81.

Gupta, V., Eapen, M., Brazauskas, R., Carreras, J., Aljurf, M., Gale, R.P., Hale, G.A., Ilhan, O., Passweg, J.R., Ringden, O., Sabloff, M., Schrezenmeier, H., Socie, G. and Marsh, J.C. (2010) 'Impact of age on outcomes after bone marrow transplantation for acquired aplastic anemia using HLA-matched sibling donors', *Haematologica*, 95(12), pp. 2119-25.

Gurdon, J.B. (1962) 'The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles', *J Embryol Exp Morphol*, 10, pp. 622-40.

Gurdon, J.B. (2006) 'From nuclear transfer to nuclear reprogramming: the reversal of cell differentiation', *Annu Rev Cell Dev Biol*, 22, pp. 1-22.

Hacein-Bey-Abina, S., de Saint Basile, G. and Cavazzana-Calvo, M. (2003) 'Gene therapy of X-linked severe combined immunodeficiency', *Methods Mol Biol*, 215, pp. 247-59.

Hackett, J.A. and Greider, C.W. (2002) 'Balancing instability: dual roles for telomerase and telomere dysfunction in tumorigenesis', *Oncogene*, 21(4), pp. 619-26.

Hanna, J., Wernig, M., Markoulaki, S., Sun, C.W., Meissner, A., Cassady, J.P., Beard, C., Brambrink, T., Wu, L.C., Townes, T.M. and Jaenisch, R. (2007) 'Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin', *Science*, 318(5858), pp. 1920-3.

Harley, C.B., Futcher, A.B. and Greider, C.W. (1990) 'Telomeres shorten during ageing of human fibroblasts', *Nature*, 345(6274), pp. 458-60.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. (1993) 'The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases', *Cell*, 75(4), pp. 805-16.

Harper, J.W., Elledge, S.J., Keyomarsi, K., Dynlacht, B., Tsai, L.H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E. and et al. (1995) 'Inhibition of cyclin-dependent kinases by p21', *Mol Biol Cell*, 6(4), pp. 387-400.

Helleday, T., Petermann, E., Lundin, C., Hodgson, B. and Sharma, R.A. (2008) 'DNA repair pathways as targets for cancer therapy', *Nat Rev Cancer*, 8(3), pp. 193-204.

Henderson, J.K., Draper, J.S., Baillie, H.S., Fishel, S., Thomson, J.A., Moore, H. and Andrews, P.W. (2002) 'Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens', *Stem cells (Dayton, Ohio)*, 20(4), pp. 329-37.

Herbig, U., Jobling, W.A., Chen, B.P., Chen, D.J. and Sedivy, J.M. (2004) 'Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a)', *Mol Cell*, 14(4), pp. 501-13.

Hills, M. and Lansdorp, P.M. (2009) 'Short telomeres resulting from heritable mutations in the telomerase reverse transcriptase gene predispose for a variety of malignancies', *Ann N Y Acad Sci*, 1176, pp. 178-90.

Hiramoto, T., Ebihara, Y., Mizoguchi, Y., Nakamura, K., Yamaguchi, K., Ueno, K., Nariai, N., Mochizuki, S., Yamamoto, S., Nagasaki, M., Furukawa, Y., Tani, K., Nakauchi, H., Kobayashi, M. and Tsuji, K. (2013) 'Wnt3a stimulates maturation of impaired neutrophils developed from severe congenital neutropenia patient-derived pluripotent stem cells', *Proc Natl Acad Sci U S A*, 110(8), pp. 3023-8.

Hochedlinger, K. and Jaenisch, R. (2006) 'Nuclear reprogramming and pluripotency', *Nature*, 441(7097), pp. 1061-7.

Hoffman, L.M. and Carpenter, M.K. (2005) 'Characterization and culture of human embryonic stem cells', *Nat Biotechnol*, 23(6), pp. 699-708.

Hotta, A. and Yamanaka, S. (2015) 'From Genomics to Gene Therapy: Induced Pluripotent Stem Cells Meet Genome Editing', *Annu Rev Genet*, 49, pp. 47-70.

Hou, P., Li, Y., Zhang, X., Liu, C., Guan, J., Li, H., Zhao, T., Ye, J., Yang, W., Liu, K., Ge, J., Xu, J., Zhang, Q., Zhao, Y. and Deng, H. (2013) 'Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds', *Science*, 341(6146), pp. 651-4.

Huang, H.P., Chen, P.H., Hwu, W.L., Chuang, C.Y., Chien, Y.H., Stone, L., Chien, C.L., Li, L.T., Chiang, S.C., Chen, H.F., Ho, H.N., Chen, C.H. and Kuo, H.C. (2011) 'Human Pompe disease-induced pluripotent stem cells for pathogenesis modeling, drug testing and disease marker identification', *Hum Mol Genet*, 20(24), pp. 4851-64.

Huang, Y., Osorno, R., Tsakiridis, A. and Wilson, V. (2012) 'In Vivo differentiation potential of epiblast stem cells revealed by chimeric embryo formation', *Cell Rep*, 2(6), pp. 1571-8.

Huber, T.L., Kouskoff, V., Fehling, H.J., Palis, J. and Keller, G. (2004) 'Haemangioblast commitment is initiated in the primitive streak of the mouse embryo', *Nature*, 432(7017), pp. 625-30.

Huffman, K.E., Levene, S.D., Tesmer, V.M., Shay, J.W. and Wright, W.E. (2000) 'Telomere shortening is proportional to the size of the G-rich telomeric 3'-overhang', *J Biol Chem*, 275(26), pp. 19719-22.

Hussein, S.M., Batada, N.N., Vuoristo, S., Ching, R.W., Autio, R., Narva, E., Ng, S., Sourour, M., Hamalainen, R., Olsson, C., Lundin, K., Mikkola, M., Trokovic, R., Peitz, M., Brustle, O., Bazett-Jones, D.P., Alitalo, K., Lahesmaa, R., Nagy,

A. and Otonkoski, T. (2011) 'Copy number variation and selection during reprogramming to pluripotency', *Nature*, 471(7336), pp. 58-62.

International Stem Cell, I., Adewumi, O., Aflatoonian, B., Ahrlund-Richter, L., Amit, M., Andrews, P.W., Beighton, G., Bello, P.A., Benvenisty, N., Berry, L.S., Bevan, S., Blum, B., Brooking, J., Chen, K.G., Choo, A.B., Churchill, G.A., Corbel, M., Damjanov, I., Draper, J.S., Dvorak, P., Emanuelsson, K., Fleck, R.A., Ford, A., Gertow, K., Gertsenstein, M., Gokhale, P.J., Hamilton, R.S., Hampl, A., Healy, L.E., Hovatta, O., Hyllner, J., Imreh, M.P., Itskovitz-Eldor, J., Jackson, J., Johnson, J.L., Jones, M., Kee, K., King, B.L., Knowles, B.B., Lako, M., Lebrin, F., Mallon, B.S., Manning, D., Mayshar, Y., McKay, R.D., Michalska, A.E., Mikkola, M., Mileikovsky, M., Minger, S.L., Moore, H.D., Mummery, C.L., Nagy, A., Nakatsuji, N., O'Brien, C.M., Oh, S.K., Olsson, C., Otonkoski, T., Park, K.Y., Passier, R., Patel, H., Patel, M., Pedersen, R., Pera, M.F., Piekarczyk, M.S., Pera, R.A., Reubinoff, B.E., Robins, A.J., Rossant, J., Rugg-Gunn, P., Schulz, T.C., Semb, H., Sherrer, E.S., Siemen, H., Stacey, G.N., Stojkovic, M., Suemori, H., Szatkiewicz, J., Turetsky, T., Tuuri, T., van den Brink, S., Vintersten, K., Vuoristo, S., Ward, D., Weaver, T.A., Young, L.A. and Zhang, W. (2007) 'Characterization of human embryonic stem cell lines by the International Stem Cell Initiative', *Nat Biotechnol*, 25(7), pp. 803-16.

Ishaq, A., Hanson, P.S., Morris, C.M. and Saretzki, G. (2016) 'Telomerase Activity is Downregulated Early During Human Brain Development', *Genes*, 7(6).

Jeong, J.Y., Levine, M.S., Abayasekara, N., Berliner, N., Laubach, J. and Vanasse, G.J. (2015) 'The non-peptide thrombopoietin receptor agonist eltrombopag stimulates megakaryopoiesis in bone marrow cells from patients with relapsed multiple myeloma', *J Hematol Oncol*, 8, p. 37.

Judson, R.L., Babiarz, J.E., Venere, M. and Blelloch, R. (2009) 'Embryonic stem cell-specific microRNAs promote induced pluripotency', *Nat Biotechnol*, 27(5), pp. 459-61.

Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P. and Woltjen, K. (2009) 'Virus-free induction of pluripotency and subsequent excision of reprogramming factors', *Nature*, 458(7239), pp. 771-5.

Kakagianni, T., Giannakoulas, N.C., Thanopoulou, E., Galani, A., Michalopoulou, S., Kouraklis-Symeonidis, A. and Zoumbos, N.C. (2006) 'A probable role for trail-induced apoptosis in the pathogenesis of marrow failure. Implications from an in vitro model and from marrow of aplastic anemia patients', *Leuk Res*, 30(6), pp. 713-21.

Kang, L., Wang, J., Zhang, Y., Kou, Z. and Gao, S. (2009) 'iPS cells can support full-term development of tetraploid blastocyst-complemented embryos', *Cell Stem Cell*, 5(2), pp. 135-8.

Kardel, M.D. and Eaves, C.J. (2012) 'Modeling human hematopoietic cell development from pluripotent stem cells', *Exp Hematol*, 40(8), pp. 601-11.

Keel, S.B., Scott, A., Sanchez-Bonilla, M., Ho, P.A., Gulsuner, S., Pritchard, C.C., Abkowitz, J.L., King, M.C., Walsh, T. and Shimamura, A. (2016) 'Genetic features of myelodysplastic syndrome and aplastic anemia in pediatric and young adult patients', *Haematologica*, 101(11), pp. 1343-1350.

Keirstead, H.S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K. and Steward, O. (2005) 'Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury', *J Neurosci*, 25(19), pp. 4694-705.

Kennedy, M., Awong, G., Sturgeon, C.M., Ditadi, A., LaMotte-Mohs, R., Zuniga-Pflucker, J.C. and Keller, G. (2012) 'T lymphocyte potential marks the emergence of definitive hematopoietic progenitors in human pluripotent stem cell differentiation cultures', *Cell Rep*, 2(6), pp. 1722-35.

Kennedy, M., D'Souza, S.L., Lynch-Kattman, M., Schwantz, S. and Keller, G. (2007) 'Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures', *Blood*, 109(7), pp. 2679-87.

Kent, W.J., Sugnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H., Zahler, A.M. and Haussler, D. (2002) 'The human genome browser at UCSC', *Genome research*, 12(6), pp. 996-1006.

Khurts, S., Masutomi, K., Delgermaa, L., Arai, K., Oishi, N., Mizuno, H., Hayashi, N., Hahn, W.C. and Murakami, S. (2004) 'Nucleolin interacts with telomerase', *J Biol Chem*, 279(49), pp. 51508-15.

Killick, S.B., Bown, N., Cavenagh, J., Dokal, I., Foukaneli, T., Hill, A., Hillmen, P., Ireland, R., Kulasekararaj, A., Mufti, G., Snowden, J.A., Samarasinghe, S., Wood, A., Marsh, J.C. and British Society for Standards in, H. (2016) 'Guidelines for the diagnosis and management of adult aplastic anaemia', *Br J Haematol*, 172(2), pp. 187-207.

Kim, D., Kim, C.H., Moon, J.I., Chung, Y.G., Chang, M.Y., Han, B.S., Ko, S., Yang, E., Cha, K.Y., Lanza, R. and Kim, K.S. (2009) 'Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins', *Cell Stem Cell*, 4(6), pp. 472-6.

Kim, K., Doi, A., Wen, B., Ng, K., Zhao, R., Cahan, P., Kim, J., Aryee, M.J., Ji, H., Ehrlich, L.I.R., Yabuuchi, A., Takeuchi, A., Cunniff, K.C., Hongguang, H., McKinney-Freeman, S., Naveiras, O., Yoon, T.J., Irizarry, R.A., Jung, N., Seita, J., Hanna, J., Murakami, P., Jaenisch, R., Weissleder, R., Orkin, S.H., Weissman, I.L., Feinberg, A.P. and Daley, G.Q. (2010) 'Epigenetic memory in induced pluripotent stem cells', *Nature*, 467(7313), pp. 285-90.

Kim, K., Zhao, R., Doi, A., Ng, K., Unternaehrer, J., Cahan, P., Huo, H., Loh, Y.-H., Aryee, M.J., Lensch, M.W., Li, H., Collins, J.J., Feinberg, A.P. and Daley, G.Q. (2011) 'Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells', *Nature biotechnology*, 29(12), pp. 1117-9.

Kirwan, M., Walne, A.J., Plagnol, V., Velangi, M., Ho, A., Hossain, U., Vulliamy, T. and Dokal, I. (2012) 'Exome sequencing identifies autosomal-dominant SRP72 mutations associated with familial aplasia and myelodysplasia', *Am J Hum Genet*, 90(5), pp. 888-92.

Kissa, K. and Herbomel, P. (2010) 'Blood stem cells emerge from aortic endothelium by a novel type of cell transition', *Nature*, 464(7285), pp. 112-5.

Klimchenko, O., Mori, M., Distefano, A., Langlois, T., Larbret, F., Lecluse, Y., Feraud, O., Vainchenker, W., Norol, F. and Debili, N. (2009) 'A common bipotent progenitor generates the erythroid and megakaryocyte lineages in embryonic stem cell-derived primitive hematopoiesis', *Blood*, 114(8), pp. 1506-17.

Knorr, D.A., Ni, Z., Hermanson, D., Hexum, M.K., Bendzick, L., Cooper, L.J., Lee, D.A. and Kaufman, D.S. (2013) 'Clinical-scale derivation of natural killer cells from human pluripotent stem cells for cancer therapy', *Stem Cells Transl Med*, 2(4), pp. 274-83.

Kobayashi, Y., Sato, K., Kibe, T., Seimiya, H., Nakamura, A., Yukawa, M., Tsuchiya, E. and Ueno, M. (2010) 'Expression of mutant RPA in human cancer cells causes telomere shortening', *Biosci Biotechnol Biochem*, 74(2), pp. 382-5.

Kondo, T., Asai, M., Tsukita, K., Kutoku, Y., Ohsawa, Y., Sunada, Y., Imamura, K., Egawa, N., Yahata, N., Okita, K., Takahashi, K., Asaka, I., Aoi, T., Watanabe, A., Watanabe, K., Kadoya, C., Nakano, R., Watanabe, D., Maruyama, K., Hori, O., Hibino, S., Choshi, T., Nakahata, T., Hioki, H., Kaneko, T., Naitoh, M., Yoshikawa, K., Yamawaki, S., Suzuki, S., Hata, R., Ueno, S., Seki, T., Kobayashi, K., Toda, T., Murakami, K., Irie, K., Klein, W.L., Mori, H., Asada, T., Takahashi, R., Iwata, N., Yamanaka, S. and Inoue, H. (2013) 'Modeling Alzheimer's disease with iPSCs reveals stress phenotypes associated with intracellular Abeta and differential drug responsiveness', *Cell Stem Cell*, 12(4), pp. 487-96.

Kordasti, S., Marsh, J., Al-Khan, S., Jiang, J., Smith, A., Mohamedali, A., Abellan, P.P., Veen, C., Costantini, B., Kulasekararaj, A.G., Benson-Quarm, N., Seidl, T., Mian, S.A., Farzaneh, F. and Mufti, G.J. (2012) 'Functional characterization of CD4+ T cells in aplastic anemia', *Blood*, 119(9), pp. 2033-43.

Kotini, A.G., Chang, C.J., Boussaad, I., Delrow, J.J., Dolezal, E.K., Nagulapally, A.B., Perna, F., Fishbein, G.A., Klimek, V.M., Hawkins, R.D., Huangfu, D., Murry, C.E., Graubert, T., Nimer, S.D. and Papapetrou, E.P. (2015) 'Functional analysis of a chromosomal deletion associated with myelodysplastic syndromes using isogenic human induced pluripotent stem cells', *Nat Biotechnol*, 33(6), pp. 646-55.

Koyanagi-Aoi, M., Ohnuki, M., Takahashi, K., Okita, K., Noma, H., Sawamura, Y., Teramoto, I., Narita, M., Sato, Y., Ichisaka, T., Amano, N., Watanabe, A., Morizane, A., Yamada, Y., Sato, T., Takahashi, J. and Yamanaka, S. (2013) 'Differentiation-defective phenotypes revealed by large-scale analyses of human pluripotent stem cells', *Proceedings of the National Academy of Sciences of the United States of America*, 110(51), pp. 20569-74.

Kulasekararaj, A.G., Jiang, J., Smith, A.E., Mohamedali, A.M., Mian, S., Gandhi, S., Gaken, J., Czepulkowski, B., Marsh, J.C. and Mufti, G.J. (2014) 'Somatic mutations identify a subgroup of aplastic anemia patients who progress to myelodysplastic syndrome', *Blood*, 124(17), pp. 2698-704.

Kwon, E.M., Connelly, J.P., Hansen, N.F., Donovan, F.X., Winkler, T., Davis, B.W., Alkadi, H., Chandrasekharappa, S.C., Dunbar, C.E., Mullikin, J.C. and Liu, P. (2017) 'iPSCs and fibroblast subclones from the same fibroblast population contain comparable levels of sequence variations', *Proc Natl Acad Sci U S A*, 114(8), pp. 1964-1969.

Kwon, J.H., Kim, I., Lee, Y.G., Koh, Y., Park, H.C., Song, E.Y., Kim, H.K., Yoon, S.S., Lee, D.S., Park, S.S., Shin, H.Y., Park, S., Park, M.H., Ahn, H.S. and Kim, B.K. (2010) 'Clinical course of non-severe aplastic anemia in adults', *Int J Hematol*, 91(5), pp. 770-5.

Kyttala, A., Moraghebi, R., Valensisi, C., Kettunen, J., Andrus, C., Pasumarthy, K.K., Nakanishi, M., Nishimura, K., Ohtaka, M., Weltner, J., Van Handel, B., Parkkonen, O., Sinisalo, J., Jalanko, A., Hawkins, R.D., Woods, N.B., Otonkoski, T. and Trokovic, R. (2016) 'Genetic Variability Overrides the Impact of Parental Cell Type and Determines iPSC Differentiation Potential', *Stem Cell Reports*, 6(2), pp. 200-12.

Lachmann, N., Ackermann, M., Frenzel, E., Liebhaber, S., Brennig, S., Happle, C., Hoffmann, D., Klimenkova, O., Luttge, D., Buchegger, T., Kuhnel, M.P., Schambach, A., Janciauskiene, S., Figueiredo, C., Hansen, G., Skokowa, J. and Moritz, T. (2015) 'Large-scale hematopoietic differentiation of human induced pluripotent stem cells provides granulocytes or macrophages for cell replacement therapies', *Stem Cell Reports*, 4(2), pp. 282-96.

Lamba, D.A., Gust, J. and Reh, T.A. (2009) 'Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice', *Cell Stem Cell*, 4(1), pp. 73-9.

Lapillonne, H., Kobari, L., Mazurier, C., Tropel, P., Giarratana, M.C., Zanella-Cleon, I., Kiger, L., Wattenhofer-Donze, M., Puccio, H., Hebert, N., Francina, A., Andreu, G., Viville, S. and Douay, L. (2010) 'Red blood cell generation from human induced pluripotent stem cells: perspectives for transfusion medicine', *Haematologica*, 95(10), pp. 1651-9.

Ledran, M.H., Krassowska, A., Armstrong, L., Dimmick, I., Renstrom, J., Lang, R., Yung, S., Santibanez-Coref, M., Dzierzak, E., Stojkovic, M., Oostendorp, R.A., Forrester, L. and Lako, M. (2008) 'Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches', *Cell Stem Cell*, 3(1), pp. 85-98.

Lee, G., Papapetrou, E.P., Kim, H., Chambers, S.M., Tomishima, M.J., Fasano, C.A., Ganat, Y.M., Menon, J., Shimizu, F., Viale, A., Tabar, V., Sadelain, M. and Studer, L. (2009) 'Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs', *Nature*, 461(7262), pp. 402-6.

Lee, J.J., Kook, H., Chung, I.J., Na, J.A., Park, M.R., Hwang, T.J., Kwak, J.Y., Sohn, S.K. and Kim, H.J. (2001) 'Telomere length changes in patients with aplastic anaemia', *Br J Haematol*, 112(4), pp. 1025-30.

Lensch, M.W. and Daley, G.Q. (2004) 'Origins of mammalian hematopoiesis: in vivo paradigms and in vitro models', *Curr Top Dev Biol*, 60, pp. 127-96.

Lensch, M.W. and Daley, G.Q. (2006) 'Scientific and clinical opportunities for modeling blood disorders with embryonic stem cells', *Blood*, 107(7), pp. 2605-12.

Li, J., Lu, S., Yang, S., Xing, W., Feng, J., Li, W., Zhao, Q., Wu, H., Ge, M., Ma, F., Zhao, H., Liu, B., Zhang, L., Zheng, Y. and Han, Z.C. (2012) 'Impaired immunomodulatory ability of bone marrow mesenchymal stem cells on CD4(+) T cells in aplastic anemia', *Results Immunol*, 2, pp. 142-7.

Liang, G. and Zhang, Y. (2013) 'Genetic and epigenetic variations in iPSCs: potential causes and implications for application', *Cell stem cell*, 13(2), pp. 149-59.

Liras, A. (2010) 'Future research and therapeutic applications of human stem cells: general, regulatory, and bioethical aspects', *J Transl Med*, 8, p. 131.

Liu, G.-H., Suzuki, K., Li, M., Qu, J., Montserrat, N., Tarantino, C., Gu, Y., Yi, F., Xu, X., Zhang, W., Ruiz, S., Plongthongkum, N., Zhang, K., Masuda, S., Nivet, E., Tsunekawa, Y., Soligalla, R.D., Goebl, A., Aizawa, E., Kim, N.Y., Kim, J., Dubova, I., Li, Y., Ren, R., Benner, C., del Sol, A., Bueren, J., Trujillo, J.P., Surralles, J., Cappelli, E., Dufour, C., Esteban, C.R. and Izpisua Belmonte, J.C. (2014) 'Modelling Fanconi anemia pathogenesis and therapeutics using integration-free patient-derived iPSCs', *Nature communications*, 5, p. 4330.

Liu, L. (2017) 'Linking Telomere Regulation to Stem Cell Pluripotency', *Trends Genet*, 33(1), pp. 16-33.

Loh, Y.H., Agarwal, S., Park, I.H., Urbach, A., Huo, H., Heffner, G.C., Kim, K., Miller, J.D., Ng, K. and Daley, G.Q. (2009) 'Generation of induced pluripotent stem cells from human blood', *Blood*, 113(22), pp. 5476-9.

Luckett, W.P. (1978) 'Origin and differentiation of the yolk sac and extraembryonic mesoderm in presomite human and rhesus monkey embryos', *Am J Anat*, 152(1), pp. 59-97.

Maciejewski, J., Selleri, C., Anderson, S. and Young, N.S. (1995) 'Fas antigen expression on CD34+ human marrow cells is induced by interferon gamma and tumor necrosis factor alpha and potentiates cytokine-mediated hematopoietic suppression in vitro', *Blood*, 85(11), pp. 3183-90.

Maciejewski, J.P., Anderson, S., Katevas, P. and Young, N.S. (1994) 'Phenotypic and functional analysis of bone marrow progenitor cell compartment in bone marrow failure', *Br J Haematol*, 87(2), pp. 227-34.

Maciejewski, J.P., Selleri, C., Sato, T., Anderson, S. and Young, N.S. (1996) 'A severe and consistent deficit in marrow and circulating primitive hematopoietic cells (long-term culture-initiating cells) in acquired aplastic anemia', *Blood*, 88(6), pp. 1983-91.

Malumbres, M. and Barbacid, M. (2001) 'To cycle or not to cycle: a critical decision in cancer', *Nat Rev Cancer*, 1(3), pp. 222-31.

Manz, C.Y., Nissen, C. and Wodnar-Filipowicz, A. (1996) 'Deficiency of CD34+ c-kit+ and CD34+38- hematopoietic precursors in aplastic anemia after immunosuppressive treatment', *Am J Hematol*, 52(4), pp. 264-74.

Marion, R.M., Strati, K., Li, H., Tejera, A., Schoeftner, S., Ortega, S., Serrano, M. and Blasco, M.A. (2009) 'Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells', *Cell Stem Cell*, 4(2), pp. 141-54.

Marrone, A., Walne, A., Tamary, H., Masunari, Y., Kirwan, M., Beswick, R., Vulliamy, T. and Dokal, I. (2007) 'Telomerase reverse-transcriptase homozygous mutations in autosomal recessive dyskeratosis congenita and Hoyeraal-Hreidarsson syndrome', *Blood*, 110(13), pp. 4198-205.

Marsh, J.C. (2005) 'Bone marrow failure syndromes', *Clin Med (Lond)*, 5(4), pp. 332-6.

Marsh, J.C., Ball, S.E., Cavenagh, J., Darbyshire, P., Dokal, I., Gordon-Smith, E.C., Keidan, J., Laurie, A., Martin, A., Mercieca, J., Killick, S.B., Stewart, R. and Yin, J.A. (2009) 'Guidelines for the diagnosis and management of aplastic anaemia', *Br J Haematol*, 147(1), pp. 43-70.

Marsh, J.C., Chang, J., Testa, N.G., Hows, J.M. and Dexter, T.M. (1990) 'The hematopoietic defect in aplastic anemia assessed by long-term marrow culture', *Blood*, 76(9), pp. 1748-57.

Marsh, J.C., Chang, J., Testa, N.G., Hows, J.M. and Dexter, T.M. (1991) 'In vitro assessment of marrow 'stem cell' and stromal cell function in aplastic anaemia', *Br J Haematol*, 78(2), pp. 258-67.

Marsh, J.C. and Mufti, G.J. (2014) 'Eltrombopag: a stem cell cookie?', *Blood*, 123(12), pp. 1774-5.

Martin-Ruiz, C.M., Gussekloo, J., van Heemst, D., von Zglinicki, T. and Westendorp, R.G.J. (2005) 'Telomere length in white blood cells is not associated with morbidity or mortality in the oldest old: a population-based study', *Aging cell*, 4(6), pp. 287-90.

Martin, G.R. (1980) 'Teratocarcinomas and mammalian embryogenesis', *Science*, 209(4458), pp. 768-76.

Martin, G.R. (1981) 'Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells', *Proc Natl Acad Sci U S A*, 78(12), pp. 7634-8.

Martinez, P. and Blasco, M.A. (2015) 'Replicating through telomeres: a means to an end', *Trends Biochem Sci*, 40(9), pp. 504-15.

Martins-Taylor, K., Nisler, B.S., Taapken, S.M., Compton, T., Crandall, L., Montgomery, K.D., Lalande, M. and Xu, R.-H. (2011) 'Recurrent copy number variations in human induced pluripotent stem cells', *Nature biotechnology*, 29(6), pp. 488-91.

Matsui, W.H., Brodsky, R.A., Smith, B.D., Borowitz, M.J. and Jones, R.J. (2006) 'Quantitative analysis of bone marrow CD34 cells in aplastic anemia and hypoplastic myelodysplastic syndromes', *Leukemia*, 20(3), pp. 458-62.

Matsui, Y., Zsebo, K. and Hogan, B.L. (1992) 'Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture', *Cell*, 70(5), pp. 841-7.
Medvinsky, A. and Dzierzak, E. (1996) 'Definitive hematopoiesis is autonomously initiated by the AGM region', *Cell*, 86(6), pp. 897-906.
Meng, A., Wang, Y., Van Zant, G. and Zhou, D. (2003) 'Ionizing radiation and busulfan induce premature senescence in murine bone marrow hematopoietic cells', *Cancer Res*, 63(17), pp. 5414-9.
Miano, M. and Dufour, C. (2015) 'The diagnosis and treatment of aplastic anemia: a review', *Int J Hematol*, 101(6), pp. 527-35.
Mills, J.A., Wang, K., Paluru, P., Ying, L., Lu, L., Galvao, A.M., Xu, D., Yao, Y., Sullivan, S.K., Sullivan, L.M., Mac, H., Omari, A., Jean, J.-C., Shen, S., Gower, A., Spira, A., Mostoslavsky, G., Kotton, D.N., French, D.L., Weiss, M.J. and Gadue, P. (2013) 'Clonal genetic and hematopoietic heterogeneity among human-induced pluripotent stem cell lines', *Blood*, 122(12), pp. 2047-51.
Mitalipov, S. and Wolf, D. (2009) 'Totipotency, pluripotency and nuclear reprogramming', *Adv Biochem Eng Biotechnol*, 114, pp. 185-99.
Mitchell, J.R., Wood, E. and Collins, K. (1999) 'A telomerase component is defective in the human disease dyskeratosis congenita', *Nature*, 402(6761), pp. 551-5.
Miyoshi, N., Ishii, H., Nagano, H., Haraguchi, N., Dewi, D.L., Kano, Y., Nishikawa, S., Tanemura, M., Mimori, K., Tanaka, F., Saito, T., Nishimura, J., Takemasa, I., Mizushima, T., Ikeda, M., Yamamoto, H., Sekimoto, M., Doki, Y. and Mori, M. (2011) 'Reprogramming of mouse and human cells to pluripotency using mature microRNAs', *Cell Stem Cell*, 8(6), pp. 633-8.
Moehrle, B.M. and Geiger, H. (2016) 'Aging of hematopoietic stem cells: DNA damage and mutations?', *Exp Hematol*, 44(10), pp. 895-901.
Montane, E., Ibanez, L., Vidal, X., Ballarin, E., Puig, R., Garcia, N. and Laporte, J.R. (2008) 'Epidemiology of aplastic anemia: a prospective multicenter study', *Haematologica*, 93(4), pp. 518-23.
Moore, M.A., Shieh, J.H. and Lee, G. (2006) 'Hematopoietic cells', *Methods Enzymol*, 418, pp. 208-42.
Morishima, T., Watanabe, K., Niwa, A., Hirai, H., Saida, S., Tanaka, T., Kato, I., Umeda, K., Hiramatsu, H., Saito, M.K., Matsubara, K., Adachi, S., Kobayashi, M., Nakahata, T. and Heike, T. (2014) 'Genetic correction of HAX1 in induced pluripotent stem cells from a patient with severe congenital neutropenia improves defective granulopoiesis', *Haematologica*, 99(1), pp. 19-27.
Muguruma, Y., Yahata, T., Miyatake, H., Sato, T., Uno, T., Itoh, J., Kato, S., Ito, M., Hotta, T. and Ando, K. (2006) 'Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment', *Blood*, 107(5), pp. 1878-87.
Muller, F.J., Goldmann, J., Loser, P. and Loring, J.F. (2010) 'A call to standardize teratoma assays used to define human pluripotent cell lines', *Cell Stem Cell*, 6(5), pp. 412-4.
Muller, L.U., Milsom, M.D., Harris, C.E., Vyas, R., Brumme, K.M., Parmar, K., Moreau, L.A., Schambach, A., Park, I.H., London, W.B., Strait, K., Schlaeger, T., Devine, A.L., Grassman, E., D'Andrea, A., Daley, G.Q. and Williams, D.A. (2012) 'Overcoming reprogramming resistance of Fanconi anemia cells', *Blood*, 119(23), pp. 5449-57.
Murakami, Y., Kosaka, H., Maeda, Y., Nishimura, J., Inoue, N., Ohishi, K., Okabe, M., Takeda, J. and Kinoshita, T. (2002) 'Inefficient response of T lymphocytes to

glycosylphosphatidylinositol anchor-negative cells: implications for paroxysmal nocturnal hemoglobinuria', *Blood*, 100(12), pp. 4116-22.
Musunuru, K. (2013) 'Genome editing of human pluripotent stem cells to generate human cellular disease models', *Dis Model Mech*, 6(4), pp. 896-904.
Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N. and Yamanaka, S. (2008) 'Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts', *Nat Biotechnol*, 26(1), pp. 101-6.
Nakagawa, M., Takizawa, N., Narita, M., Ichisaka, T. and Yamanaka, S. (2010) 'Promotion of direct reprogramming by transformation-deficient Myc', *Proc Natl Acad Sci U S A*, 107(32), pp. 14152-7.
Nakajima-Takagi, Y., Osawa, M., Oshima, M., Takagi, H., Miyagi, S., Endoh, M., Endo, T.A., Takayama, N., Eto, K., Toyoda, T., Koseki, H., Nakauchi, H. and Iwama, A. (2013) 'Role of SOX17 in hematopoietic development from human embryonic stem cells', *Blood*, 121(3), pp. 447-58.
Nakajima, F., Tokunaga, K. and Nakatsuji, N. (2007) 'Human leukocyte antigen matching estimations in a hypothetical bank of human embryonic stem cell lines in the Japanese population for use in cell transplantation therapy', *Stem Cells*, 25(4), pp. 983-5.
Narayan, A.D., Chase, J.L., Lewis, R.L., Tian, X., Kaufman, D.S., Thomson, J.A. and Zanjani, E.D. (2006) 'Human embryonic stem cell-derived hematopoietic cells are capable of engrafting primary as well as secondary fetal sheep recipients', *Blood*, 107(5), pp. 2180-3.
Nashun, B., Hill, P.W. and Hajkova, P. (2015) 'Reprogramming of cell fate: epigenetic memory and the erasure of memories past', *EMBO J*, 34(10), pp. 1296-308.
Nayak, R.C., Trump, L.R., Aronow, B.J., Myers, K., Mehta, P., Kalfa, T., Wellendorf, A.M., Valencia, C.A., Paddison, P.J., Horwitz, M.S., Grimes, H.L., Lutzko, C. and Cancelas, J.A. (2015) 'Pathogenesis of ELANE-mutant severe neutropenia revealed by induced pluripotent stem cells', *J Clin Invest*, 125(8), pp. 3103-16.
Newman, A.M. and Cooper, J.B. (2010) 'Lab-specific gene expression signatures in pluripotent stem cells', *Cell Stem Cell*, 7(2), pp. 258-62.
Nishino, K., Toyoda, M., Yamazaki-Inoue, M., Fukawatase, Y., Chikazawa, E., Sakaguchi, H., Akutsu, H. and Umezawa, A. (2011) 'DNA methylation dynamics in human induced pluripotent stem cells over time', *PLoS genetics*, 7(5), p. e1002085.
Nishizawa, M., Chonabayashi, K., Nomura, M., Tanaka, A., Nakamura, M., Inagaki, A., Nishikawa, M., Takei, I., Oishi, A., Tanabe, K., Ohnuki, M., Yokota, H., Koyanagi-Aoi, M., Okita, K., Watanabe, A., Takaori-Kondo, A., Yamanaka, S. and Yoshida, Y. (2016) 'Epigenetic Variation between Human Induced Pluripotent Stem Cell Lines Is an Indicator of Differentiation Capacity', *Cell Stem Cell*, 19(3), pp. 341-54.
Nistico, A. and Young, N.S. (1994) 'gamma-Interferon gene expression in the bone marrow of patients with aplastic anemia', *Ann Intern Med*, 120(6), pp. 463-9.
Niwa, A., Heike, T., Umeda, K., Oshima, K., Kato, I., Sakai, H., Suemori, H., Nakahata, T. and Saito, M.K. (2011) 'A novel serum-free monolayer culture for orderly hematopoietic differentiation of human pluripotent cells via mesodermal progenitors', *PLoS One*, 6(7), p. e22261.
Niwa, H., Miyazaki, J. and Smith, A.G. (2000) 'Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells', *Nat Genet*, 24(4), pp. 372-6.

O'Sullivan, R.J. and Karlseder, J. (2010) 'Telomeres: protecting chromosomes against genome instability', *Nat Rev Mol Cell Biol*, 11(3), pp. 171-81.
Ohi, Y., Qin, H., Hong, C., Blouin, L., Polo, J.M., Guo, T., Qi, Z., Downey, S.L., Manos, P.D., Rossi, D.J., Yu, J., Hebrok, M., Hochedlinger, K., Costello, J.F., Song, J.S. and Ramalho-Santos, M. (2011) 'Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells', *Nat Cell Biol*, 13(5), pp. 541-9.
Ohnuki, M. and Takahashi, K. (2015) 'Present and future challenges of induced pluripotent stem cells', *Philos Trans R Soc Lond B Biol Sci*, 370(1680), p. 20140367.
Okita, K., Ichisaka, T. and Yamanaka, S. (2007) 'Generation of germline-competent induced pluripotent stem cells', *Nature*, 448(7151), pp. 313-7.
Olivier, E.N., Marenah, L., McCahill, A., Condie, A., Cowan, S. and Mountford, J.C. (2016) 'High-Efficiency Serum-Free Feeder-Free Erythroid Differentiation of Human Pluripotent Stem Cells Using Small Molecules', *Stem Cells Transl Med*, 5(10), pp. 1394-1405.
Olivier, E.N., Qiu, C., Velho, M., Hirsch, R.E. and Bouhassira, E.E. (2006) 'Large-scale production of embryonic red blood cells from human embryonic stem cells', *Exp Hematol*, 34(12), pp. 1635-42.
Olnes, M.J., Scheinberg, P., Calvo, K.R., Desmond, R., Tang, Y., Dumitriu, B., Parikh, A.R., Soto, S., Biancotto, A., Feng, X., Lozier, J., Wu, C.O., Young, N.S. and Dunbar, C.E. (2012) 'Eltrombopag and improved hematopoiesis in refractory aplastic anemia', *N Engl J Med*, 367(1), pp. 11-9.
Osborn, M.J., Gabriel, R., Webber, B.R., DeFeo, A.P., McElroy, A.N., Jarjour, J., Starker, C.G., Wagner, J.E., Joung, J.K., Voytas, D.F., von Kalle, C., Schmidt, M., Blazar, B.R. and Tolar, J. (2015) 'Fanconi anemia gene editing by the CRISPR/Cas9 system', *Hum Gene Ther*, 26(2), pp. 114-26.
Paes, B., Moco, P.D., Pereira, C.G., Porto, G.S., de Sousa Russo, E.M., Reis, L.C.J., Covas, D.T. and Picanco-Castro, V. (2017) 'Ten years of iPSC: clinical potential and advances in vitro hematopoietic differentiation', *Cell Biol Toxicol*, 33(3), pp. 233-250.
Palis, J. (2014) 'Primitive and definitive erythropoiesis in mammals', *Front Physiol*, 5, p. 3.
Park, H.S., Park, S.N., Kyoungok, I., Kim, J.A., Hwang, S.M. and Lee, D.S. (2016) 'Telomere Length and Somatic Mutation in Aplastic Anemia- Correlation with Response to Treatement', *Blood*, 128, p. 1504.
Park, I.H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W. and Daley, G.Q. (2008) 'Reprogramming of human somatic cells to pluripotency with defined factors', *Nature*, 451(7175), pp. 141-6.
Pera, M.F., Reubinoff, B. and Trounson, A. (2000) 'Human embryonic stem cells', *J Cell Sci*, 113 (Pt 1), pp. 5-10.
Perlingeiro, R.C., Kyba, M., Bodie, S. and Daley, G.Q. (2003) 'A role for thrombopoietin in hemangioblast development', *Stem Cells*, 21(3), pp. 272-80.
Petermann, E., Orta, M.L., Issaeva, N., Schultz, N. and Helleday, T. (2010) 'Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair', *Mol Cell*, 37(4), pp. 492-502.
Pick, M., Azzola, L., Mossman, A., Stanley, E.G. and Elefanty, A.G. (2007) 'Differentiation of human embryonic stem cells in serum-free medium reveals distinct roles for bone morphogenetic protein 4, vascular endothelial growth

factor, stem cell factor, and fibroblast growth factor 2 in hematopoiesis', *Stem Cells*, 25(9), pp. 2206-14.

Plath, K. and Lowry, W.E. (2011) 'Progress in understanding reprogramming to the induced pluripotent state', *Nat Rev Genet*, 12(4), pp. 253-65.

Polo, J.M., Liu, S., Figueroa, M.E., Kulalert, W., Eminli, S., Tan, K.Y., Apostolou, E., Stadtfeld, M., Li, Y., Shioda, T., Natesan, S., Wagers, A.J., Melnick, A., Evans, T. and Hochedlinger, K. (2010) 'Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells', *Nat Biotechnol*, 28(8), pp. 848-55.

Pu, J.J., Hu, R., Mukhina, G.L., Carraway, H.E., McDevitt, M.A. and Brodsky, R.A. (2012) 'The small population of PIG-A mutant cells in myelodysplastic syndromes do not arise from multipotent hematopoietic stem cells', *Haematologica*, 97(8), pp. 1225-33.

Pulecio, J., Nivet, E., Sancho-Martinez, I., Vitaloni, M., Guenechea, G., Xia, Y., Kurian, L., Dubova, I., Bueren, J., Laricchia-Robbio, L. and Izpisua Belmonte, J.C. (2014) 'Conversion of human fibroblasts into monocyte-like progenitor cells', *Stem Cells*, 32(11), pp. 2923-38.

Qian, H., Buza-Vidas, N., Hyland, C.D., Jensen, C.T., Antonchuk, J., Mansson, R., Thoren, L.A., Ekblom, M., Alexander, W.S. and Jacobsen, S.E. (2007) 'Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells', *Cell Stem Cell*, 1(6), pp. 671-84.

Quillen, K., Wong, E., Scheinberg, P., Young, N.S., Walsh, T.J., Wu, C.O. and Leitman, S.F. (2009) 'Granulocyte transfusions in severe aplastic anemia: an eleven-year experience', *Haematologica*, 94(12), pp. 1661-8.

Rabbani, B., Mahdieh, N., Hosomichi, K., Nakaoka, H. and Inoue, I. (2012) 'Next-generation sequencing: impact of exome sequencing in characterizing Mendelian disorders', *J Hum Genet*, 57(10), pp. 621-32.

Raghupathy, R. and Derman, O. (2012) 'Response of paroxysmal nocturnal hemoglobinuria clone with aplastic anemia to rituximab', *Case Rep Hematol*, 2012, p. 106182.

Ramos-Mejia, V., Montes, R., Bueno, C., Ayllon, V., Real, P.J., Rodriguez, R. and Menendez, P. (2012) 'Residual expression of the reprogramming factors prevents differentiation of iPSC generated from human fibroblasts and cord blood CD34+ progenitors', *PLoS One*, 7(4), p. e35824.

Ran, D., Shia, W.J., Lo, M.C., Fan, J.B., Knorr, D.A., Ferrell, P.I., Ye, Z., Yan, M., Cheng, L., Kaufman, D.S. and Zhang, D.E. (2013) 'RUNX1a enhances hematopoietic lineage commitment from human embryonic stem cells and inducible pluripotent stem cells', *Blood*, 121(15), pp. 2882-90.

Raval, A., Behbehani, G.K., Nguyen le, X.T., Thomas, D., Kusler, B., Garbuzov, A., Ramunas, J., Holbrook, C., Park, C.Y., Blau, H., Nolan, G.P., Artandi, S.E. and Mitchell, B.S. (2015) 'Reversibility of Defective Hematopoiesis Caused by Telomere Shortening in Telomerase Knockout Mice', *PLoS One*, 10(7), p. e0131722.

Raya, A., Rodriguez-Piza, I., Guenechea, G., Vassena, R., Navarro, S., Barrero, M.J., Consiglio, A., Castella, M., Rio, P., Sleep, E., Gonzalez, F., Tiscornia, G., Garreta, E., Aasen, T., Veiga, A., Verma, I.M., Surralles, J., Bueren, J. and Izpisua Belmonte, J.C. (2009) 'Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells', *Nature*, 460(7251), pp. 53-9.

Real, P.J., Ligero, G., Ayllon, V., Ramos-Mejia, V., Bueno, C., Gutierrez-Aranda, I., Navarro-Montero, O., Lako, M. and Menendez, P. (2012) 'SCL/TAL1

regulates hematopoietic specification from human embryonic stem cells', *Mol Ther*, 20(7), pp. 1443-53.
Remold-O'Donnell, E., Zimmerman, C., Kenney, D. and Rosen, F.S. (1987) 'Expression on blood cells of sialophorin, the surface glycoprotein that is defective in Wiskott-Aldrich syndrome', *Blood*, 70(1), pp. 104-9.
Ren, J., Hou, X.Y., Ma, S.H., Zhang, F.K., Zhen, J.H., Sun, L., Sun, Y.X., Hao, Y.L., Cheng, Y.F., Hou, M., Xu, C.G., Zhang, M.H. and Peng, J. (2014) 'Elevated expression of CX3C chemokine receptor 1 mediates recruitment of T cells into bone marrow of patients with acquired aplastic anaemia', *J Intern Med*, 276(5), pp. 512-24.
Resnick, J.L., Bixler, L.S., Cheng, L. and Donovan, P.J. (1992) 'Long-term proliferation of mouse primordial germ cells in culture', *Nature*, 359(6395), pp. 550-1.
Rio, P., Banos, R., Lombardo, A., Quintana-Bustamante, O., Alvarez, L., Garate, Z., Genovese, P., Almarza, E., Valeri, A., Diez, B., Navarro, S., Torres, Y., Trujillo, J.P., Murillas, R., Segovia, J.C., Samper, E., Surralles, J., Gregory, P.D., Holmes, M.C., Naldini, L. and Bueren, J.A. (2014) 'Targeted gene therapy and cell reprogramming in Fanconi anemia', *EMBO Mol Med*, 6(6), pp. 835-48.
Risitano, A.M., Maciejewski, J.P., Green, S., Plasilova, M., Zeng, W. and Young, N.S. (2004) 'In-vivo dominant immune responses in aplastic anaemia: molecular tracking of putatively pathogenetic T-cell clones by TCR beta-CDR3 sequencing', *Lancet*, 364(9431), pp. 355-64.
Risueno, R.M., Sachlos, E., Lee, J.H., Lee, J.B., Hong, S.H., Szabo, E. and Bhatia, M. (2012) 'Inability of human induced pluripotent stem cell-hematopoietic derivatives to downregulate microRNAs in vivo reveals a block in xenograft hematopoietic regeneration', *Stem Cells*, 30(2), pp. 131-9.
Rivera, T., Haggblom, C., Cosconati, S. and Karlseder, J. (2017) 'A balance between elongation and trimming regulates telomere stability in stem cells', *Nat Struct Mol Biol*, 24(1), pp. 30-39.
Rizzo, S., Scopes, J., Draycott, G.S., Pocock, C., Foukaneli, T., Rutherford, T.R., Gordon-Smith, E.C. and Gibson, F.M. (2004) 'Quiescent (5-fluorouracil-resistant) aplastic anemia hematopoietic cells in vitro', *Exp Hematol*, 32(7), pp. 665-72.
Rizzo, S., Scopes, J., Elebute, M.O., Papadaki, H.A., Gordon-Smith, E.C. and Gibson, F.M. (2002) 'Stem cell defect in aplastic anemia: reduced long term culture-initiating cells (LTC-IC) in CD34+ cells isolated from aplastic anemia patient bone marrow', *Hematol J*, 3(5), pp. 230-6.
Robin, C., Bollerot, K., Mendes, S., Haak, E., Crisan, M., Cerisoli, F., Lauw, I., Kaimakis, P., Jorna, R., Vermeulen, M., Kayser, M., van der Linden, R., Imanirad, P., Verstegen, M., Nawaz-Yousaf, H., Papazian, N., Steegers, E., Cupedo, T. and Dzierzak, E. (2009) 'Human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development', *Cell Stem Cell*, 5(4), pp. 385-95.
Rodrigues, N.P., Tipping, A.J., Wang, Z. and Enver, T. (2012) 'GATA-2 mediated regulation of normal hematopoietic stem/progenitor cell function, myelodysplasia and myeloid leukemia', *Int J Biochem Cell Biol*, 44(3), pp. 457-60.
Rohani, L., Johnson, A.A., Arnold, A. and Stolzing, A. (2014) 'The aging signature: a hallmark of induced pluripotent stem cells?', *Aging Cell*, 13(1), pp. 2-7.

Ronn, R.E., Guibentif, C., Moraghebi, R., Chaves, P., Saxena, S., Garcia, B. and Woods, N.B. (2015) 'Retinoic acid regulates hematopoietic development from human pluripotent stem cells', *Stem Cell Reports*, 4(2), pp. 269-81.

Rouhani, F., Kumasaka, N., de Brito, M.C., Bradley, A., Vallier, L. and Gaffney, D. (2014) 'Genetic background drives transcriptional variation in human induced pluripotent stem cells', *PLoS genetics*, 10(6), p. e1004432.

Roundy, K.M., Jacobson, A.C., Weis, J.J. and Weis, J.H. (2010) 'The in vitro derivation of phenotypically mature and diverse B cells from immature spleen and bone marrow precursors', *European journal of immunology*, 40(4), pp. 1139-49.

Saeki, K., Nakahara, M., Matsuyama, S., Nakamura, N., Yogiashi, Y., Yoneda, A., Koyanagi, M., Kondo, Y. and Yuo, A. (2009) 'A feeder-free and efficient production of functional neutrophils from human embryonic stem cells', *Stem Cells*, 27(1), pp. 59-67.

Sakaguchi, H., Nishio, N., Hama, A., Kawashima, N., Wang, X., Narita, A., Doisaki, S., Xu, Y., Muramatsu, H., Yoshida, N., Takahashi, Y., Kudo, K., Moritake, H., Nakamura, K., Kobayashi, R., Ito, E., Yabe, H., Ohga, S., Ohara, A., Kojima, S. and Japan Childhood Aplastic Anemia Study, G. (2014) 'Peripheral blood lymphocyte telomere length as a predictor of response to immunosuppressive therapy in childhood aplastic anemia', *Haematologica*, 99(8), pp. 1312-6.

Samarasinghe, S. and Webb, D.K. (2012) 'How I manage aplastic anaemia in children', *Br J Haematol*, 157(1), pp. 26-40.

Sandler, V.M., Lis, R., Liu, Y., Kedem, A., James, D., Elemento, O., Butler, J.M., Scandura, J.M. and Rafii, S. (2014) 'Reprogramming human endothelial cells to haematopoietic cells requires vascular induction', *Nature*, 511(7509), pp. 312-8.

Sankaran, V.G. and Orkin, S.H. (2013) 'The switch from fetal to adult hemoglobin', *Cold Spring Harb Perspect Med*, 3(1), p. a011643.

Savage, S.A., Calado, R.T., Xin, Z.T., Ly, H., Young, N.S. and Chanock, S.J. (2006) 'Genetic variation in telomeric repeat binding factors 1 and 2 in aplastic anemia', *Exp Hematol*, 34(5), pp. 664-71.

Savage, S.A., Giri, N., Baerlocher, G.M., Orr, N., Lansdorp, P.M. and Alter, B.P. (2008) 'TINF2, a component of the shelterin telomere protection complex, is mutated in dyskeratosis congenita', *Am J Hum Genet*, 82(2), pp. 501-9.

Sayed, N., Liu, C. and Wu, J.C. (2016) 'Translation of Human-Induced Pluripotent Stem Cells: From Clinical Trial in a Dish to Precision Medicine', *J Am Coll Cardiol*, 67(18), pp. 2161-76.

Scheinberg, P. and Chen, J. (2013) 'Aplastic anemia: what have we learned from animal models and from the clinic', *Semin Hematol*, 50(2), pp. 156-64.

Scheinberg, P., Cooper, J.N., Sloand, E.M., Wu, C.O., Calado, R.T. and Young, N.S. (2010) 'Association of telomere length of peripheral blood leukocytes with hematopoietic relapse, malignant transformation, and survival in severe aplastic anemia', *JAMA*, 304(12), pp. 1358-64.

Schrezenmeier, H., Jenal, M., Herrmann, F., Heimpel, H. and Raghavachar, A. (1996) 'Quantitative analysis of cobblestone area-forming cells in bone marrow of patients with aplastic anemia by limiting dilution assay', *Blood*, 88(12), pp. 4474-80.

Scopes, J., Bagnara, M., Gordon-Smith, E.C., Ball, S.E. and Gibson, F.M. (1994) 'Haemopoietic progenitor cells are reduced in aplastic anaemia', *Br J Haematol*, 86(2), pp. 427-30.

Senju, S., Haruta, M., Matsumura, K., Matsunaga, Y., Fukushima, S., Ikeda, T., Takamatsu, K., Irie, A. and Nishimura, Y. (2011) 'Generation of dendritic cells and macrophages from human induced pluripotent stem cells aiming at cell therapy', *Gene Ther*, 18(9), pp. 874-83.

Shamblott, M.J., Axelman, J., Wang, S., Bugg, E.M., Littlefield, J.W., Donovan, P.J., Blumenthal, P.D., Huggins, G.R. and Gearhart, J.D. (1998) 'Derivation of pluripotent stem cells from cultured human primordial germ cells', *Proc Natl Acad Sci U S A*, 95(23), pp. 13726-31.

Shi, Y., Inoue, H., Wu, J.C. and Yamanaka, S. (2017) 'Induced pluripotent stem cell technology: a decade of progress', *Nat Rev Drug Discov*, 16(2), pp. 115-130.

Shimamura, A. and Alter, B.P. (2010) 'Pathophysiology and management of inherited bone marrow failure syndromes', *Blood Rev*, 24(3), pp. 101-22.

Silva, J., Nichols, J., Theunissen, T.W., Guo, G., van Oosten, A.L., Barrandon, O., Wray, J., Yamanaka, S., Chambers, I. and Smith, A. (2009) 'Nanog is the gateway to the pluripotent ground state', *Cell*, 138(4), pp. 722-37.

Singh, A. and Xu, Y.J. (2016) 'The Cell Killing Mechanisms of Hydroxyurea', *Genes (Basel)*, 7(11).

Sloand, E., Kim, S., Maciejewski, J.P., Tisdale, J., Follmann, D. and Young, N.S. (2002) 'Intracellular interferon-gamma in circulating and marrow T cells detected by flow cytometry and the response to immunosuppressive therapy in patients with aplastic anemia', *Blood*, 100(4), pp. 1185-91.

Slukvin, I.I. (2013) 'Hematopoietic specification from human pluripotent stem cells: current advances and challenges toward de novo generation of hematopoietic stem cells', *Blood*, 122(25), pp. 4035-46.

Smith, Z.D., Chan, M.M., Humm, K.C., Karnik, R., Mekhoubad, S., Regev, A., Eggan, K. and Meissner, A. (2014) 'DNA methylation dynamics of the human preimplantation embryo', *Nature*, 511(7511), pp. 611-5.

Soldner, F. and Jaenisch, R. (2012) 'Medicine. iPSC disease modeling', *Science (New York, N Y)*, 338(6111), pp. 1155-6.

Solomou, E.E., Rezvani, K., Mielke, S., Malide, D., Keyvanfar, K., Visconte, V., Kajigaya, S., Barrett, A.J. and Young, N.S. (2007) 'Deficient CD4+ CD25+ FOXP3+ T regulatory cells in acquired aplastic anemia', *Blood*, 110(5), pp. 1603-6.

Solter, D. (2006) 'From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research', *Nat Rev Genet*, 7(4), pp. 319-27.

Song, J.Y., Kuang, L.P., Wang, Y., Li, Y.H., Wu, J.L., Zhang, H., Li, L., Wang, Y.C., Jiang, Z.J. and Xiao, Y. (2013) '[The relationship of telomere and telomerase activity with outcome of aplastic anemia after immunosuppressive therapy]', *Zhonghua Xue Ye Xue Za Zhi*, 34(9), pp. 771-6.

Sridharan, R., Tchieu, J., Mason, M.J., Yachechko, R., Kuoy, E., Horvath, S., Zhou, Q. and Plath, K. (2009) 'Role of the murine reprogramming factors in the induction of pluripotency', *Cell*, 136(2), pp. 364-77.

Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G. and Hochedlinger, K. (2008) 'Induced pluripotent stem cells generated without viral integration', *Science*, 322(5903), pp. 945-9.

Staerk, J., Dawlaty, M.M., Gao, Q., Maetzel, D., Hanna, J., Sommer, C.A., Mostoslavsky, G. and Jaenisch, R. (2010) 'Reprogramming of human peripheral blood cells to induced pluripotent stem cells', *Cell Stem Cell*, 7(1), pp. 20-4.

Stevens, L.C. (1967) 'The biology of teratomas', *Adv Morphog*, 6, pp. 1-31.

Stevens, L.C. and Little, C.C. (1954) 'Spontaneous Testicular Teratomas in an Inbred Strain of Mice', *Proc Natl Acad Sci U S A*, 40(11), pp. 1080-7.

Sturgeon, C.M., Ditadi, A., Awong, G., Kennedy, M. and Keller, G. (2014) 'Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells', *Nat Biotechnol*, 32(6), pp. 554-61.

Sugimura, R., Jha, D.K., Han, A., Soria-Valles, C., da Rocha, E.L., Lu, Y.F., Goettel, J.A., Serrao, E., Rowe, R.G., Malleshaiah, M., Wong, I., Sousa, P., Zhu, T.N., Ditadi, A., Keller, G., Engelman, A.N., Snapper, S.B., Doulatov, S. and Daley, G.Q. (2017) 'Haematopoietic stem and progenitor cells from human pluripotent stem cells', *Nature*, 545(7655), pp. 432-438.

Sumi, T., Tsuneyoshi, N., Nakatsuji, N. and Suemori, H. (2008) 'Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, Activin/Nodal and BMP signaling', *Development*, 135(17), pp. 2969-79.

Sun, H., Tsai, Y., Nowak, I., Liesveld, J. and Chen, Y. (2012) 'Eltrombopag, a thrombopoietin receptor agonist, enhances human umbilical cord blood hematopoietic stem/primitive progenitor cell expansion and promotes multi-lineage hematopoiesis', *Stem Cell Res*, 9(2), pp. 77-86.

Sureda, A., Bader, P., Cesaro, S., Dreger, P., Duarte, R.F., Dufour, C., Falkenburg, J.H., Farge-Bancel, D., Gennery, A., Kroger, N., Lanza, F., Marsh, J.C., Nagler, A., Peters, C., Velardi, A., Mohty, M. and Madrigal, A. (2015) 'Indications for allo- and auto-SCT for haematological diseases, solid tumours and immune disorders: current practice in Europe, 2015', *Bone Marrow Transplant*, 50(8), pp. 1037-56.

Suzuki, N., Yamazaki, S., Yamaguchi, T., Okabe, M., Masaki, H., Takaki, S., Otsu, M. and Nakauchi, H. (2013) 'Generation of engraftable hematopoietic stem cells from induced pluripotent stem cells by way of teratoma formation', *Mol Ther*, 21(7), pp. 1424-31.

Taapken, S.M., Nisler, B.S., Newton, M.A., Sampsell-Barron, T.L., Leonhard, K.A., McIntire, E.M. and Montgomery, K.D. (2011) 'Karotypic abnormalities in human induced pluripotent stem cells and embryonic stem cells', *Nat Biotechnol*, 29(4), pp. 313-4.

Tachibana, M., Amato, P., Sparman, M., Gutierrez, N.M., Tippner-Hedges, R., Ma, H., Kang, E., Fulati, A., Lee, H.S., Sritanaudomchai, H., Masterson, K., Larson, J., Eaton, D., Sadler-Fredd, K., Battaglia, D., Lee, D., Wu, D., Jensen, J., Patton, P., Gokhale, S., Stouffer, R.L., Wolf, D. and Mitalipov, S. (2013) 'Human embryonic stem cells derived by somatic cell nuclear transfer', *Cell*, 153(6), pp. 1228-38.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007) 'Induction of pluripotent stem cells from adult human fibroblasts by defined factors', *Cell*, 131(5), pp. 861-72.

Takahashi, K. and Yamanaka, S. (2006) 'Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors', *Cell*, 126(4), pp. 663-76.

Takahashi, K. and Yamanaka, S. (2016) 'A decade of transcription factor-mediated reprogramming to pluripotency', *Nat Rev Mol Cell Biol*, 17(3), pp. 183-93.

Takai, H., Smogorzewska, A. and de Lange, T. (2003) 'DNA damage foci at dysfunctional telomeres', *Curr Biol*, 13(17), pp. 1549-56.

Takashima, Y., Guo, G., Loos, R., Nichols, J., Ficz, G., Krueger, F., Oxley, D., Santos, F., Clarke, J., Mansfield, W., Reik, W., Bertone, P. and Smith, A. (2014)

'Resetting transcription factor control circuitry toward ground-state pluripotency in human', *Cell*, 158(6), pp. 1254-69.

Tavian, M., Hallais, M.F. and Peault, B. (1999) 'Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo', *Development*, 126(4), pp. 793-803.

Tavian, M., Robin, C., Coulombel, L. and Peault, B. (2001) 'The human embryo, but not its yolk sac, generates lympho-myeloid stem cells: mapping multipotent hematopoietic cell fate in intraembryonic mesoderm', *Immunity*, 15(3), pp. 487-95.

Thatava, T., Kudva, Y.C., Edukulla, R., Squillace, K., De Lamo, J.G., Khan, Y.K., Sakuma, T., Ohmine, S., Terzic, A. and Ikeda, Y. (2013) 'Intrapatient variations in type 1 diabetes-specific iPS cell differentiation into insulin-producing cells', *Mol Ther*, 21(1), pp. 228-39.

Theunissen, T.W., Friedli, M., He, Y., Planet, E., O'Neil, R.C., Markoulaki, S., Pontis, J., Wang, H., Iouranova, A., Imbeault, M., Duc, J., Cohen, M.A., Wert, K.J., Castanon, R., Zhang, Z., Huang, Y., Nery, J.R., Drotar, J., Lungjangwa, T., Trono, D., Ecker, J.R. and Jaenisch, R. (2016) 'Molecular Criteria for Defining the Naive Human Pluripotent State', *Cell stem cell*, 19(4), pp. 502-515.

Theunissen, T.W., Powell, B.E., Wang, H., Mitalipova, M., Faddah, D.A., Reddy, J., Fan, Z.P., Maetzel, D., Ganz, K., Shi, L., Lungjangwa, T., Imsoonthornruksa, S., Stelzer, Y., Rangarajan, S., D'Alessio, A., Zhang, J., Gao, Q., Dawlaty, M.M., Young, R.A., Gray, N.S. and Jaenisch, R. (2014) 'Systematic identification of culture conditions for induction and maintenance of naive human pluripotency', *Cell stem cell*, 15(4), pp. 471-87.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. and Jones, J.M. (1998) 'Embryonic stem cell lines derived from human blastocysts', *Science*, 282(5391), pp. 1145-7.

Tian, X., Hexum, M.K., Penchev, V.R., Taylor, R.J., Shultz, L.D. and Kaufman, D.S. (2009) 'Bioluminescent imaging demonstrates that transplanted human embryonic stem cell-derived CD34(+) cells preferentially develop into endothelial cells', *Stem Cells*, 27(11), pp. 2675-85.

Tian, X., Woll, P.S., Morris, J.K., Linehan, J.L. and Kaufman, D.S. (2006) 'Hematopoietic engraftment of human embryonic stem cell-derived cells is regulated by recipient innate immunity', *Stem Cells*, 24(5), pp. 1370-80.

Tilgner, K., Neganova, I., Moreno-Gimeno, I., Al-Aama, J.Y., Burks, D., Yung, S., Singhapol, C., Saretzki, G., Evans, J., Gorbunova, V., Gennery, A., Przyborski, S., Stojkovic, M., Armstrong, L., Jeggo, P. and Lako, M. (2013) 'A human iPSC model of Ligase IV deficiency reveals an important role for NHEJ-mediated-DSB repair in the survival and genomic stability of induced pluripotent stem cells and emerging haematopoietic progenitors', *Cell Death Differ*, 20(8), pp. 1089-100.

Timmermans, F., Velghe, I., Vanwalleghem, L., De Smedt, M., Van Coppernolle, S., Taghon, T., Moore, H.D., Leclercq, G., Langerak, A.W., Kerre, T., Plum, J. and Vandekerckhove, B. (2009) 'Generation of T cells from human embryonic stem cell-derived hematopoietic zones', *J Immunol*, 182(11), pp. 6879-88.

Togarrati, P.P. and Suknuntha, K. (2012) 'Generation of mature hematopoietic cells from human pluripotent stem cells', *Int J Hematol*, 95(6), pp. 617-23.

Townsley, D.M., Dumitriu, B., Liu, D., Biancotto, A., Weinstein, B., Chen, C., Hardy, N., Mihalek, A.D., Lingala, S., Kim, Y.J., Yao, J., Jones, E., Gochuico, B.R., Heller, T., Wu, C.O., Calado, R.T., Scheinberg, P. and Young, N.S. (2016a)

'Danazol Treatment for Telomere Diseases', *N Engl J Med*, 374(20), pp. 1922-31.

Townsley, D.M., Dumitriu, B. and Young, N.S. (2014) 'Bone marrow failure and the telomeropathies', *Blood*, 124(18), pp. 2775-83.

Townsley, D.M., Dumitriu, B. and Young, N.S. (2016b) 'Danazol Treatment for Telomere Diseases', *N Engl J Med*, 375(11), pp. 1095-6.

Trautmann, K., Jakob, C., von Grunhagen, U., Schleyer, E., Brummendorf, T.H., Siegert, G., Ehninger, G. and Platzbecker, U. (2012) 'Eltrombopag fails to improve severe thrombocytopenia in late-stage dyskeratosis congenita and diamond-blackfan-anaemia', *Thromb Haemost*, 108(2), pp. 397-8.

Tsankov, A.M., Akopian, V., Pop, R., Chetty, S., Gifford, C.A., Daheron, L., Tsankova, N.M. and Meissner, A. (2015) 'A qPCR ScoreCard quantifies the differentiation potential of human pluripotent stem cells', *Nat Biotechnol*, 33(11), pp. 1182-92.

Tseng, S.Y., Nishimoto, K.P., Silk, K.M., Majumdar, A.S., Dawes, G.N., Waldmann, H., Fairchild, P.J., Lebkowski, J.S. and Reddy, A. (2009) 'Generation of immunogenic dendritic cells from human embryonic stem cells without serum and feeder cells', *Regen Med*, 4(4), pp. 513-26.

Tulpule, A., Kelley, J.M., Lensch, M.W., McPherson, J., Park, I.H., Hartung, O., Nakamura, T., Schlaeger, T.M., Shimamura, A. and Daley, G.Q. (2013) 'Pluripotent stem cell models of Shwachman-Diamond syndrome reveal a common mechanism for pancreatic and hematopoietic dysfunction', *Cell Stem Cell*, 12(6), pp. 727-36.

Turton, J.A., Havard, A.C., Robinson, S., Holt, D.E., Andrews, C.M., Fagg, R. and Williams, T.C. (2000) 'An assessment of chloramphenicol and thiamphenicol in the induction of aplastic anaemia in the BALB/c mouse', *Food Chem Toxicol*, 38(10), pp. 925-38.

Uccelli, A., Moretta, L. and Pistoia, V. (2008) 'Mesenchymal stem cells in health and disease', *Nat Rev Immunol*, 8(9), pp. 726-36.

Uenishi, G., Theisen, D., Lee, J.H., Kumar, A., Raymond, M., Vodyanik, M., Swanson, S., Stewart, R., Thomson, J. and Slukvin, I. (2014) 'Tenascin C promotes hematoendothelial development and T lymphoid commitment from human pluripotent stem cells in chemically defined conditions', *Stem Cell Reports*, 3(6), pp. 1073-84.

Vodyanik, M.A., Bork, J.A., Thomson, J.A. and Slukvin, II (2005) 'Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential', *Blood*, 105(2), pp. 617-26.

Vodyanik, M.A., Thomson, J.A. and Slukvin, II (2006) 'Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures', *Blood*, 108(6), pp. 2095-105.

Vodyanik, M.A., Yu, J., Zhang, X., Tian, S., Stewart, R., Thomson, J.A. and Slukvin, II (2010) 'A mesoderm-derived precursor for mesenchymal stem and endothelial cells', *Cell Stem Cell*, 7(6), pp. 718-29.

Vulliamy, T., Marrone, A., Goldman, F., Dearlove, A., Bessler, M., Mason, P.J. and Dokal, I. (2001) 'The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita', *Nature*, 413(6854), pp. 432-5.

Vulliamy, T.J., Marrone, A., Knight, S.W., Walne, A., Mason, P.J. and Dokal, I. (2006) 'Mutations in dyskeratosis congenita: their impact on telomere length and the diversity of clinical presentation', *Blood*, 107(7), pp. 2680-5.

Vulliamy, T.J., Walne, A., Baskaradas, A., Mason, P.J., Marrone, A. and Dokal, I. (2005) 'Mutations in the reverse transcriptase component of telomerase (TERT) in patients with bone marrow failure', *Blood Cells Mol Dis*, 34(3), pp. 257-63.

Wahlster, L. and Daley, G.Q. (2016) 'Progress towards generation of human haematopoietic stem cells', *Nat Cell Biol*, 18(11), pp. 1111-1117.

Walasek, M.A., van Os, R. and de Haan, G. (2012) 'Hematopoietic stem cell expansion: challenges and opportunities', *Ann N Y Acad Sci*, 1266, pp. 138-50.

Walne, A.J. and Dokal, I. (2009) 'Advances in the understanding of dyskeratosis congenita', *Br J Haematol*, 145(2), pp. 164-72.

Walne, A.J., Vulliamy, T., Kirwan, M., Plagnol, V. and Dokal, I. (2013) 'Constitutional mutations in RTEL1 cause severe dyskeratosis congenita', *Am J Hum Genet*, 92(3), pp. 448-53.

Walne, A.J., Vulliamy, T., Marrone, A., Beswick, R., Kirwan, M., Masunari, Y., Al-Qurashi, F.H., Aljurf, M. and Dokal, I. (2007) 'Genetic heterogeneity in autosomal recessive dyskeratosis congenita with one subtype due to mutations in the telomerase-associated protein NOP10', *Hum Mol Genet*, 16(13), pp. 1619-29.

Wang, F., Yin, Y., Ye, X., Liu, K., Zhu, H., Wang, L., Chiourea, M., Okuka, M., Ji, G., Dan, J., Zuo, B., Li, M., Zhang, Q., Liu, N., Chen, L., Pan, X., Gagos, S., Keefe, D.L. and Liu, L. (2012) 'Molecular insights into the heterogeneity of telomere reprogramming in induced pluripotent stem cells', *Cell Res*, 22(4), pp. 757-68.

Wang, L., Menendez, P., Shojaei, F., Li, L., Mazurier, F., Dick, J.E., Cerdan, C., Levac, K. and Bhatia, M. (2005) 'Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression', *J Exp Med*, 201(10), pp. 1603-14.

Wang, X. and Dai, J. (2010) 'Concise review: isoforms of OCT4 contribute to the confusing diversity in stem cell biology', *Stem cells (Dayton, Ohio)*, 28(5), pp. 885-93.

Wang, Y. and Nakayama, N. (2009) 'WNT and BMP signaling are both required for hematopoietic cell development from human ES cells', *Stem Cell Res*, 3(2-3), pp. 113-25.

Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., Daley, G.Q., Brack, A.S., Collins, J.J., Cowan, C., Schlaeger, T.M. and Rossi, D.J. (2010) 'Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA', *Cell Stem Cell*, 7(5), pp. 618-30.

Weinberger, L., Ayyash, M., Novershtern, N. and Hanna, J.H. (2016) 'Dynamic stem cell states: naive to primed pluripotency in rodents and humans', *Nature reviews Molecular cell biology*, 17(3), pp. 155-69.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H. (1997) 'Viable offspring derived from fetal and adult mammalian cells', *Nature*, 385(6619), pp. 810-3.

Winkler, T., Hong, S.G., Decker, J.E., Morgan, M.J., Wu, C., Hughes, W.M.t., Yang, Y., Wangsa, D., Padilla-Nash, H.M., Ried, T., Young, N.S., Dunbar, C.E. and Calado, R.T. (2013) 'Defective telomere elongation and hematopoiesis from telomerase-mutant aplastic anemia iPSCs', *J Clin Invest*, 123(5), pp. 1952-63.

Woll, P.S., Morris, J.K., Painschab, M.S., Marcus, R.K., Kohn, A.D., Biechele, T.L., Moon, R.T. and Kaufman, D.S. (2008) 'Wnt signaling promotes

hematoendothelial cell development from human embryonic stem cells', *Blood*, 111(1), pp. 122-31.
Wong, M.S., Wright, W.E. and Shay, J.W. (2014) 'Alternative splicing regulation of telomerase: a new paradigm?', *Trends Genet*, 30(10), pp. 430-8.
Woo, D.H., Chen, Q., Yang, T.L., Glineburg, M.R., Hoge, C., Leu, N.A., Johnson, F.B. and Lengner, C.J. (2016) 'Enhancing a Wnt-Telomere Feedback Loop Restores Intestinal Stem Cell Function in a Human Organotypic Model of Dyskeratosis Congenita', *Cell Stem Cell*, 19(3), pp. 397-405.
Xie, H., Ye, M., Feng, R. and Graf, T. (2004) 'Stepwise reprogramming of B cells into macrophages', *Cell*, 117(5), pp. 663-76.
Xu, Y., Takahashi, Y., Yoshimi, A., Tanaka, M., Yagasaki, H. and Kojima, S. (2009) 'Immunosuppressive activity of mesenchymal stem cells is not decreased in children with aplastic anemia', *Int J Hematol*, 89(1), pp. 126-7.
Yamaguchi, H., Baerlocher, G.M., Lansdorp, P.M., Chanock, S.J., Nunez, O., Sloand, E. and Young, N.S. (2003) 'Mutations of the human telomerase RNA gene (TERC) in aplastic anemia and myelodysplastic syndrome', *Blood*, 102(3), pp. 916-8.
Yamaguchi, H., Calado, R.T., Ly, H., Kajigaya, S., Baerlocher, G.M., Chanock, S.J., Lansdorp, P.M. and Young, N.S. (2005) 'Mutations in TERT, the gene for telomerase reverse transcriptase, in aplastic anemia', *N Engl J Med*, 352(14), pp. 1413-24.
Yamanaka, S. and Blau, H.M. (2010) 'Nuclear reprogramming to a pluripotent state by three approaches', *Nature*, 465(7299), pp. 704-12.
Yang, D., Zhang, Z.J., Oldenburg, M., Ayala, M. and Zhang, S.C. (2008) 'Human embryonic stem cell-derived dopaminergic neurons reverse functional deficit in parkinsonian rats', *Stem Cells*, 26(1), pp. 55-63.
Yoshihara, M., Hayashizaki, Y. and Murakawa, Y. (2017) 'Genomic Instability of iPSCs: Challenges Towards Their Clinical Applications', *Stem Cell Rev*, 13(1), pp. 7-16.
Yoshizato, T., Dumitriu, B., Hosokawa, K., Makishima, H., Yoshida, K., Townsley, D., Sato-Otsubo, A., Sato, Y., Liu, D., Suzuki, H., Wu, C.O., Shiraishi, Y., Clemente, M.J., Kataoka, K., Shiozawa, Y., Okuno, Y., Chiba, K., Tanaka, H., Nagata, Y., Katagiri, T., Kon, A., Sanada, M., Scheinberg, P., Miyano, S., Maciejewski, J.P., Nakao, S., Young, N.S. and Ogawa, S. (2015) 'Somatic Mutations and Clonal Hematopoiesis in Aplastic Anemia', *N Engl J Med*, 373(1), pp. 35-47.
Young, M.A., Larson, D.E., Sun, C.W., George, D.R., Ding, L., Miller, C.A., Lin, L., Pawlik, K.M., Chen, K., Fan, X., Schmidt, H., Kalicki-Veizer, J., Cook, L.L., Swift, G.W., Demeter, R.T., Wendl, M.C., Sands, M.S., Mardis, E.R., Wilson, R.K., Townes, T.M. and Ley, T.J. (2012) 'Background mutations in parental cells account for most of the genetic heterogeneity of induced pluripotent stem cells', *Cell Stem Cell*, 10(5), pp. 570-82.
Young, N.S. (2005) 'Paroxysmal nocturnal hemoglobinuria: current issues in pathophysiology and treatment', *Curr Hematol Rep*, 4(2), pp. 103-9.
Young, N.S. (2013) 'Current concepts in the pathophysiology and treatment of aplastic anemia', *Hematology Am Soc Hematol Educ Program*, 2013, pp. 76-81.
Young, N.S., Bacigalupo, A. and Marsh, J.C. (2010) 'Aplastic anemia: pathophysiology and treatment', *Biol Blood Marrow Transplant*, 16(1 Suppl), pp. S119-25.
Young, N.S., Calado, R.T. and Scheinberg, P. (2006) 'Current concepts in the pathophysiology and treatment of aplastic anemia', *Blood*, 108(8), pp. 2509-19.

Young, N.S. and Kaufman, D.W. (2008) 'The epidemiology of acquired aplastic anemia', *Haematologica*, 93(4), pp. 489-92.
Young, N.S., Scheinberg, P. and Calado, R.T. (2008) 'Aplastic anemia', *Curr Opin Hematol*, 15(3), pp. 162-8.
Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, R., Slukvin, II and Thomson, J.A. (2009) 'Human induced pluripotent stem cells free of vector and transgene sequences', *Science*, 324(5928), pp. 797-801.
Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, II and Thomson, J.A. (2007) 'Induced pluripotent stem cell lines derived from human somatic cells', *Science*, 318(5858), pp. 1917-20.
Yung, S.K., Tilgner, K., Ledran, M.H., Habibollah, S., Neganova, I., Singhapol, C., Saretzki, G., Stojkovic, M., Armstrong, L., Przyborski, S. and Lako, M. (2013) 'Brief report: human pluripotent stem cell models of fanconi anemia deficiency reveal an important role for fanconi anemia proteins in cellular reprogramming and survival of hematopoietic progenitors', *Stem Cells*, 31(5), pp. 1022-9.
Zalzman, M., Falco, G., Sharova, L.V., Nishiyama, A., Thomas, M., Lee, S.L., Stagg, C.A., Hoang, H.G., Yang, H.T., Indig, F.E., Wersto, R.P. and Ko, M.S. (2010) 'Zscan4 regulates telomere elongation and genomic stability in ES cells', *Nature*, 464(7290), pp. 858-63.
Zambidis, E.T., Peault, B., Park, T.S., Bunz, F. and Civin, C.I. (2005) 'Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development', *Blood*, 106(3), pp. 860-70.
Zeigler, F.C., de Sauvage, F., Widmer, H.R., Keller, G.A., Donahue, C., Schreiber, R.D., Malloy, B., Hass, P., Eaton, D. and Matthews, W. (1994) 'In vitro megakaryocytopoietic and thrombopoietic activity of c-mpl ligand (TPO) on purified murine hematopoietic stem cells', *Blood*, 84(12), pp. 4045-52.
Zeman, M.K. and Cimprich, K.A. (2014) 'Causes and consequences of replication stress', *Nat Cell Biol*, 16(1), pp. 2-9.
Zeng, W., Chen, G., Kajigaya, S., Nunez, O., Charrow, A., Billings, E.M. and Young, N.S. (2004) 'Gene expression profiling in CD34 cells to identify differences between aplastic anemia patients and healthy volunteers', *Blood*, 103(1), pp. 325-32.
Zeng, W., Miyazato, A., Chen, G., Kajigaya, S., Young, N.S. and Maciejewski, J.P. (2006) 'Interferon-gamma-induced gene expression in CD34 cells: identification of pathologic cytokine-specific signature profiles', *Blood*, 107(1), pp. 167-75.
Zeng, Y. and Katsanis, E. (2015) 'The complex pathophysiology of acquired aplastic anaemia', *Clin Exp Immunol*, 180(3), pp. 361-70.
Zhang, C.C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C. and Lodish, H.F. (2006) 'Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells', *Nat Med*, 12(2), pp. 240-5.
Zhang, M.Y., Keel, S.B., Walsh, T., Lee, M.K., Gulsuner, S., Watts, A.C., Pritchard, C.C., Salipante, S.J., Jeng, M.R., Hofmann, I., Williams, D.A., Fleming, M.D., Abkowitz, J.L., King, M.C. and Shimamura, A. (2015) 'Genomic analysis of bone marrow failure and myelodysplastic syndromes reveals phenotypic and diagnostic complexity', *Haematologica*, 100(1), pp. 42-8.
Zhang, P., Li, J., Tan, Z., Wang, C., Liu, T., Chen, L., Yong, J., Jiang, W., Sun, X., Du, L., Ding, M. and Deng, H. (2008) 'Short-term BMP-4 treatment initiates

mesoderm induction in human embryonic stem cells', *Blood*, 111(4), pp. 1933-41.

Zhang Wendy, Y., de Almeida Patricia, E., and Wu Joseph, C., (June 10, 2012), StemBook, ed. The Stem Cell Research Community, StemBook, doi/ 10.3824/ .1.53.1, http://www.stembook.org. (June 10, 2012) *Teratoma formation: A tool for monitoring pluripotency in stem cell research*. StemBook. Available at: doi/ 10.3824/ .1.53.1, http://www.stembook.org.

Zhang, Y. and Kolesar, J.M. (2011) 'Eltrombopag: an oral thrombopoietin receptor agonist for the treatment of idiopathic thrombocytopenic purpura', *Clin Ther*, 33(11), pp. 1560-76.

Zhu, L., Gomez-Duran, A., Saretzki, G., Jin, S., Tilgner, K., Melguizo-Sanchis, D., Anyfantis, G., Al-Aama, J., Vallier, L., Chinnery, P., Lako, M. and Armstrong, L. (2016) 'The mitochondrial protein CHCHD2 primes the differentiation potential of human induced pluripotent stem cells to neuroectodermal lineages', *J Cell Biol*, 215(2), pp. 187-202.

Zimmermann, S. and Martens, U.M. (2008) 'Telomeres, senescence, and hematopoietic stem cells', *Cell Tissue Res*, 331(1), pp. 79-90.

Zovein, A.C., Hofmann, J.J., Lynch, M., French, W.J., Turlo, K.A., Yang, Y., Becker, M.S., Zanetta, L., Dejana, E., Gasson, J.C., Tallquist, M.D. and Iruela-Arispe, M.L. (2008) 'Fate tracing reveals the endothelial origin of hematopoietic stem cells', *Cell Stem Cell*, 3(6), pp. 625-36.

APPENDIX A: Gating strategies for flow cytometric analysis

Positive population for SSEA-4 and TRA-1-60 markers on day 0 of haematopoietic differentiation was gated using Fluorescence Minus One controls for each specific marker (**Figure 58**).

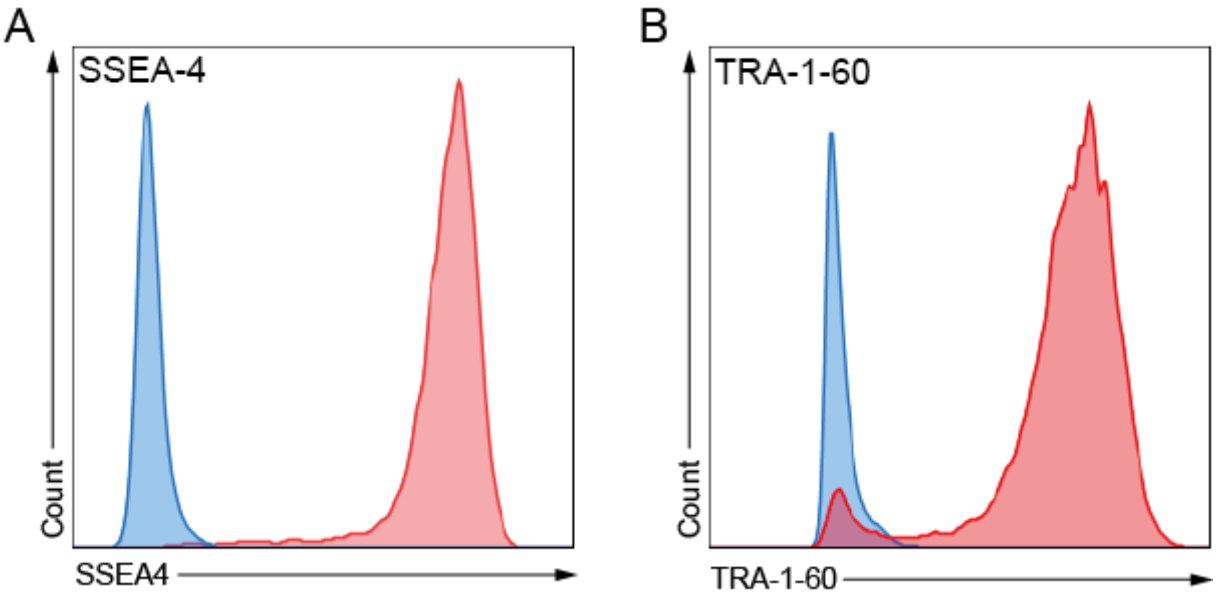

Figure 58. Gating strategy for analysis of SSEA-4 and TRAA-1-60 markers on day 0 of haematopoietic differentiation.

(A) iPSC Fluorescence Minus One control for SSEA-4 marker (blue) and iPSC cells stained with PerCPCy™5.5 anti-human SSEA-4 (red); (B) iPSC Fluorescence Minus One control for TRA-1-60 marker (blue) and iPSC cells stained with FITC anti-human SSEA-4 (red).

Positive population for KDR marker on day 3 of haematopoietic differentiation was gated using PE-Mouse IgG1 isotype control to identify unspecific staining (**Figure 59**).

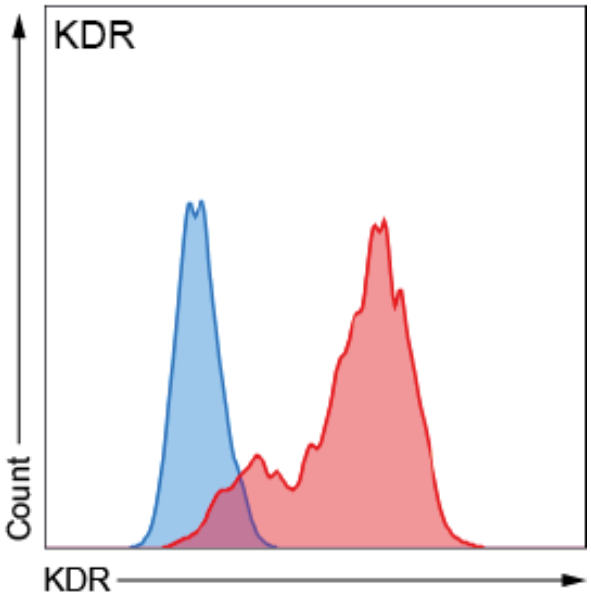

Figure 59. Gating strategy for analysis of KDR marker on day 3 of haematopoietic differentiation. Differentiated iPSC on day 3 were stained with either PE mouse IgG1 k Isotype Control (blue) or PE anti-human KDR (red).

Positive population for CD34, CD43, CD41a and CD235a markers on day 6 and day 12 of haematopoietic differentiation was gated using Fluorescence Minus One controls for each specific marker (**Figure 60A-D**).

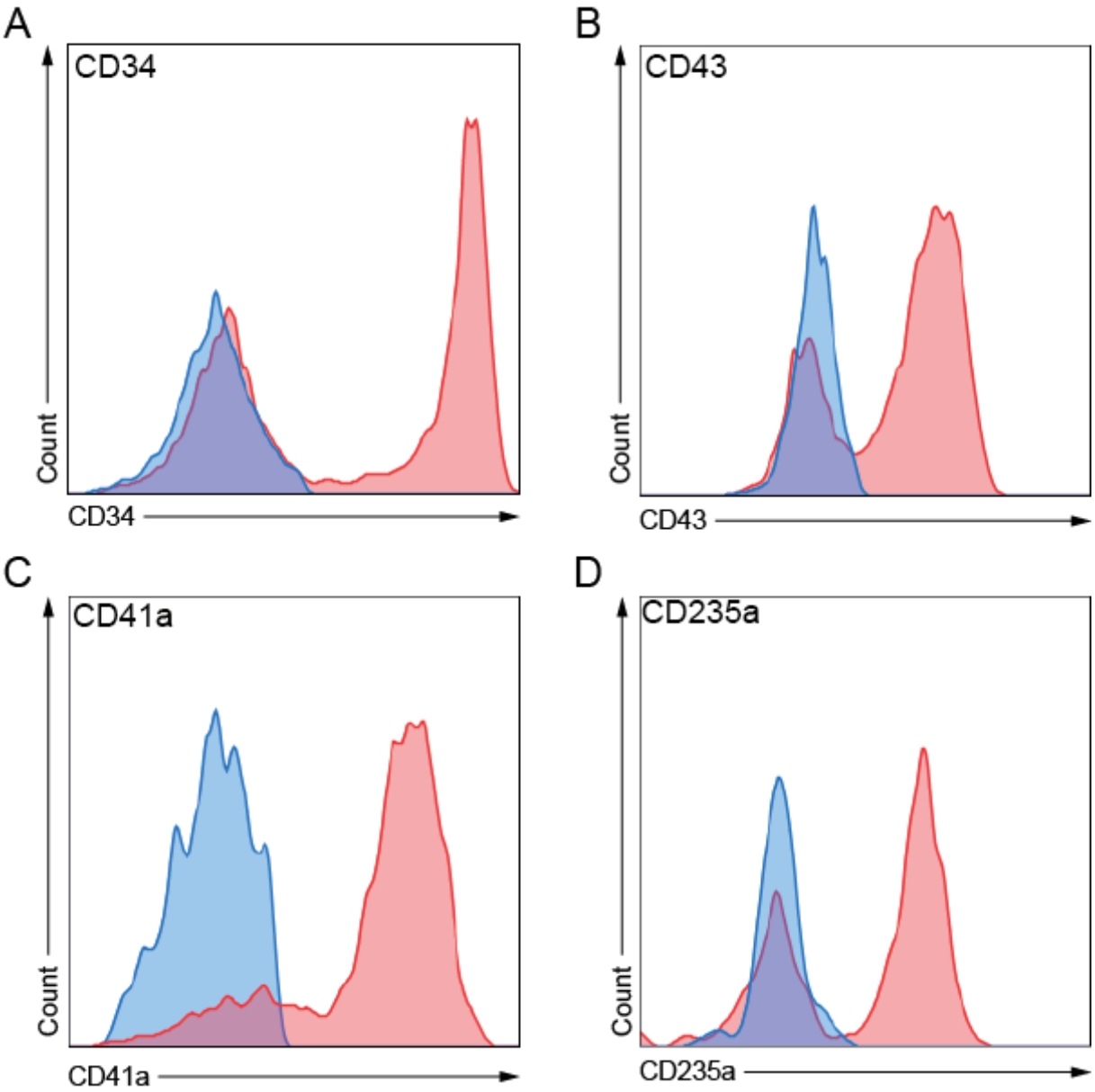

Figure 60. Gating strategy for analysis of CD34, CD43, CD41a and CD235a markers on day 6 and 12 of haematopoietic differentiation.

(A) Differentiated iPSC Fluorescence Minus One control for CD34 marker (blue) and differentiated iPSC cells stained with APC anti-human CD34 (red); (B) Differentiated iPSC Fluorescence Minus One control for CD43 marker (blue) and differentiated iPSC cells stained with FITC anti-human CD43 (red); (C) Differentiated iPSC Fluorescence Minus One control for CD41a marker (blue) and differentiated iPSC cells stained with APCH7 anti-human CD41a (red); (D) Differentiated iPSC Fluorescence Minus One control for CD235a marker (blue) and differentiated iPSC cells stained with BV421 anti-human CD235a (red).

Positive population for CD43, BrdU, γH2AX and Cleaved PARP markers on day 14 of haematopoietic differentiation for analysis of proliferation, DNA damage and apoptosis of haematopoietic progenitors was gated using Fluorescence Minus One controls for each specific marker (**Figure 61A-D**).

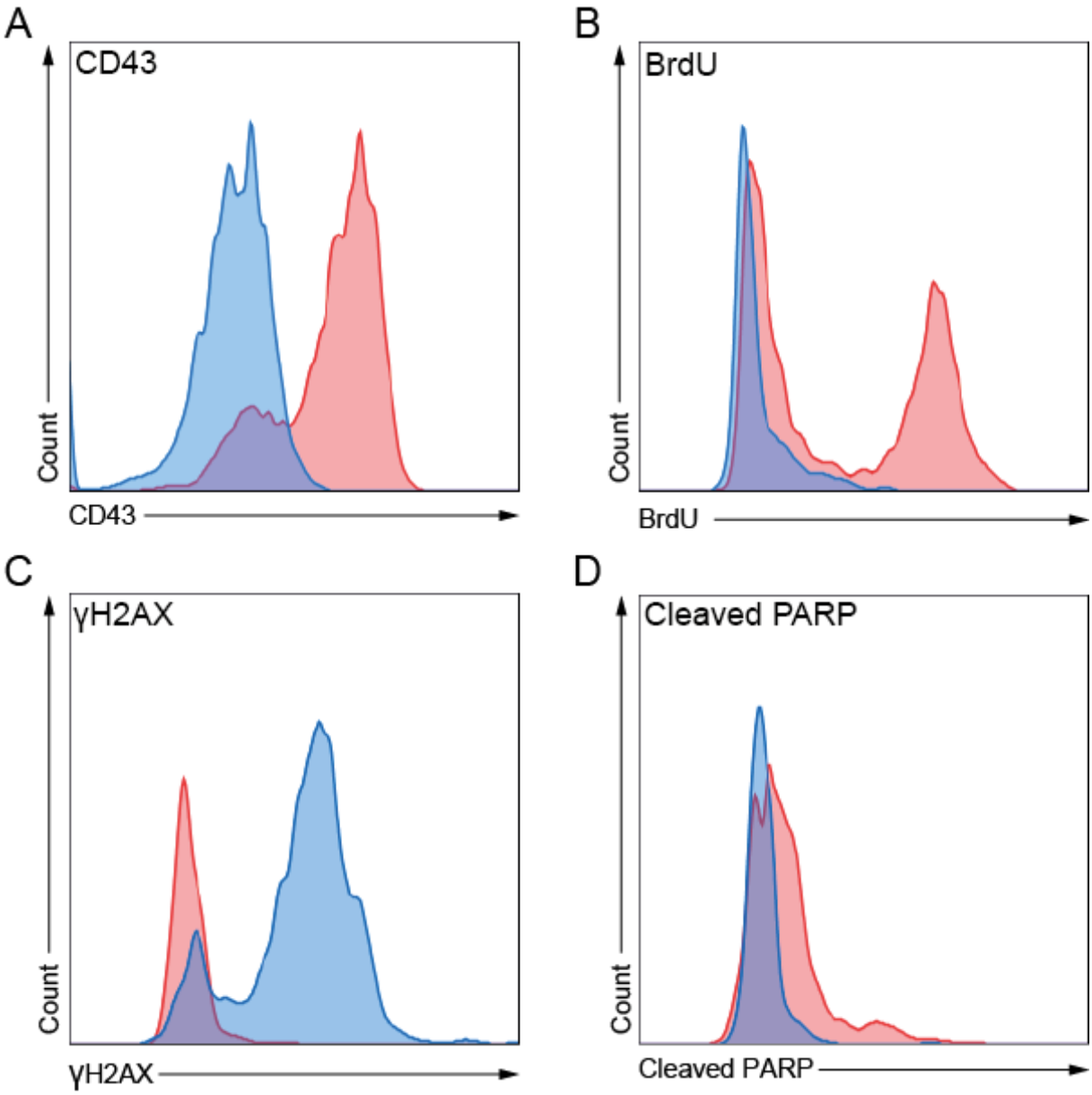

Figure 61. Gating strategy for analysis of CD43, BrdU, γH2AX and Cleaved-PARP markers on day 14 of haematopoietic differentiation.

(A) Differentiated iPSC Fluorescence Minus One control for CD43 marker (blue) and differentiated iPSC cells stained with FITC anti-human CD43 (red); (B) Differentiated iPSC Fluorescence Minus One control for BrdU marker (blue) and differentiated iPSC cells stained with PerCPCy™5.5 anti-human BrdU (red); (C) Differentiated iPSC Fluorescence Minus One control for γH2AX marker (red) and differentiated iPSC cells stained with Alexa Fluor® 647 anti-human γH2AX (blue); (D) Differentiated iPSC Fluorescence Minus One control for Cleaved-PARP marker (blue) and differentiated iPSC cells stained with PE anti-human Cleaved-PARP (red).

APPENDIX B: Data used for analysis of variation in differentiation of iPSC into haematopoietic progenitors

Variable	Sample	% CD43+	% Ery/MkP	% MkP	% EryP	% MyeP
Differentiation experiment	WT3-iPSC Clone 1 p42 #1	59	10.5	15.4	15.6	17.5
	WT3-iPSC Clone 1 p42 #2	78.3	19.5	23.7	15.3	19.8
	WT3-iPSC Clone 1 p42 #3	77.9	22.2	24.4	14.7	16.5
Passage	WT3-iPSC Clone 1 p32 #1	86.8	15.7	24.2	31.7	15.2
	WT3-iPSC Clone 1 p32 #2	79.9	13.5	23	23.5	19.8
	WT3-iPSC Clone 1 p32 #3	88.9	20.3	21.3	32.4	15
	WT3-iPSC Clone 1 p42 #1	59	10.5	15.4	15.6	17.5
	WT3-iPSC Clone 1 p42 #2	78.3	19.5	23.7	15.3	19.8
	WT3-iPSC Clone 1 p42 #3	77.9	22.2	24.4	14.7	16.5
	WT3-iPSC Clone 1 p52 #1	60.7	11.4	26.8	14.9	7.6
	WT3-iPSC Clone 1 p52 #2	60.1	12.6	26.5	12.9	8.16
	WT3-iPSC Clone 1 p52 #3	41.5	8.19	19	9.14	5.22
Clone	WT3-iPSC Clone 1 p42 #1	79.5	18.3	26.8	14.3	20.1

	WT3-iPSC Clone 1 p42 #2	73.9	16.8	20.1	17.4	19.6
	WT3-iPSC Clone 1 p42 #3	69.8	16.4	20.2	14.1	19.1
	WT3-iPSC Clone 4 p42 #1	64	4.26	31.4	19.6	4.77
	WT3-iPSC Clone 4 p42 #2	75.9	9.78	24.8	35.1	6.17
	WT3-iPSC Clone 4 p42 #3	79.4	11.1	26	36.5	5.88
	WT3-iPSC Clone 5 p42 #1	47.1	12.7	10.9	10.9	12.7
	WT3-iPSC Clone 5 p42 #2	57.6	17.4	12.4	16.1	11.7
	WT3-iPSC Clone 5 p42 #3	59.2	15.8	13.1	13.5	16.8
Genetic background	WT1-iPSC Clone 1 p42 #1	76.1	22	29.1	6.57	18.5
	WT1-iPSC Clone 1 p42 #2	70.8	24.6	22.9	7.15	16.1
	WT1-iPSC Clone 1 p42 #3	71.7	20.8	24.1	8.27	18.5
	WT2-iPSC Clone 1 p42 #1	57.6	1.82	10.8	37	8.06
	WT2-iPSC Clone 1 p42 #2	43.1	1.97	7.88	29	4.23
	WT3-iPSC Clone 1 p42 #1	65.8	18.3	26.8	14.3	20.1
	WT3-iPSC Clone 1 p42 #2	57.3	16.8	20.1	17.4	19.6
	WT3-iPSC Clone 1 p42 #3	57.2	16.4	20.2	14.1	19.1

Table 12. Percentages of the different populations of haematopoietic progenitors obtained for the different parameters analysed in the variation during haematopoietic differentiation of iPSC

APPENDIX C: Publications

Melguizo Sanchis, D., Xu, Y., Taheem, D., Yu M., Tilgner, K., Barta, T., Gassner, K., Anyfantis, G, Wan, T., Elango, R., Alharthi, S., El-Harouni, A., Przyborski, S., Adam, S., Saretzki, G., Samarasinghe, S., Armstrong, L. and Lako, M. (2017) 'iPSC modelling of severe aplastic anemia shows impaired differentiation and telomere shortening in blood progenitors' *Cell death and disease*, accepted for publication.

Meader E, Barta T, **Melguizo-Sanchis D**., Tilgner K, Montaner D, El-Harouni AA, Armstrong L, Lako M. (2017) 'Brief Report: Pluripotent Stem Cell-Derived Hematopoietic Progenitors Are Unable to Downregulate Key Epithelial-Mesenchymal Transition-Associated miRNAs' *Stem Cells,* ahead of print.

Adam, S., **Melguizo Sanchis, D.**, El-Kamah, G., Samarasinghe, S., Alharthi, S., Armstrong, L. and Lako, M. (2017) 'Concise Review: Getting to the Core of Inherited Bone Marrow Failures', *Stem Cells,* 35(2), pp. 284-298

Zhu, L., Gomez-Duran, A., Saretzki, G., Jin, S., Tilgner, K., **Melguizo-Sanchis, D.**, Anyfantis, G., Al-Aama, J., Vallier, L., Chinnery, P., Lako, M. and Armstrong, L. (2016) 'The mitochondrial protein CHCHD2 primes the differentiation potential of human induced pluripotent stem cells to neuroectodermal lineages', *The Jounal of Cell Biology*, 215(2), pp. 187-202