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The expansion and transduction of hematopoietic stem cells for gene therapy

Doctor of Philosophy

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I thank my heavenly Father for His Spirit that transforms us and prepares us for eternity. All the glory and honour be to the Lord from everlasting to everlasting.
Amen!

SUMMARY

Gene therapy is a field in medical research that is being investigated for the treatment of genetic, non-communicable and infectious diseases. The results obtained from clinical trials that make use of gene therapy show promise; however, it has been determined that several procedural limitations hinder convincing outcomes. The genetic modification of host cells can be achieved using transfection (transfer of DNA fragments into the chromosomes of cells using chemical methods) or transduction (transfer of DNA fragments into the chromosomes of cells using viruses) procedures. Considerable work has been done to improve the safety of the viral vectors used to transfer genes, that are capable of providing therapeutic effects, into target cells and to improve the efficiency of the therapeutic genes. Differentiated cells can be used for a transient therapeutic effect or alternatively stem cells can be used which would provide a more long-term effect. The use of stem cells requires the isolation, gene modification and reintroduction of the cells into the patient without the stem cells losing their engraftment potential which is their ability to return to the original location and self-renew as well as differentiate. The majority of gene therapy clinical trials make use of hematopoietic stem cells (HSCs) that have been modified using molecular techniques; however, poor engraftment and low transduction efficiencies of long-term repopulating HSCs leads to minimal therapeutic effects. The majority of clinical trials make use of HSCs that were released from the bone marrow of patients or donors using mobilization techniques. Poor engraftment of genetically modified HSCs can be attributed to insufficient numbers of mobilized bone marrow CD34+ cells being obtained from some patients and to CD34+ cells that lose their repopulating potential during the transduction process.

The transduction procedures used in gene therapy clinical trials do not promote the transduction of long-term repolulating HSCs. This study aimed to develop techniques that facilitate the improved transduction and expansion of the primitive HSC populations. The use of hypoxia and Stemregenin 1 (SR1) in the culturing of HSCs has been investigated to determine their benefit in the expansion of HSC cellular subpopulations. Also, the use of SR1 in the transduction protocol was studied to determine if it can benefit the engraftment potential of transduced HSCs. It was found that SR1 can be used to improve the expansion of the various HSC subpopulations, determined *in vitro* and *in vivo*. The use of SR1 with 1% O_2 led to an increase in the number and ability of cells to repopulate NOD SCID gamma mice (NSG, NOD.Cg-Prkdc<scid> Il2rg<tm1Wjl>/SzJ). Also, SR1 promotes the transduction and expansion of the primitive HSC population during the transduction process and significantly increases the engraftment of HSCs transduced with therapeutic genes. SR1 may be used to benefit the field of gene therapy where HSC transduction is used.

Gene therapy can be used in the treatment of monogenic disorders such as hemaglobinopathy or infectious diseases such as HIV-AIDS. South Africa is highly burdened with HIV-AIDS which can potentially be treated with gene therapy. However, the complexity of gene therapy requires the need for highly specialized expertise, infrastructure and supportive legislation that should be put into place in South Africa before gene therapy can be implemented. The barriers and opportunities in the implementing of gene therapy in South Africa have been investigated in this study and recommendations are made.

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7-AAD	7-Aminoactinomycin
ADA-SCID	Adenosine deaminase severe combined immunodeficiency
AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
BCNU	Bis-chloroethylnitrosourea
BIV	Bovine immunodeficiency virus
BM-HSC	Bone marrow HSC
BM-MSC	Bone marrow MSC
BMP2	Bone morphogenetic protein 2
Bu	Busulfan
CBMPs	Cell-based Medicinal Products
CFC	Colony forming cell (CFC)
CGD	Chronic granulomatous disease
cGMP	Current good manufacturing practices
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats
Cy	Cyclophosphamide
DMEM	Dulbecco's Modified Eagle Medium
DMOG	Dimethyloxaloylglycine
DMSO	Dimethylsulfoxide
EFS	Etablissement Français du Sang
ESC	Epithelial stem cells
EGFP	Enhanced green fluorescent protein

EIAV	Equine infectious anemia virus
FACS	Fluorescence Activated cell sorter
FBS	Fetal bovine serum
FIV	Feline immunodeficiency virus
Flu	Fludarabine
GCI	Global Competitiveness Index
HAART	Highly active anti-retroviral therapy
HEK-293T	Human embryonic kidney 293 cells
hG-CSF	Human Granulocyte Colony Stimulating Factor
HIF	Hypoxia inducible factor
hIL-3	Human interleukin 3
HIV	Human immunodeficiency virus
HRE	Hypoxia response elements
HSC	Hematopoietic stem cell
hSCF	Human stem cell factor
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem and progenitor cell
HSV-TK	Herpes simplex thymidine kinase
ICGEB	International Centre for Genetic Engineering and Biotechnology
IMDM	Iscove's Modified Dulbecco's Medium
IP	Intellectual property
IPR-PFRD	Intellectual Property Regulations from Publicly Funded Research and Development
iPS	Induced ploripotent sell
LT-HSC	Long-term hematopoietic stem cells

LV	Lentiviral vector
Mel	Melphalan
MFI	Mean fluorescence intensity
MLD	Metachromatic leukodistophy
MLV	Murine leukemia virus
MMLV	Murine Moloney leukemia retrovirus
MOI	Multiplicity of infection
MSC	Mesenchymal stromal cell
NBS	National Biotechnology Strategy
NHA	National Health Act
NIPMO	National Intellectual Property Management Office
NSG	NOD.Cg-Prkdc<scid> Il2rg<tm1Wjl>/SzJ
O6BG	O^6-benzylguanine
PB-HSC	Peripheral blood HSC
PFA	Paraformaldehyde
PHDs	Prolyl-hydroxylases
PPT/CTS	Polypurine tract/central termination sequence
PS	Penicillin/streptomycin
RCR	Replication competent recombination
REE	Rev response elements
RNAi	RNA interference
RRV	Ross River Virus
SCID	Severe combined immune deficiency
SIN	Self-inactivating
siRNA	Small interference RNA

SIV	Simian immunodeficiency virus
SP	Side population
SR1	Stemregenin 1
SRC	SCID repopulating cell
ST-HSC	Short-term hematopoietic stem cells
TALENs	Transcription activator-like effector nucleases
TIA	Technology Innovation Agency
TMTX	Trimethotrexate
TPO	Thrombopoietin
VCN	Vector copy number
VDC	Vybrant® DyeCycle™ Violet
VpR	Viral accessory protein R
VSV-G	Vesicle stomatituc virus protein glykoprotein
WAS	Wiskott-Aldrich syndrome
WEF	World Economic Forum
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
B-thal	β-thalassemia

Chapter 1

Introduction

Gene therapy is an exciting new field that has the potential to contribute to the treatment of many incurable diseases. Gene therapy is mostly performed in the *ex-vivo* setting on either adult cells that have a temporary lifespan (Rosa et al. 1999; Rossig and Brenner 2004) or stem cells that have a more permanent nature (Burnett et al. 2012; Penn and Mangi 2008). Autologous and allogeneic hematopoietic stem cell (HSC) transplantation has been performed in patients with various, but a limited number ofhematological and other disorders for more than 50 years (Appelbaum 2007; Copelan 2006). Genetic modification of HSCs prior to transplantation has opened new therapeutic avenues for genetic and infectious diseases (Watts et al. 2012). However, the development of stem cell gene therapies is complex and requires optimization of several facets (Young et al. 2006). Several obstacles need to be overcome prior to the application of HSC-mediated gene therapy into more widespread clinical practice. In the early years of gene therapy, the possibility that integration of genetic elements might cause endogenous gene activation or deactivation that in turn may lead to aberrant cell proliferation and tumour formation, has been a concern (Hacein-Bey-Abina et al. 2003). Many improvements have been made in vector design aimed at overcoming this problem such as the development of self inactivating vectors, but the optimization of other aspects of the process including improved transduction rates could further increase safety by reducing the number of vector copies per cell and thereby reducing the likelihood of causing aberrant affects (Anderson et al. 2007a). Local and international consortia have been established to provide support and facilitate the entry of these technologies into the clinic.

The CD34+ cells that are mobilized from the bone marrow of patients using the G-CSF cytokine in gene therapy clinical trials are not always sufficient in number for transplantation (Biffi et al. 2006; Amado et al. 2004). The patients in whom low CD34+ cells were obtained

would forfeit the treatment or the treatment would not be successful due to the cells that do not engraft properly. Hematopoietic CD34+ cells can be expanded; however, they differentiate spontaneously in culture and lose their engraftment potential which is their ability to return to the original location and self-renew as well as differentiate (Glimm et al. 2014). Culture conditions should ideally favor the *in vitro* expansion of hematopoietic progenitor cells (HPCs) and the self-renewal of long-term HSCs (Huang et al. 2012; Mikkola and Orkin 2006). It is hypothesized that *in vitro* conditions that mimic the hypoxic conditions of the bone marrow from which the cells came would be most suitable (Takubo, 2012), although other conditions such as the use of Stemregenin 1 (SR1) have proven to provide superior results (Boitano et al., 2010). The enzymatic pathways that regulate hematopoiesis have been characterized and the hypoxia inducible factor (HIF) subunits play an important function in keeping cells in a state of quiescence. The self-renewal of HSCs mainly takes place when the cells are quiescent (Takubo, 2012). The aryl hydrogenase receptor (AHR), however, promotes differentiation and the depletion of the stem cell population (Nie et al. 2001). By stabilizing HIF subunits and inhibiting AHR, the enzymatic pathways could be manipulated to promote HSC expansion (Boitano et al. 2010; Nie et al. 2001).

The lentivirus transduction procedures currently used in clinical trials are inadequate for transducing high percentages of CD34+ cells and do not favour the preservation or expansion of primitive HSCs (Kang et al. 2010; Digiusto et al. 2010). The number of transduced primitive HSCs in the sample after transduction is crucial for long-term engraftment in order to provide a sufficient therapeutic affect (Huang et al. 2012; Mikkola and Orkin 2006). A transduction technique that improves the number of transduced primitive HSCs will benefit many gene therapies. In this study we have investigated how hypoxia, dimethyloxaloylglycine (DMOG) (which mimics hypoxia by stabilizing HIF-1α) and

SR1 affect the various HSC subpopulations in vitro and whether hypoxia, SR1 and the combination thereof improve the engraftment of expanded HSCs into NOD SCID gamma mice (NSG, NOD.Cg-Prkdc<scid> Il2rg<tm1Wjl>/SzJ), It was demonstrated that the engraftment of cells that were cultured in the presence of hypoxia and SR1 provided similar engraftment success as cells cultured with only SR1 while significantly less cells were introduced into the mice. Also, we investigated whether SR1, DMOG, genistein and spinoculation improve the transduction of primitive HSCs. We demonstrated that the use of SR1 in the transduction medium promotes that transduction and expansion of the primitive HSC population. The improved engraftment of transduced HSCs was demonstrated following the transplantation of NSG mice with HSCs transduced in the presence of SR1. This finding has the potential to provide a way to overcome the limitations related to the limited ability of transduced HSCs to engraft.

Gene therapy has the potential to provide significant relief to many patients with diseases such as HIV, once the limitations have been overcome. However, it is an intricate procedure and needs proper coordination and infrastructure to be successful (Jackson and Pepper 2013). The end goal is to contribute to the development of a technique that can be provided to HIV patients to improve their quality of life and reduce mortality. South Africa has one of the highest HIV prevalence rates and is severely affected by HIV (Schwab, 2011). A feasibility assessment should be conducted to determine if an anti-HIV gene therapy could be successfully implemented in South Africa. Recommendations to overcome barriers in establishing a gene therapy platform in South Africa have been made.

Aims and objectives

The aim of this study was to improve the efficiency of hematopoietic stem cells expansion and transduction.

Objectives

1. The first objective was to investigate all registered clinical trials that have made use of transduced HSCs in gene therapy to identify where previously used techniques have failed to provide desired results and need improvement. This objective was addressed in chapter 3.
2. The second objective was to investigate what effect hypoxia and SR1 could have on HSPC expansion. This objective was addressed in chapter 4.
3. The third objective was to determine if the use of hypoxia and SR1 could improve the engraftment and repopulation of HSCs in the NSG mouse model. This objective was addressed in chapter 5.
4. The fourth objective was to investigate what efficiency the methods, previously used to improve lentivirus transduction of mammalian cells and to expand HSCs, have in the transduction of the long-term repopulating HSCs. This objective was addressed in chapter 6.
5. The fifth objective was to investigate if the use of SR1 during lentivirus transduction of HSCs could improve the long-term engraftment capacity of transduced cells. This objective was addressed in chapter 7.

6. The sixth objective was to investigate the feasibility of setting up a cell therapy program in South Africa by assessing the opportunities and barriers a cell therapy program would face. This objective was addressed in chapter 8.

Chapter 2

Clinical safety and applications of stem cell gene therapy

2.1. Introduction

Gene therapy is becoming an increasingly recognized field in modern medicine and has the potential to become an important option for treating certain communicable and non-communicable diseases. Gene therapy is performed by modifying the expression of genes in the cells of patients to obtain a specific therapeutic effect. Techniques used to accomplish this include knocking down gene expression, introduction of functional genes and gene repair (Gould & Favorov, 2003). Initially, monogenic disorders such as adenosine deaminase severe combined immunodeficiency (ADA-SCID) were considered for gene therapy (Mullen et al., 1996). Subsequently, more complex diseases have been addressed, including the disruption of the human CCR5 gene and HIV replication genes using RNA interference to suppress viral replication in HIV-positive patients (DiGiusto et al., 2010).

The first gene therapy clinical trial was performed in the 1990s (Mullen et al., 1996), and the first success was reported in 2002 for the treatment of X-linked SCID (Hacein-Bey-Abina et al. 2002). However, the excitement was soon dampened when two patients developed leukemia due to the inserted therapeutic gene that caused the activation of endogenous genes which in turn lead to the formation of tumors. This was a major setback for the field and resulted in a loss of enthusiasm for this technology. However, in-depth investigation into the cause of the oncogenesis and attempts to improve the safety of the vectors has led to the development of much-improved techniques elaborated on in detail below (Check, 2002).

The use of genetically manipulated cells that are already differentiated provides a transient therapeutic effect, due to their short life span and the inability of these cells to replace

untreated cells. Genetically manipulated stem cells on the other hand have the ability to populate a niche in the body and self-renew. This provides a potential long-term supply of cells that can express the therapeutic gene(s) (Burnett et al. 2012). Pluripotent and adult stem cells are the two major categories of stem cell types. Pluripotent cells include embryonic stem cells (ES cells - isolated from a blastocyst) or induced pluripotent stem (iPS) cells. Adult stem cells include hematopoietic, skeletal, mesenchymal, neural and epithelial stem cells and are found in various tissues where they maintain a steady number of mature cells (Figure 1, page 11).

Hematopoietic stem cells (HSCs) were the first multipotent stem cell type to be used for gene therapy purposes, given that they had been extensively used in the clinical setting for transplantation purposes (Hacein-Bey-Abina et al. 2002). Hematopoietic stem cells can be harvested directly from the bone marrow, or indirectly through mobilizing HSCs from the bone marrow into the peripheral blood which is then collected. The mobilization technique is used most often. The population of HSCs within a harvested sample is relatively rare, but can be identified and enriched from this mixed population using cluster of differentiation (CD) cell surface markers such as CD34 (Weissman and Shizuru 2008). Therapeutic genes can be introduced into stem cells with the use of vectors that transfer the DNA fragments into the nucleus of cells. Viral vectors are most commonly used, due to the fact that gene transduction is most efficient with these techniques (Neff et al. 1997). The virus backbone originally used was the γ-retrovirus vector, and later adenovirus vectors were adopted (Gould & Favorov, 2003). A major drawback of using these vectors is the potential for the therapeutic gene to be inserted into or close to endogenous genes and thereby to modify the activity of these genes (Hacein-Bey-Abina et al. 2003). The γ-retrovirus vector was found to frequently insert therapeutic genes into or close to oncogenes causing insertional mutagenesis (Blumenthal et al., 2007). A further problem with these vectors is that primitive

HSCs, which are mostly quiescent or slow-dividing cells, are not easily transduced or are transduced at lower efficiencies (Horn et al., 2015). Lentivirus vectors are currently the preferred vector choice and a series of improvements have been made to increase safety and efficacy (Gould & Favorov, 2003). Gene therapy has rapidly expanded to include the use of various vector types and the treatment of numerous hematological disorders. Recently, genetically manipulated stem cells types, other than HSCs, have also been explored for use in treating non-hematologic disorders.

Many of the safety and efficiency concerns with stem cell gene therapy have been resolved, but there are still some uncertainties. Those that have been identified and that can hinder the application of stem cell gene therapy are discussed, as are the issues that have the potential to cause harm. It is a priority to pursue methods that will lead to safer and more efficient gene therapies.

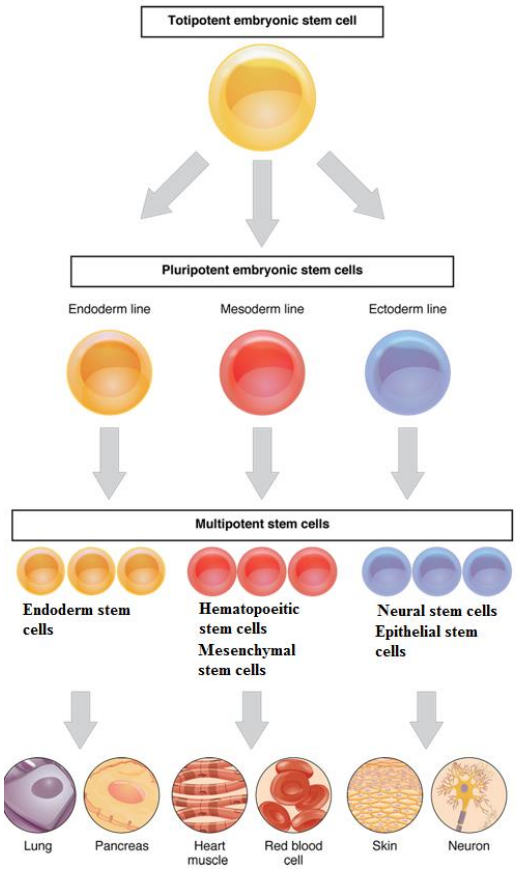

Figure 1: Pluripotent, totipotent and multipotent stem cells. Adult stem cells are found in the body including in the lung, pancreas, heart muscle, blood cells, skin and neurons. http://oerpub.github.io/epubjs-demo-book/resources/422_Feature_Stem_Cell_new.png

Applications

The introduction of a therapeutic transgene into target cells or tissues can be accomplished using either a direct delivery strategy or cell-based delivery (Figure 2, page 13) (Mohit & Rafati, 2013). With the direct delivery strategy, the therapeutic gene is cloned into a plasmid construct. This plasmid construct can be directly administered to the target site for a transient therapeutic effect or packaged into delivery vehicles such as adenoviruses or lentiviruses to have a long-term effect. The virus vectors used in this strategy are usually replication competent and are targeted to specifically enter and replicate in the desired cell types (Mohit & Rafati, 2013). The use of replication competent virus vectors in patients faces various challenges and the preferred delivery strategy is cell-based. In such cases, adult stem cells, ES cells, or iPS cells can be used. Adult stem cells, isolated from the patient or donor and propagated in the laboratory, are currently used in clinical trials and have been proven to be safe (Table 1). The genetically modified stem cells are then reintroduced into the patient to engraft and populate a particular site specific to each cell type (Figure 2, page 13) (Mohit & Rafati, 2013). Autologous adult stem cells are preferred to allogeneic stem cells due to the elimination of the risk of transplant rejection (Harris, 2014).

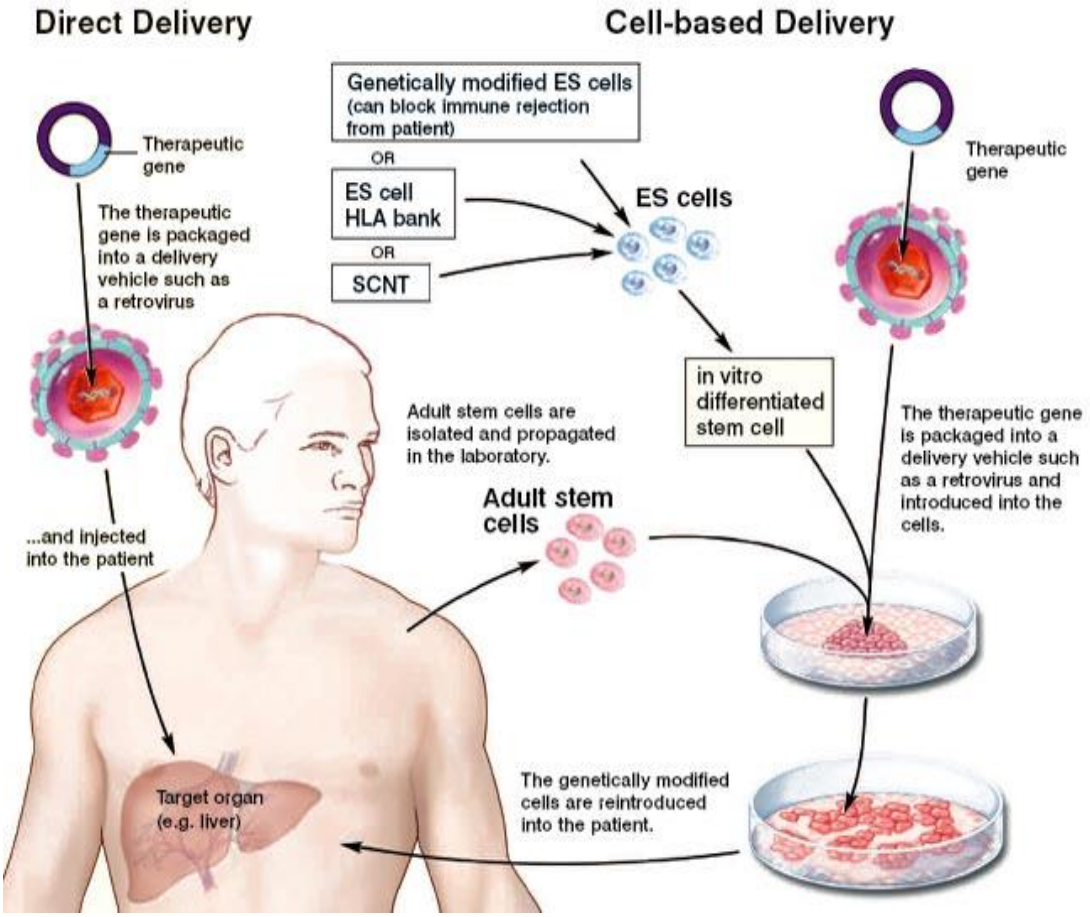

Figure 2: Strategies for delivering therapeutic transgenes into patients (Image used with permission from Terese Winslow LLC).

Table 1 Selection of clinical trials that have made use of gene therapy and stem cells to date

Stem cell type	Disease	Tissue source	NCT Number	Vector	References
HSC	**Primary immunodeficiencies**				
	X-SCID	PB-HSC	NCT00028236	Retro	(Cavazzana-Calvo et al. 2000)
	X-SCID	BM-HSC	NCT01306019	Lenti	(Buckley 2004; Cavazzana-Calvo et al. 2000)
	X-CGD	PB-HSC	NCT00927134	γ-Retro	(Ott et al. 2006; Stein et al. 2010)
	ADA-SCID	BM-HSC	NCT01852071	MMLV	(Candotti et al., 2012)
	WAS	BM-HSC	NCT01515462	Retro	(Aiuti et al. 2013)
	CGD	PB-HSC	NCT00394316	MMLV	(Kang et al. 2010)
	MLD	PB-HSC	NCT01560182	Lenti	(Biffi et al. 2006; Capotondo et al. 2007)
	Hemoglobinopathies				
	β-Thal	BM-HSC	-	Lenti	(Cavazzana-Calvo et al. 2010)
	β-Thal	PB-HSC	NCT01639690	Lenti	(Boulad et al., 2014)
	Sickle cell anaemia	BM-HSC	NCT02247843	Lenti	(Romero et al., 2013)
	Other single-gene disorders				
	Gaucher's disease	BM-HSC	NCT00001234	Retro	(Fink, Correll, Perry, Brady, & Karlsson, 1990)
	Fanconi anemia	BM-HSC	NCT00001399	Retro	(Walsh et al. 1994)
	Infectious diseases				
	HIV	BM-HSC	-	MMLV	(Bauer et al. 1997; John J Rossi 2000)
	HIV	BM-HSC	-	MMLV	(Kohn et al. 1999)
	HIV	BM-HSC	-	Retro	(Kang et al. 2002; Mautino et al 2001)
	HIV	PB-HSC	NCT01769911	Lenti	(DiGiusto et al., 2010)
MSC	Tumors	BM-MSC	NCT01844661	Adeno	(Martı et al., 2010)
	Tumors	BM-MSC	NCT02008539	γ-Retro	(Niess et al., 2015)
ESC	**Dermatological diseases**				
	Netherton	Epithelial stem cells	NCT01545323	Lenti	(Di et al., 2013)
	Junctional epidermolysis bullosa	Epithelial stem cells	-	MMLV	(Rosa et al. 2014)
NSC	High Grade Gliomas	Neural stem cells	NCT01172964	Adeno	(Kaliberov et al., 2007)

Severe combined immunodeficiency (SCID), Wiskott-Aldrich syndrome (WAS), β-thalassemia (β-thal), metachromatic leukodystrophy (MLD), human immunodeficiency virus (HIV), *Chronic granulomatous disease* (CGD), murine Moloney leukemia retrovirus (MMLV), bone marrow HSC (BM-HSC), peripheral blood HSC (PB-HSC), bone marrow MSC (BM-MSC), neural stem cell (NSC). (Adapted from Mohit and Rafati 2013).

Clinical trials using adult stem cells and gene therapy have provided several success stories but this has generally been limited to the use of HSCs. However, other studies that investigate the use of mesenchymal stromal cells (MSCs) are in the pipeline (Myers et al., 2011). Indications explored in clinical trials include SCID, Wiskott-Aldrich syndrome, β-thalassemia, metachromatic leukodystrophy (MLD), high grade gliomas and HIV (Watts et al. 2012) (Table 1). Cavazzana-Calvo et al. (2000) demonstrated that full correction of the SCID-X1 disease phenotype is possible, given that eight of the nine patients that received autologous gene modified cells displayed good health nine years after treatment compared to non-treated patients (Cavazzana-Calvo et al. 2000; Hacein-Bey-Abina et al. 2011). Full immune reconstitution has also been reported for 30 patients that received autologous gene modified cells for ADA-SCID (Ferrua et al. 2010). Cavazzana-Calvo and colleagues have also demonstrated that gene therapy can provide transfusion independence for patients with severe β-thalassemia, a common inherited disorder that is not easily treated with gene therapy. Decreased β-globin synthesis results in an excess of alpha globin in erythroid precursors which results in red cell membrane damage and ineffective erythropoiesis. A high expression of functional forms of the β-hemoglobin chain is needed to achieve a therapeutic effect by binding to the excess alpha globin (Cavazzana-Calvo et al. 2010; Watts et al. 2012). Other therapeutic targets of corrective stem cell gene therapy that have been investigated include Hurler's disease, Hemophilia A, B and Alpha-1 antitrypsin deficiency (Herzog, 2015; Mueller & Flotte, 2015; Ou, 2015; Vollweiler, Zielske, Reese, & Gerson, 2003).

The potential use of MSCs for gene therapy was recognized during the early characterization of these cells (Myers et al., 2011). It was determined that MSCs are hypo-immunogenic and immunomodulatory, and have the ability to home to damaged tissues to

initiate repair processes (Martinez-Quintanilla et al., 2013). MSCs are found in very low numbers in adult tissues, but can easily be isolated from bone marrow, adipose tissue and umbilical cord. To obtain desired MSC numbers for clinical applications, the isolated MSC population needs to be expanded (Fossett and Khan 2012). Unlike HSCs, it is possible to expand MSCs *ex vivo* without differentiation; however, factors such as the age and gender of the donor as well as the seeding density and culture conditions can effect expansion (Fossett and Khan 2012). MSCs can be differentiated into osteo-, chondro- and adipogenic cellular lineages (Choudhery, Badowski, Muise, & Harris, 2013) and have been investigated for their use in treatments of musculoskeletal, vascular, hematological and neurological diseases, and neoplasms (Akram et al. 2012; Fossett and Khan 2012; Harris 2014).

The usefulness of MSCs in gene therapy has been demonstrated in animal models. MSCs transduced with modified human bone morphogenetic protein 2 (BMP2), under the regulation of an inducible activation system using the adenoviral delivery system, and transplanted into the muscle surrounding the lumbar spine leads to ectopic bone formation in mice (Martinez-Quintanilla et al., 2013). This demonstrates the potential usefulness of genetically manipulated MSCs in bone regeneration and repair (Martinez-Quintanilla et al., 2013). Chondrogenesis was obtained in rabbit, horse and pig arthritis models when MSCs transduced with BMP2 and TGFβ were transplanted, resulting in reduced progression of osteoarthritis (Cucchiarini et al., 2005). The ability of MSCs, isolated from patients with osteogenesis imperfecta, to produce collagen fibrils was restored following a genetic disruption of the mutant collagen genes (Chamberlain et al. 2004). MSCs transduced with the anti-apoptotic proteins oxygenase-1, angiogenin (Tsubokawa et al., 2010), Bcl2, adrenomedullin (Copland et al., 2008) and integrin-linked kinase (Song et al. 2009) or the angiogenic protein angiopoeitin-1 in combination with the prosurvival protein Akt1 (Shujia et

al., 2008) improve heart function in animal models following myocardial infarction (Myers et al., 2011).

Genetically engineered MSCs have been used to treat cancers in rodent xenograft models by modulating the immune system with the expression of IL-2, -7, -12 and -18 or by the expression of TNF, a ligand of the TNF receptors expressed on many types of tumors, to cause cancer-specific apoptosis (Myers et al., 2011). MSCs that deliver the suicide gene herpes simplex thymidine kinase (HSV-TK) in combination with ganciclovir treatment have been used to suppress tumor growth and metastasis in mice (Carcinoma, 2011). The expressed TK enzyme preferentially monophosphorylates ganciclovir to a toxic compound. This toxic compound can be transferred to adjacent cells by diffusion through gap junctions and mediates neighboring cell death.

The first clinical trial that made use of genetically modified MSCs (NCT01844661) was initiated in 2007 (Martı et al., 2010) (Table 1). MSCs obtained from the bone marrow of four patients with metastatic neuroblastoma were transduced with an oncolytic adenovirus vector called CELYVER that specifically replicates in cancer cells. The metastatic tumors disappeared within weeks in one patient who was in complete remission 36 months after treatment (Martı et al., 2010). Another clinical trial (NCT02008539) made use of genetically modified MSCs and an HSV-TK suicide gene therapy strategy in combination with ganciclovir to suppress cancer metastasis (Mátrai et al 2009).

Epithelial stem cells renew and repair the epidermis. Holoclones, generated by epithelial stem cells are colonies with long-term regeneration potential, and can restore large epithelial defects such as skin and ocular burns. Epidermal stem cell holoclones,

transduced with a murine leukemia virus (MLV)-based retroviral vector expressing LAMB3 have been applied in clinical trials for the treatment of junctional epidermolysis bullosa, a serious skin disease (De Rosa et al. 2014) (Table 1). Functional laminin-332 was observed in newly formed epidermis that was firmly adherent and stable in the absence of blisters, infections or inflammation. A long-term follow up indicated the safety and efficacy of using epidermal stem cells in a gene therapy for the treatment of skin diseases (De Rosa et al. 2014). Five patients with Netherton skin disease have received epithelial stem cells transduced with lentivirus vectors expressing the serine protease inhibitor Kazal type 5 gene in an ongoing clinical trial (SPINK5)(Di et al., 2013).

Further stem cell types that are being considered include ES cells, iPS cells and multipotent adult progenitor cells (MAPCs) (Kazuki et al. 2010; Narsinh et al. 2009). The MAPC population can be isolated from tissue types such as bone marrow and adipose tissue and differentiate into MSCs, endothelial cells, epithelial cells and hematopoietic cells, which makes it potentially very useful for future gene therapy procedures (Reiser et al. 2006). Undifferentiated ES and iPS cells are said to hold great promise, but safety and ethical hurdles are still to be overcome as they have the potential to form teratocarcinomas. Safety precautions are therefore necessary and no clinical trials that use ES and iPS cells in gene therapy have been approved (Yu et al. 2014). The three dimensional development of organoids *ex vivo* from ES cells or tissue-resident adult stem cells are being used to study organogenesis and stem cell behavior. These organoids can potentially be used to generate functional organs for transplants into patients (Huch & Koo, 2015; Xinaris, Brizi, & Remuzzi, 2015).

2.2. Safety

The safety of administering gene therapy products is of utmost importance, and should not be compromised to obtain greater therapeutic efficiency (Hadaczek et al., 2010). Given that the preparation of gene therapy products is complex, there are various facets that could possibly compromise patient safety which are discussed in detail below.

2.2.1. Proof of concept

Each gene therapy application has specific requirements, including whether a single or multiple genes are to be targeted for being “knocked in”, “knocked out”, ”knocked down” or upregulated, the need for transient or long-term transgene expression and the target cell type. The majority of diseases that are currently being studied in clinical trials are monogenic disorders, which require the corrected expression of a single gene in target cells (Scaramuzza et al., 2009). The demonstration of effective rescuing of the phenotype is usually straight forward and does not require much optimization. The correct version of the gene with its promoter is amplified from a healthy patient, cloned into a virus vector and transduced into the target cells (Tsuruta, 2013). Regarding the treatment of β-thalassemia and ADA-SCID, the therapeutic genes are functional forms of the β-hemoglobin chain and adenosine deaminase genes, respectively (Kalle et al. 2004). The only matter of consideration in a proof of concept study is the type of vector that will be used to obtain the safest integration profile (Corrigan-curay et al., 2009).

The gene therapy treatment of some diseases requires the suppression of host genes and/or pathogens, which is more complicated (Bunnell, 1998). Genes can be suppressed by a range of techniques. The most commonly used is RNA interference (RNAi), which includes short hairpin RNA (shRNA), micro RNA (miRNA) and ribozymes to disrupt the post-transcriptional expression of the target gene(s) (Rossi 2009). Although shRNA and

miRNA are very efficient, the use of several consecutive copies could be required to fully suppress target gene expression and this requires significant optimization, especially when multiple genes are targeted (Myburgh et al., 2014; Ter Brake et al., 2008). With the treatment of HIV, host and virus genes can be silenced with various RNAi techniques. Care should be taken not to use an RNAi technique that nonspecifically targets other genes with off-target effects (Tiscornia et al. 2007). Gene editing techniques that disrupt target genes at the genomic level, include zinc finger nucleases, transcription activator-like effector nucleases (TALENs) (Bobis-Wozowicz et al., 2014) and the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system (Cong et al., 2013). These techniques, except for the CRISPR/Cas9 system, require lengthy optimization. The advantage is that the constructs can be transiently expressed and do not require insertion into cellular genomes. Gene editing techniques can be used to “knock-in” genes at specific loci; however, the insertion efficiency is low and is only useful in certain therapies (Bobis-Wozowicz et al., 2014). The *ex vivo* cloning of selected cells, where the DNA insert is in a desired location and no undesired secondary insertions have occurred, can be performed and used in therapies.

Various methods have been developed to transfer therapeutic gene(s) into target cells, each with its own advantages and disadvantages. Non-viral vectors (e.g., plasmids) used in direct delivery methods, are easy to produce, capable of delivering synthetic compounds such as oligonucleotides or small interfering RNA (siRNA), do not have the risk of vector infection and have reduced inflammatory complications (Seow and Wood 2009). However, the transfection efficiency obtained with non-viral vectors is poor compared to viral vectors, transgene expression is transient and cell specificity is low (Molas et al. 2003). Replication-deficient viral vectors (e.g., adenovirus, adeno-associated virus (AAV), retrovirus, lentivirus,

poxvirus, HSV) are more difficult to produce, and have the risk of causing insertional mutagenesis (Blumenthal et al., 2007). However, cellular specificity can be adjusted, transduction efficiency is high and stable transgene expression can be obtained in the long-term. Replication-competent oncolytic vectors (e.g., measles, reovirus, vesicular stomatitis virus, vaccinia) have the advantage of being able to spread to and transduce multiple cells *in vivo*. However, immune activation as well as strict control over cellular specificity is problematic (Seow and Wood 2009). Microbial vectors (e.g., listeria, salmonella, E. coli, bacteriophage) can be used to deliver therapeutic genes *in vivo*; however, they are currently limited to the targeting of cancer cells and face safety and efficacy problems (Baban et al. 2010) (Table 2).

Table 2 Comparison of gene therapy vectors (Gould and Favorov 2003; Nayerossadat Maedeh and Abas 2012).

Vector	Transgene capacity	Immunogenicity	Genome integration	Long-term expression	Transfer into dividing (D) and quiescent (Q) cells
Plasmid-naked	Unlimited	Low	No	Only in muscle	D and Q
Plasmid-complex	Unlimited	Low	No	No	D and Q
AAV	4 kb	High	Yes and episomal	Yes	D and Q
HSV	35 kb	High	No	Yes	D and Q
Retrovirus	Up to 8 kb	Low	Yes	Yes	D
Lentivirus	Up to 8 kb	Low	Yes	Yes	D and Q
Bacteria	Unlimited	High	No	No	D and Q

Adeno-associated virus *(AAV),* herpes *simplex virus (HSV)*. With a plasmid-complex the plasmid is allowed to complex with cationic lipids which improves the transfer to the nucleus.

Early clinical trials (Table 1) demonstrated that the use of γ-retroviruses for stem cell genetic engineering has major safety issues and does not provide efficient transduction (Doering et al. 2011). The insertion sites of therapeutic genes in two patients that developed leukemia with γ-retroviral vector stem cell gene therapy were found to be near oncogenes. It was found that the integration profile of gamma-retrovirus vectors generally favors oncogenes. This initiated the search for other virus vector options (Hacein-bey-abina et al., 2008a). Clinical trials have been conducted to test the use of viruses constructed from the backbone of the human foamy virus, murine Moloney leukemia retrovirus (MMLV), adenovirus and lentivirus (Bouard et al. 2009). Retrovirus vectors and lentivirus vectors were further improved; however, lentivirus vectors have the advantage of having a large packaging capacity (10kb from long terminal repeat (LTR) to LTR), the ability to transduce non-dividing cells and to facilitate stable integration and long-term expression of transgenes, low immunogenicity of the virus particles and an integration profile that does not favor oncogenes (Naldini et al. 1996) (Table 2). The latest self-inactivating (SIN) lentivirus vectors reduce the risk of activating oncogenes or deactivating anti-oncogenes and limit interference of endogenous gene regulation (Baum, 2008). The safe and efficient transduction of a wide range of cell types has made lentivirus the vector of choice (Throm et al. 2009). Lentiviral vectors can be derived from a range of viruses, but those derived from HIV are the most widely used. The safety and efficacy of lentivirus vectors are progressively being improved to prevent replication competent recombination (RCR) (http://lentilab.unique.ch/) and insertional oncogene activation. The polypurine tract/central termination sequence, the woodchuck hepatitis virus post-transcriptional regulatory element, the rev response element, the tat-independent self-inactivating and the Rous sarcoma virus promoter elements have been incorporated in lentivirus constructs to improve safety and transduction efficiency (Giry et al. 2011).

Although pre-clinical proof of concept studies may indicate a potentially significant benefit, translation to the clinic may be hindered by low transgene efficiency, risk for the patients and ethical constraints (Sanchez & Silberstein, 2013). After proof of concept has been demonstrated, techniques will need to be adapted for clinical purposes (Baoutina, Alexander, Rasko, & Emslie, 2007). It would be unethical to test experimental medical therapies on humans if clear benefit and safety have not been demonstrated in the pre-clinical setting (American Cancer Society, 2014). Most experimental procedures are tested either in tissue culture or in animals; however, predicted outcomes are not always representative of actual outcomes in humans (Seok et al., 2013). In order to bridge the gap between tissue culture experiments and patients, mice have been genetically manipulated to be able to receive human tissue without rejection (American Cancer Society, 2014). Various genes involved in the immune system of these mice have been functionally compromised to achieve a mouse strain that provides *in vivo* conditions comparable to those found in humans. One of the most widely used immunodeficient mouse strains is the nod scid gamma (NSG) mouse (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ)(Brehm et al., 2015). Human HSCs engraft well into irradiated newborn NSG mice or after myeloablation of adult mice, which then provide a fully functional human immune system (Ishikawa et al., 2014). These “humanized mice” can be used to investigate cancer, infectious human blood borne diseases, transplantation of cells and tissues and even genetically modified stem cells (Shultz et al., 2013). The infection and replication properties of HIV have been investigated in humanized NSG mice and were found to be similar to infection and replication in humans (Singh et al. 2012). It has further been demonstrated that the engraftment of human HSCs, genetically modified with lentivirus constructs to contain genes that prevent HIV replication, reduces HIV viral load and increases human CD4+ T-cell counts in these mice (Kiem et al.,

2012). This was accomplished and demonstrated using various techniques that include short interference RNA, micro RNA and nucleases that disrupt the expression of CCR5 (DiGiusto et al., 2010, Myburgh, 2015), a cell surface receptor that is used by HIV as a co-receptor to enter the cell.

2.2.2. The preclinical testing program

The Center for Biologics Evaluation and Research (CBER), the Office of Cellular, Tissue and Gene Therapies (OCTGT) as well as the European Medicines Agency (EMA) have produced guidelines that should to be considered when gene therapy techniques are being developed for clinical application (GTWP, 2008; OCTGT, 2013). The selection of a species/model for investigating gene therapy products must be relevant and best suited to the provision of data comparable to the clinical setting. Immunodeficient animals provide information on the potential for adverse immune responses to the *ex vivo* genetically modified cells, the vector, or the expressed transgene (GTWP, 2008). Dogs and non-human primates offer good preclinical models for HSC-related protocols with non-human primates being better, due to the fact that they are largely homologous with humans, allowing for nearly all human cytokines to be functional in this model (Watts et al., 2012). Appropriate animal models allow for accurate analysis of the potential toxicity generated by a vector, transgene and cell type, as well as the potential risks of the delivery procedure prior to clinical trials (Ciurea & Andersson, 2009). The route of administration and the procedures used to administer the product may nullify the therapeutic effect seen *in vitro* and in animal models. For example, when compared to intravenous delivery, intra-arterial delivery of MSCs avoids the accumulation of the cells in the lungs, allowing the cells more time to migrate to intended tissues (Kean, Lin, Caplan, & Dennis, 2013). The delivery procedure of the manipulated stem cells may require bone marrow conditioning which has an associated morbidity and mortality risk; this risk can however be reduced by carefully selecting an

appropriate conditioning regimen (Ciurea & Andersson, 2009). The *in vivo* behavior and activity of the transduced cells must be determined, including distribution, localization, trafficking and persistence (GTWP, 2008). Scaramuzza et al. (2009) found that CD34+ cells from a WAS patient, intended for gene therapy, proliferated slower *in vitro* with reduced cytokine receptor production compared with healthy donor cells, thereby affecting the efficacy of the gene therapy (Scaramuzza et al., 2009).

The risk of aberrant gene expression caused by the transgene and the integration profile of the vector should be thoroughly investigated and characterized in animal models to prevent further treatment related fatalities. Cavazzana-Calvo et al. 2010 determined that dominant clonal expansion is possible due to truncated cellular transcripts of a proto-oncogene that can be spliced to an acceptor site within the vector (Cavazzana-Calvo et al. 2010). Low cell viability, high cell proliferation and abnormal cytokine production of the engrafted cells can affect the safety and efficacy of the therapy (Gnecchi, Zhang, Ni, & Dzau, 2008).

Controlled procedures are to be followed during virus stock production and the transduction of HSCs to prevent contamination with pathogens. Furthermore the large scale production of vector particles must not be adversely affected by the therapeutic genes in the vector construct (Logan et al. 2002). Virus vector production- and concentration protocols can cause cellular toxicity. For example, virus concentration with polyethylene glycol 6000 precipitation provides high vector titers with significantly reduced toxicities compared to ultracentrifugation due to reduced mechanical stress on the cells (Kutner et al. 2009). The target gene that is either knocked in or knocked out should not interfere with cellular activities. A siRNA can cause non-specific gene silencing or cellular toxicity if over expressed (Tiscornia et al. 2007). Care must thus be taken with the selection of an appropriate promoter to drive the transgene as well as the transgene itself. Promoters such

as the U6 Pol-III promoter that are used to drive the strong expression of shRNA can cause toxicities *in vivo* due to the saturation of the endogenous miRNA pathway (Ely et al. 2008; Grimm et al., 2006). Furthermore, a low transduction rate can be sufficient to provide proof of efficacy *in vitro*; however, it has been demonstrated that a relatively pure transduced population of cells may be necessary to obtain effective results in mouse models. Myburgh et al. (2015) observed that the transplantation of CD34+ cells, with 20-30% positively transduced cells containing an anti-CCR5 miRNA construct, did not provide HIV modulation in NSG mice. However, HIV modulation was obtained with a CD34 cell population enriched for positively transduced cells (Myburgh et al., 2015).

2.2.3. Suicide genes

There is always the risk that stem cells used in gene therapy behave abnormally and have off-target effects, which in turn may become detrimental to the patient. The history of gene therapy demonstrates the potential for treating patients, but also shows that the gene therapy itself may be detrimental to patients and may lead to significant morbidity and mortality (Check, 2002). Hence it would be beneficial if the transplanted cells could be eliminated in the event of a life threatening situation. Additionally, the techniques used to eliminate transplanted cells should do so exclusively and not affect the recipient's cells. This can be achieved with the use of "suicide genes" (Zhan et al., 2013). The first clinical trials testing suicide genes were initiated in 2002, and since then many have followed (Table 3). The trials listed in Table 4 used T-cells and CD34+ cells transduced with suicide genes using replication competent virus vectors to target cancer cells in patients (Zhan et al., 2013). The field of suicide gene therapy has made significant progress (Blumenthal et al., 2007). Cells intended for transplantation for gene therapy can be transduced with a suicide gene, in combination with the therapeutic gene, which can be activated to specifically eliminate the transduced cells in cases of unintended oncogenesis or graft vs host disease

(GVHD) (Blumenthal et al., 2007; Zhan et al., 2013). The two most widely investigated suicide genes are caspase-9 and Herpes simplex thymidine kinase (HSV-TK) (NCT01204502, NCT01744223). The inducible caspase-9 (iCasp9) protein is activated to dimerise using a specific chemical inducer of dimerization (CID), which in turn causes apoptosis to the specific cells (Riviere et al. 2012).

Table 3 Suicide genes tested in clinical trial

NCT Number	Suicide gene	Virus vector	Conditions	Start Date
NCT00423124	HSVTK	Retro	Hematological malignancies	Jul 2002
NCT00844623	HSVTK	Adeno	Hepatocellular carcinoma,	Dec 2002
NCT00415454	HSVTK	Adeno	Pancreatic cancer	Nov 2006
NCT00710892	iCasp9	Retro	Acute lymphoblastic leukemia / Non-Hodgkin's lymphoma / Myelodysplastic syndrome / Chronic myeloid leukemia	Dec 2008
NCT00964756	HSVTK	Adeno	Ovarian cancer	Aug 2009
NCT01086735	HSVTK	Retro	Hematological malignancies	Feb 2010
NCT01204502	HSVTK	Adeno	Haploidentical stem cell transplantation	Jan 2011
NCT01744223	iCasp9	Retro	Acute lymphoblastic leukemia / Acute myelogenous leukemia / Lymphoma	Mar 2013
NCT01822652	iCasp9	Retro	Neuroblastoma	Aug 2013
NCT01875237	iCasp9	Retro	Leukemia / Myeloma / Myeloproliferative diseases	Dec 2013

Human herpes simplex virus thymidine kinase gene (HSVTK), inducible caspase 9 (iCasp9)

2.3. Efficacy

The success of a particular gene therapy depends mainly on the ability to achieve a safe level of gene dosage *in vivo* and an effective therapeutic level of gene modified stem cells (Watts et al. 2012). The transgene should be stably but not excessively expressed. Stable expression of the transgene is affected by promoter and vector choice (Taylor et al. 2013). Obtaining an effective therapeutic level of gene modified stem cells depends on the selective advantage and engraftment capacity of the modified cells.

2.3.1. Promoter selection

The expression of the therapeutic transgene is differentially driven by the nature of the promoter (Johnston and Denning 2013). Viral promoters such as the CMV promoter are commonly used in *in vitro* experiments to obtain strong constitutive protein expression. However, viral promoters are prone to long-term inactivation *in vivo* (Papadakis et al. 2004). Therefore, human promoters like the human elongation factor-1 alpha and the phosphoglycerate kinase promoters are preferentially used in vectors for gene therapy (Johnston and Denning 2013). Tissue specific promoters reduce the risk of gene expression in unwanted tissues and thereby increase the safety of the therapy. Table 4 lists a selection of tissue specific promoters which can be used to selectively express a transgene in a given tissue type such as hepatocytes, Langerhans cells and chondrocytes. These are important considerations as the promoter choice influences virus titer and transduction rates, which in turn will impact on the efficacy of the gene therapy (Giry-et al. 2011).

Table 4 Tissue specific promoters (adapted from Gould and Favorov, 2003)

Promoter	Target tissue	Disease
Salivary gland amylase promoter	Salivary gland acinar epithelial cells	Sjogren's syndrome
Kallikrein promoter	Salivary gland ductal epithelial cells	Sjogren's syndrome
Involucrin promoter	Keratinocytes	Scleroderma
Keratin 14 promoter	Basal layer of epidermis	Scleroderma
L-type pyruvate kinase promoter	Liver (hepatocytes)	Diabetes and other autoimmune diseases
Rat insulin promoter	Pancreatic β-islet cells	Diabetes
Collagen II promoter	Joints (chondrocytes)	Rheumatoid arthritis
Human Glial fibrillary acidic protein promoter	Brain (astrocytes)	Multiple sclerosis
Neuron-specific enolase promoter	Brain (neurons)	Multiple sclerosis
Interleukin-2 promoter	Activated T cells	All autoimmune diseases
MHC-II specific HLA-DRα promoter	Antigen presenting cells	All autoimmune diseases
Dectin-2 promoter	Langerhans cells	All autoimmune diseases
GATA-1 enhancer	Erythroid cells	All autoimmune diseases

2.3.2. Transduction efficiency

Transduction efficiency has a significant impact on the success of a gene therapy (Griensven et al., 2005). Only the positively transduced stem cells, which are generally a minor proportion, provide a therapeutic effect. However, the population of untransduced cells will also engraft, but will not contribute to providing the therapeutic effect. In fact, these cells may even inhibit the therapeutic effect of the successfully transduced cells (Myburgh et al., 2015). With an isolated HSC population, the primitive population of HSCs within the HSC population that has long-term engraftment capacity is very small (Griensven et al., 2005; Vollweiler et al., 2003). Therefore, with a high transduction rate, the probability of obtaining a long-term therapeutic effect from transduced HSCs is low (Watts et al., 2012). This has indeed been demonstrated in clinical trials, where short term engraftment in the majority of trials was satisfactory, but long-term engraftment was poor (Watts et al., 2012). This effect may also be important in other stem cell types such as MSCs that contain small primitive stem cell proportions (Myers et al., 2011). Since the transduced cells engraft at extremely low rates (Müller et al., 2008), the number of engrafted cells that contain the transgene is insufficient to effectively convey the efficacy of the gene therapy. Examples of where this has been observed are in treatments for Fanconi anemia and Gaucher disease. The therapeutic benefit of the transgenes was not obtained due to the low number of primitive HSCs that were manipulated (Sidransky et al. 2007). The transduction efficiency of the target cell population is thus important and is affected by parameters that include the time period of pre-stimulation, transduction duration and post transduction cell culture (Liu et al. 2009).

In order to increase the proportion of transduced cells, and hence select for a purer population, a selection marker can be used. The selection of positively transduced cells *in vitro* and *in vivo* has been accomplished with human genes such as $DHFR_{L22Y}$ which

provide resistance to the antifolate drug trimethotrexate (TMTX), and $\alpha 1_{Q118R/N129D}$ that provides resistance to ouabain, a selective Na^+/K^+-ATPase inhibitor (Rajab, Nelson, Keung, & Conrad, 2013; Treschow et al., 2007). Chemotherapy resistance genes, such as O^6-benzylguanine (O6BG), bis-chloroethylnitrosourea (BCNU) and cytidine deaminase have also been used (Momparler et al. 2002). This type of selection can greatly increase engraftment; however, a large initial number of cells is needed due to the removal of the non-transduced proportion of the cells (Biffi et al. 2013; Kang et al. 2010).

2.3.3. Engraftment efficiency

Selective advantage of the gene-modified cells and pre-transplant conditioning is important for obtaining successful treatments. Genetic correction of HSCs in the treatment of SCID provides a selective advantage over mutant cells allowing a small number of manipulated cells to achieve a therapeutic effect without a transplant conditioning regimen (Aiuti and Roncarolo 2009; Fischer et al. 2010). However, in the treatment of Fanconi anemia and Gaucher disease, the therapeutic efficacy of the HSC gene therapy was insufficient due to the small numbers of manipulated cells obtained (mentioned in section 4.2) that had no selective advantage over non-manipulated cells, and no preparative regimen was provided to overcome this limitation (Liu et al. 1999; Sidransky et al. 2007). Finding a balance between *in vivo* selection of cells and pre-transplant conditioning is difficult and is one of the current limitations in gene therapy protocols (Watts et al. 2012). If the gene-corrected cells have a selective advantage that allows them to persist and function, then no preparative regimen is provided. However, in cases where there is no selective advantage, bone marrow conditioning is to be provided to improve the repopulation of the cells. Increasing the number or proportion of gene-modified cells by *in vitro* or *in vivo* selection and/or *ex vivo* expansion will improve the therapeutic effect of the treatment and provide engraftment with less or no conditioning (Watts et al. 2012).

The success of gene therapy is generally affected by the number of stem cells used, and the efficacy of the stem cells themselves can further be affected by donor variables such as age and health status at the time of collection (Harris, 2014). Due to the difficulty of expanding HSCs without differentiation, it is important to have a high number of isolated cells at the outset of transduction (Biffi et al. 2013; Kang et al. 2010). The transplantation of expanded HSCs is being investigated in non-gene therapy clinical trials (NCT01474681, NCT01816230). The expansion of gene-modified HSCs will improve transplant success by increasing cell numbers. During the expansion of gene-modified HSCs, transduced cells can be screened for mutagenic integration sites and this will increase the safety of the treatment (Watts et al. 2012).

The number of MSCs required in gene therapies varies greatly depending on the application, site of injection and research group (Lewis & Suzuki, 2014). The advantage of MSCs is that they can be expanded in culture to obtain the desired cell number. The time it takes to expand these cells should be taken into account, which could take up to several weeks (Choudhery et al., 2013). The success of a treatment that used MSCs does not depend as much on the engraftment capacity of the cells, as with HSCs, but rather on cell number and the site of delivery. Cryopreservation of stem cells in stem cell banks offers the opportunity to preserve freshly isolated or expanded stem cells prior to the onset of disease. Stem cell isolation can be performed at a time of good health to obtained large numbers of cells which can be cryopreserved to provide a population of stem cells for gene therapy when needed (Harris, 2014).

2.4. Gene therapy procedures

Providing stem cell gene therapies to patients comes with many safety, ethical and socioeconomic concerns, all of which affect the potential success of the therapy (Vattemi & Claudio, 2009). Manipulated stem cells are considered drugs in the USA and in the European Union are classified as Cell-based Medicinal Products (CBMPs) or Advanced Therapeutic Products (Giancola et al. 2012). The handling of these cells has special requirements concerning equipment, safety standard and the training of personnel (Bosse et al. 1997). Appropriate handling conditions include the use of clean room facilities operated according to current good manufacturing practices (cGMP) and require a quality control system (Giancola et al 2012). The production protocols and clinical use of the gene-modified cells serve as a basis for the preparation of a risk management plan and the production and distribution of CBMPs. These regulations depend on the relevant national authorities and legislations (Giancola et al. 2012). A cGMP facility is specifically designed as a production facility for the manufacturing of pharmaceutical or cellular products which include the manufacturing space, the raw and finished product storage warehouse and support laboratory areas (Giancola et al. 2012). Furthermore, the vectors used (mostly viral vectors) require an accredited vector production platform that can provide high titer vector samples free of pathogenic contamination and traces of replication competent virus vectors (Bosse et al. 1997; Spencer et al. 2009). The costs involved in establishing these facilities, together with the maintenance and consumable costs, make gene therapy procedures very expensive. This creates uncertainties regarding the financial feasibility of the applications (Mavilio 2012; Soares et al. 2005). From a cost-benefit perspective, the feasibility of such therapies is measured against the costs of current therapies (some of which are life-long) and the life expectancy of patients suffering from the disease to be treated (Jackson and Pepper 2013). Gene therapy can for example be used to potentially “cure” HIV with a once

off intervention and thereby allow infected patients to reduce or discontinue highly active antiretroviral treatments (HAART). Although the once off gene therapy treatment is expensive, compared to lifelong HAART treatment it may be more cost effective (Bollinger & Stover, 1999). The economic impact of HIV on countries with high infection rates could be positively impacted (Bollinger and Stover 1999; Jackson and Pepper 2013). The cost of these gene therapies is also anticipated to decrease as the technology develops (Soares et al. 2005; Tremblay 2013).

The re-introduction of genetically modified stem cells into the patient can be associated with significant risk. In cases where allogeneic stem cells are used, there is a the risk of GVHD, which is regarded as the most lethal complication of HSC transplantation (Zaia and Forman 2013). Since bone marrow ablation is often required to free up a niche for the transplanted HSCs, an additional risk of chemotherapy-related mortality is present (Biffi et al. 2013; Kuramoto et al. 2004). The appropriate patient population should therefore be selected based on prognostic determinants, hence limiting the application of certain gene therapies to specific patient populations. Alternative techniques to total body irradiation are being investigated to provide safer procedures for creating space in the bone marrow where the HSCs can migrate to and repopulate the space. These techniques include the use of reduced intensity and combined chemotherapy regimens, anti c-Kit antibodies (Czechovicz et al. 2008) and the use of G-CSF prior to transplantation (Ugarte & Forsberg, 2013).

Close monitoring of patients receiving gene therapy is extremely important. The Committee for Medicinal Products for Human Use at the EMA has provided guidelines for follow-up of patients who have received gene therapy medicinal products (Agency, 2009). Delayed adverse reactions such as oncogenesis and immunological reactions as well as the delayed

effectiveness such as vector reactivation should be taken into account. This is due to the long life-span or persistence of the modified cells, the bio-distribution and delayed effects associated with the integrated vector and product expression. Finally, the route of administration also influences bio-distribution and the potential for serious delayed reactions. A change in the route of administration could lead to an increase in the dose of cells that gets distributed to tissues not represented in safety studies (Agency, 2009). For example, when the administration of MSCs is changed from a local to systemic route, the cells will be filtered out of circulation in the lungs and cause adverse effects (Turner et al. 2011).

2.5. Way forward

Significant progress has been made in the field of stem cell gene therapy with isolated cases of benefit having been reported to date. Due to an increase in confidence in the genetic manipulation techniques, a large number of disease types are being investigated and included in clinical trials (Myers et al., 2011). Many research groups are able to produce proof of concept data on novel gene therapy techniques in cell and animal models, but may lack the capability (expertise, facilities and funding) to translate their strategies to the clinic (Galis et al. 2015). Fragmented gene therapy research efforts are a significant hurdle to clinical translation, which supports the need for the establishment of an international gene therapy cooperative. Such a consortium would provide the platform for smaller groups to benefit from broader expertise and infrastructure, and hence enable more effective translation of gene therapies into the clinical trial stages of development. Following the example of other international consortia such as the Human Genome Project and the ENCODE projects, large budgets could be secured from governments and industries to make more funding available for gene therapy research (Tremblay, 2013; U.S. Congress, 1988). Smaller funded gene therapy consortia such as the Transatlantic Gene Therapy Consortium and the EU Seventh Framework program already exist, and they have successfully translated gene therapy strategies from techniques performed *in vitro* to clinical trials (Tremblay, 2013). These consortia have demonstrated that the sharing of expertise, the cost effectiveness and the increased probability of success are some of the benefits that can be expected when forming an international gene therapy consortium (Tremblay, 2013).

2.6. Conclusion

Stem cell gene therapies are being investigated for the treatment of many diseases. However, as with any new form of therapy, only by careful administration of the therapies and thorough evaluation of the techniques, can success be ensured. Significant progress has been made in the safety and efficacy of gene therapy procedures since the first gene therapy clinical trial was performed 26 years ago. Gene therapies are currently dominated by the use of HSCs to treat hematological diseases; however, other cell types such as MSCs and epithelial stem cells are expected to be used more commonly. The translation of a potential therapy from the proof-of-concept phase to the clinic is a long journey and many factors need to be addressed to ensure efficacy and safety. It is important to be aware of each of these factors before a subsequent phase of the process is initiated in order to be able to thoroughly investigate and improve the techniques used. The future of stem cell gene therapy lies in combining the experience obtained from clinical trials and the advances made in basic research to provide the safest and most efficacious product for administration.

Chapter 3

Clinical applications of genetically modified hematopoietic stem cells

3.1. Introduction

Gene engineering cells of the hematopoietic system has great potential for the treatment of a diverse range of genetic and infectious diseases (Somia and Verma, 2000). Although gene engineered differentiated hematopoietic cells, such as T-cells, have been used in clinical trials, this only provides a transient effect as the life span of adult cells is relatively short (Rosa et al. 1999, Rossig and Brenner, 2004). In contrast to differentiated cells, hematopoietic stem and progenitor cells (HSPCs) have the potential to engraft and self-renew, thus providing long term benefits to patients (Burnett et al. 2012; Penn and Mangi, 2008). This chapter focuses on advances made in the field of using genetically manipulated HSCs for the treatment of diseases such as severe combined immunodeficiency (SCID), Wiskott-Aldrich syndrome (WAS), metachromatic leukodystrophy (MLD) and human immunodeficiency virus infection (HIV) (Genovese et al. 2014; Ye at al. 2008). The process followed by most clinical trials that make use of genetically modified HSCs is shown in Figure 3. This process includes isolating HSPCs from peripheral blood that contains CD34+ cells that were mobilized from the bone marrow of patients using the G-CSF cytokine, transduction with a viral vector carrying the transgene of interest and infusion of transduced cells back into the same patient following myeloablative cytoreduction. However, the beneficial effects and safety of protein, shRNA or miRNA transgene products need to be demonstrated *in vitro* and in *in vivo* before entering into phase I clinical trials. This is a very costly and time consuming process (Snoy, 2010). The success is further dependent on the ability to reconstitute the hematopoietic system with enough cells that contain the transgene (Genovese et al., 2014; Liu et al., 1999; Sidransky et al., 2007). In order to do this, a large number of HSCs must be transduced, preferably with only one copy per cell without the cells losing their engraftment capacity (Genovese et al., 2014; Liu et al., 1999; Sidransky et al., 2007); however, obtaining one vector copy per cell is not critical. The transduction rate

of HSCs is much lower than other cell types and spontaneous differentiation occurs under standard culture conditions (Briones et al. 1999; Ivanovic, 2009). A high transduction rate can be obtained by using a high multiplicity of infection (MOI), but doing so increases the number of integrated transgene copies per cell (Kustikova et al. 2003). Caution thus needs to be exercised when using high virus titers due to an increased risk of oncogenesis (Hacein-Bey-Abina et al. 2003, Kustikova et al. 2003). Virus vectors that were initially used were derived from γ-retrovirus, a subclass of the retrovirus family, which had a high rate of associated oncogenesis (Riviere at al. 2012). Lentivirus vectors, derived from the lentivirus subclass, were developed in the 1990s. Although their use reduces this risk due to non-preferential random insertion, it does not eliminate it (Naldini et al. 1996). The potential for random insertion of the DNA fragment into the cellular genome resulting in oncogensis is reduced by eliminating the viral promoter and enhancer from the integrated vector (Baum, 2008). Endogenous human genes can therefore not be induced by these viral factors. It is also possible to include a suicide gene in the vector designed (Table 3) to allow for the selective killing of cells that behave in an aberrant manner (see section 2.3.2) (Duarte et al. 2012). Many clinical trials have demonstrated that the use of HSCs in gene therapy is safe and does provide benefit. However, various facets of the gene therapy process which have been improved in the experimental setting have not yet been implemented in clinical trials.

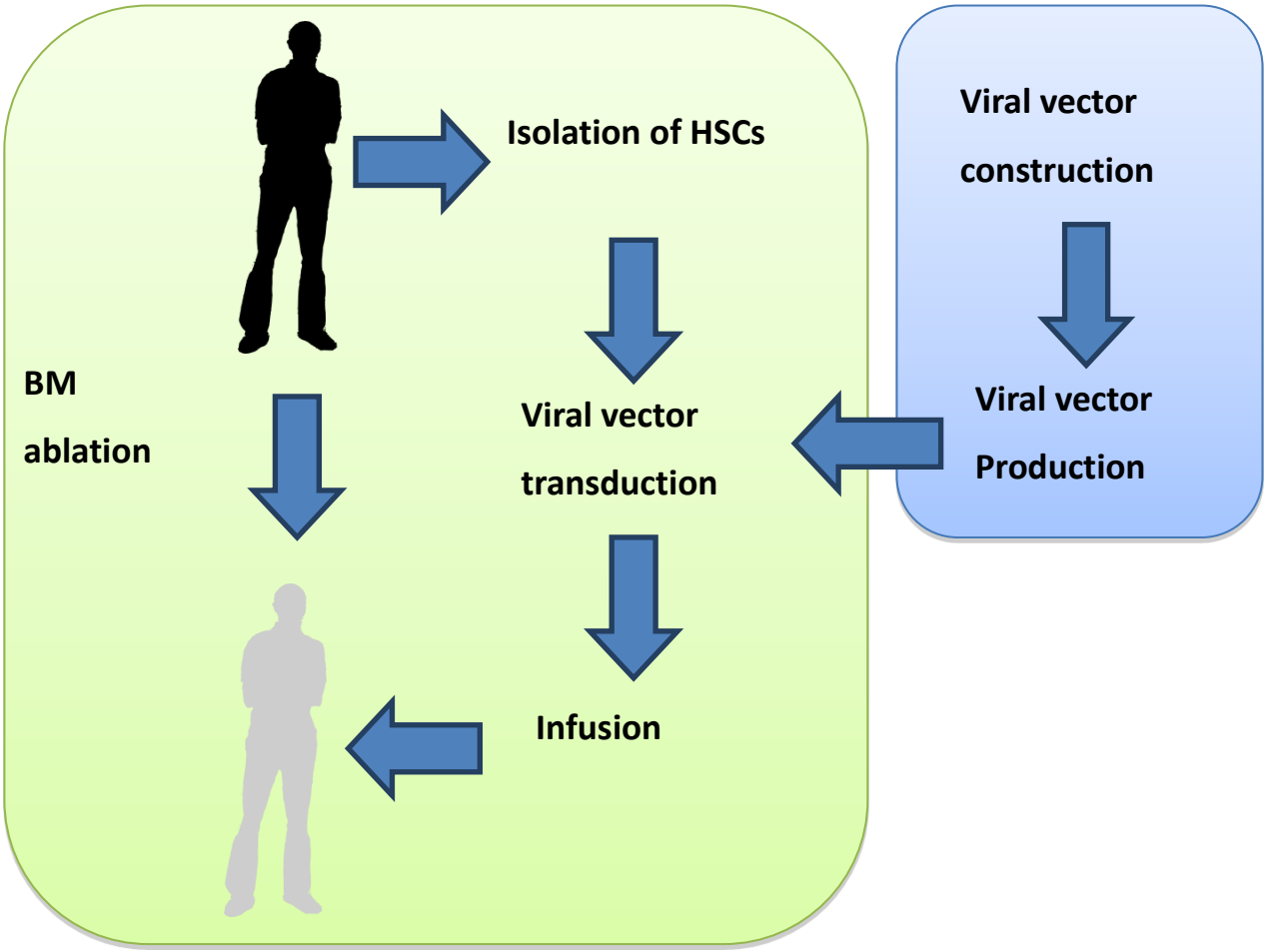

Figure 3. Gene therapy process. Steps in the gene therapy process are shown. The indicated process can only be followed in a clinical trial setup once the trial is accepted by the relevant regulatory body which usually requires sufficient data from a pre-clinical animal model. During a cell therapy treatment, a donor (black) provides HSCs obtained directly from the bone marrow or indirectly from HSCs mobilized from the bone marrow into the peripheral blood. The patient (grey) needs to undergo bone marrow ablation before receiving the donor's HSCs. During a stem cell gene therapy, HSCs are obtained from the donor and subjected to transduction using a viral vector construct prior to the treatment. Thereafter the transduced cells are infused into the patient.

3.2. Clinical trials

Genetic engineering aims to alter the genotype of a population of cells with the objective of introducing a change in the health of the recipient (Singer and Cohen, 2001). Fifteen phase I/II clinical trials are currently registered on the clinicaltrials.gov website, which make use of transduced HSCs in the treatment of various diseases including HIV, SCID and WAS. However, many non-registered clinical trials were identified on PubMed using “gene therapy”, “cd34+ cells” and “clinical trial” as key words (Table 5). The types of diseases treated in this way include immunodeficiency diseases as well as genetic and infectious diseases. Over a hundred patients within the listed clinical trials have received gene-modified HSCs (Riviere et al. 2012) and gene engineering has been successful in improving the symptoms of the underlying disorder in a growing number of diseases. For example, in 2010 three patients with beta-thalassemia major received gene therapy that partially corrected the disease (Boulad et al. 2014). In 2012, five out of twenty patients with adenosine deaminase severe combined immunodeficiency (ADA-SCID) showed positive results (Cavazzana-Calvo et al. 2012). In 2013, it was reported that three patients with metachromatic leukodystrophy (MLD) (Biffi et al. 2013) and three with WAS (Aiuti et al. 2013) showed improvement.

Previous clinical trials attempted to alleviate the symptoms of particular diseases by inserting therapeutic genes into the host DNA that either produce the desired proteins (Nienhuis, 2013) or permanently down-regulate undesired genes with the use of shRNA, miRNA or post transcriptional regulation (Digiusto et al. 2010). The first attempt to use genetic manipulation as a therapy to treat humans was during 1990 at the National Institutes of Health, where T cells were isolated from two patients and were treated withretroviral vectors containing a corrected ADA gene. The transduced T cells were

reinfused into the patients and both patients experienced partial improvements of disease symptoms (Blaese et al. 2015). However, it was not until 2000 that therapy provided an observed improvement of the disease phenotype when two patients with severe combined immunodeficiency-X1 (SCID-X1) received CD34+ cells treated with Moloney-retrovirus (MLV) derived vectors expressing the gamma c chain of the IL-2 receptor (Cavazzana-Calvo et al. 2000). However, four out of nine treated patients in this trial developed leukemia. In retrospect, the success of this treatment is not surprising. Gamma-chain X-SCID is probably the only lympho-hematological defect that can be treated by gamma-retroviral gene therapy. This is because, despite the very low overall efficiency of stable gene correction in HSCs (approximately 0.1 % corrected cells) (Cavazzana-Calvo et al. 2000; Rivat et al. 2012, Aiuti 2002), corrected T-cells benefit from a massive proliferation phase in the thymus due to the presence of a functional gamma c-chain (Gougeon and Chiodi, 2010). The end result is the replacement of nearly all peripheral T-cells with corrected cells. The final T-cell population is constituted by only a few clones that surprisingly and luckily cover a repertoire that is diverse enough to ensure an overall good immune response (Cavazzana-Calvo et al. 2000; Gougeon and Chiodi, 2010). Due to gamma-retroviruses that have a low transduction efficiency, it uses cell division to access genomic DNA (Yamashita and Emerman 2006), and since cell division has a dramatic impact on the “stemness” of human HSCs (Briones et al. 1999; Ivanovic, 2009), it is not surprising to find so few corrected HSCs in the bone marrow of patients in the X-SCID trial.

The leukemia cells of the 4 SCID-X patients who developed T-cell leukemia contained the MLV integrated vector near proto-oncogenes such as LMO2, Bmi-1 or Cyclin D2 (Hacein-bey-abina et al. 2008a). These malignant proliferations are most likely the result of activation of the proto-oncogenes by the fully active LTR present in the MLV vector.

Interestingly, forced expression of LMO2 activates the expression of several genes typical of HSCs, suggesting that LMO2 can reactivate a HSC program (McCormack et al., 2010). Also, forced expression of Bmi-1 leads to an increase in the repopulating capacity of HSCs (Iwama et al. 2004). Altogether, it seems that activation of these oncogenes assisted transduced cells to remain in a stem-like state, and/or repopulate the bone marrow of the recipients, and thus compensated for the overall very low efficiency of the MLV procedure. Although this trial showed the dramatic effect of gene therapy in lympho-hematologic defects (the treated children are now living a nearly normal life outside of their plastic bubble (Baum, 2011)), it also showed that gamma-retroviruses have safety issues associated with their use in gene therapy applications due to proto-oncogenic activation.

The most commonly used vectors today are lentivirus vectors. Recent findings suggest that they overcome previously experienced safety and gene-transfer issues relating to oncogenesis (Flight, 2013). Several studies related to the clinical trials mentioned have determined vector copy number (VCN) of the transgene per CD34 positive cell. The average VCN/cell ranged between 0.0001 and 4.4 and is strongly dependent on the multiplicity of infection (MOI) used (Table 5) (Biffi et al. 2013; Charrier et al. 2011; Ronen et al. 2011). The insertional mutagenic implications thereof were investigated using next generation sequencing (Table 5) (Aiuti et al. 2013; Hacein-bey-abina et al. 2008b). No adverse effects have yet been reported for lentivirus vector insertions. Transduction efficiencies with lentiviral vectors observed in clinical trials vary considerably (Table 5, column 5) which affects the number of engrafted cells that express the transgene (Table 5, column 7) and the number of transduced cells capable of long-term engraftment (Table 5, column 8). Potential benefits of the transgenes may be too small to be observed and rendered obsolete due to a the small number of transduced cells that engrafted. Due to the

large numbers of CD34+ cells needed for the transduction procedure (Table 5, column 4), the source of CD34+ cells is mainly from bone marrow mobilized peripheral blood (Nienhuis, 2013).

Although clinical trials conducted with gene modified HSCs are based on their undeniable therapeutic potential (Riviere et al. 2012), meaningful successes has thus far only been obtained in one clinical trial (NCT00028236). The protocols used to repopulate patients' bone marrow with genetically modified HSCs have generally not provided the desired results. The ideal situation would be (a) to obtain a large number of primitive HSCs from a patient; (b) to transduce 100% of the cells with a vector that integrates at one VCN per cell; and (c) for the transduced cells to maintain their primitive HSC characteristics. The integration of the transgene should not affect the expression of surrounding genes and expression of the transgene itself should be at a desired level to obtain a therapeutic effect. Vector constructs must preferably contain a mechanism to eliminate transduced cells *in vivo* should undesirable effects arise. Areas requiring careful consideration in the gene therapy process are discussed below.

Table 5 Gene therapy clinical trials for patients with hematopoietic disorders.

	Disease	NCT Number	Initial CD34 cells (cells/kg)	Percentage transduced CD34+ cells or cells/kg	VCNs/cell	Short-term engraftment (% of total cells)	Long-term engraftment (% of total cells)	References
1	X-SCID	NCT00028236	$1x10^6$	36%-40%	-	-	-	(Cavazzana-Calvo et al. 2000)
2	X-CGD	NCT00927134	9.11-11.3 $x10^6$	5.1, $3.6x10^6$	2.6, 1.5	-	-	(Ott et al. 2006; Stein et al. 2010)
3	β-Thal	NCT00336362	$2x10^6$	-	-	-	-	(Yannaki et al., 2012)
4	CGD	NCT00394316	$5x10^6$	4%	1.335	0.03%, 1.1%	-	(Kang et al. 2010)
5	β-Thal	-	$2.05x10^6$	50, 33%	0.6	23%, 25%	1%	(Cavazzana-Calvo et al. 2010)
6	MLD	NCT01560182	1.35 - $9.25x10^6$	90-97%	2.2-4.4	4%	-	(Biffi et al. 2006; Capotondo et al. 2007)
7	WAS	NCT01515462	$5x10^6$	>90%	2.3	>90%	25%-50%	(Aiuti et al. 2013)
8	X-SCID	NCT01306019	$4.8\text{-}9.9x10^6$	20-40%	0.2-1.5	-	-	(Buckley 2004; Cavazzana-Calvo et al. 2000)
9	β-Thal	NCT01639690	$2\text{-}10x10^6$	$2\text{-}12x10^6$ cells/kg	0.4-0.6	-	-	(Boulad et al., 2014)
10	ADA-SCID	NCT01852071	$0.7\text{-}9.8x10^6$	-	0.1, 1.8	<0.01%	Detected > 9 years	(Candotti et al., 2012)
11	HIV	NCT01769911	$7x10^6$	4.5×10^6, 18%	-	1%	-	(DiGiusto et al., 2010)
12	HIV	-	1.29-$10.1X10^6$	7-57%	0.0001-0.00001	0.01%	0.005%,	(Amado et al., 2004; Mitsuyasu et al., 2009)
13	HIV	-	$17X10^6$	48%	-	<0.1%	-	(Kang et al. 2002)(Mautino, Keiser, and Morgan 2001)
14	HIV	-	-	17.6-33.5%	-	-	-	(Bauer et al. 1997; Rossi 2000)
15	HIV	-	$1.2\text{-}3.2x10^6$	7-30%	-	<0.03%	0.001%	Kohn et al. 1999)

Disease type and National Clinical Trial (NCT) number are mentioned, the initial number of CD34 cells (which affects engraftment potential), the percentage transduced cells or total number of cells (which also affects engraftment of transduced cells), vector copy number (VCN) (which can possible affect mutagenesis), and the percentage of cells that engrafted in the short-term and long-term are indicated.

Table 5 (Continued)

	Vector	Ablation	Assisted transduction	Cytokines used with transduction	MOI
1	Retro	No ablation	Retronectin	50ng/mL SCF, 50ng/mL TPO, 25ng/mL IL-6, 5ng/mL IL-3, 50ng/mL Flt-3L	3x 1 MOI
2	γ-Retro	4mg/kg Busulfan	Retronectin	300ng/ml SCF, 100 ng/ml TPO, 60 ng/ml IL-3 , 300 ng/ml FLt-3L	-
3	Lenti	-	-	-	-
4	MML	10mg/kg Busulfan	Retronectin	50ng/ml SCF, 50ng/ml TPO, 5ng/ml, IL3, 50ng/ml FLt-3L	2 X ? MOI
5	Lenti	4x 3.2mg/kg Busulfan	Retronectin, protamine sulfate	300ng/ml SCF, 10ng/ml TPO, 100ng/ml IL-3, 300ng/ml Flt-3L	1 X55 MOI
6	Lenti	-	Retronectin	300ng/ml SCF, 100ng/ml TPO, 60ng/ml IL-3, 300ng/ml Flt-3L	2 X 100 MOI
7	Retro	-	-	300ng/ml SCF, 100ng/ml TPO, 60ng/ml, IL-3, 300ng/ml Flt-3L	2 X 100 MOI
8	Lenti	-	-	100ng/ml SCF, 100ng/ml TPO, 100ng/ml, IL-3, 100ng/ml Flt-3L	-
9	Lenti	8mg/kg Busulfan	-	-	-
10	MML	4mg/kg Busulfan	Retronectin	MGDF, SCF, FLt-3L	3 X ? MOI
11	Lenti	BCNU/VP16/Cytoxan	-	-	-
12	Retro	-	Retronectin	100ng/ml SCF, MGDF 50ng/ml	-
13	Retro	60mg/kg Cyclophosphamide	Fibronectin	-	-
14	Retro MMLV	-	-	100ng/ml SCF, 50ng/ml IL-6, 10ng/ml IL-3	1 X 2 - 25 MOI
15	Retro MMLV	None	Retronectin, protoamine sulfate	100ng/ml SCF, 50ng/ml IL-6, 20ng/ml IL-3	3 X ? MOI

Vector type used is indicated, the ablation regime indicated affects engraftment, assisted transduction affects transduction efficiency, cytokines used affect transduction efficiency and engraftment potential of cells and MOI affect transduction efficiancy.

3.3 Transgene efficacy and safety

The indication for the gene therapy will determine if an exogenous protein needs to be expressed in the target cells or be secreted by the cells, or if selected endogenous genes need to be inactivated. Stable expression of the transgene is important for a long term therapeutic effect and should not interfere with cellular function.

3.3.1. Efficacy

The nature of the promoter that drives the expression of the therapeutic transgene is one of the most important factors that will affect the efficacy of the transgene (Johnston et al, 2013). Constitutive viral promoters such as the CMV promoter, that is used for protein expression, results in higher levels of expression than endogenous promoters such as the human elongation factor-1 alpha (EF1α) and the phosphoglycerate kinase (PGK) promoters (Johnston et al, 2013), but are prone to long term inactivation (Lee et al, 2011). The simultaneous expression from one construct of two genes such as a therapeutic gene and a reporter gene has been described. The two genes can be driven either by two separate promoters or by a single promoter in which case they would be connected via an IRES (Internal Ribosome Entry Site) element or a cleavage peptide. The first gene in the bicistronic IRES arrangement is prone to stronger expression while the use of two promoters ensures strong expression of both genes (Giry-laterrière et al., 2011). The optimal orientation of transgenes used in constructs depends strongly on the choice of promoter and it might be necessary to consider moving one gene to the negative strand. Promoter choice has also been found to affect virus packaging and thus the titer and transduction rates (Giry-laterrière et al., 2011).

The optimization of expression of non-coding RNAs such as shRNAs and miRNAs is not trivial. U1, U6, H1, SK7 and tRNA lysine 3 promoters have been used to drive expression of shRNAs and miRNAs (Ter Brake et al., 2008). Usually, expression of shRNAs has been achieved by using Pol III promoters, the reason being that the addition of polyA tails is incompatible with further steps of shRNA processing (Abbas-terki, Blanco-bose, Déglon, & Pralong, 2002; An et al., 2003). The advantages of Pol III promoters are their simple and compact structure together with their high levels of expression. However, this high level of activity is potentially toxic due to competition with expression of endogenous non-coding RNA species resulting from the over-saturation of the RNAi machinery, specifically karyopherin exportin-5 (An et al., 2006; Grimm et al., 2006). miRNAs can be expressed from Pol II promoters allowing for tissue specific and/or inducible expression of the transgene (Boudreau, Martins, & Davidson, 2009). Multiple siRNAs can be expressed with a single promoter or with multiple promoters. However, DNA sequence repeats need to be avoided to prevent siRNAs from being cut out during the packaging of the virions. Thus, it might be more ideal for multiple siRNAs to be expressed from different promoters. The alternative is that the shRNA loops contain nucleotide mismatches in the stem section that do not affect the expressed siRNAs.

3.3.2. Safety

Allogeneic haematopoietic cells, when transplanted, can contain or produce white blood cells that recognize the recipient as foreign and attack the host's cells; this commonly manifests as GVHD (Zhan et al., 2013). A further major safety concern for gene therapy is the disruption of endogenous gene transcription and function with the potential of causing oncogenesis; multiple insertions in random positions in a cell's genome

increases this potential (Blumenthal et al., 2007). A technique that has been developed to overcome these concerns includes the transduction of cells intended for transplantation with a vector which also contains a suicide gene that can be activated to eliminate the transduced cells when severe GVHD occurs (Zhan et al., 2013) or when oncogenesis is activated (Blumenthal et al., 2007). Clinical trials that have made use of this strategy in HSCs are shown in Table 3; they either use inducible Caspase-9 or the Herpes simplex thymidine kinase (HSV-TK) suicide genes applied to the cells via transduction with adenovirus or retrovirus vectors (www.clinicaltrials.gov). Cells used in the mentioned clinical trials to treat patients that received chemotherapy include T-cells transduced with a suicide gene to induce a graft vs. tumour reaction together with CD34+ cells that are needed for bone marrow reconstitution (Alton et al. 2007; Zhan et al. 2013). The inability to obtain 100% transduced cells is a limiting factor. This is because it is only the transduced cells that will be killed when the suicide genes are activated. The non-transduced cells will still be in circulation and be able to cause GVHD as normal. Improved transduction procedures will thus greatly benefit this technology (Bordignon et al. 1999). Attempts to further increase gene therapy safety include the use of Zinc finger nucleases, CRISPRs (clustered regularly interspaced short palindromic repeats) (Cong et al., 2013) or transcription activator-like effector nucleases (TALENs) that can be used to facilitate a more specific insertion of the transgene with less risk of insertional activation of oncogenes (Bobis-Wozowicz et al., 2014). Each of these techniques has its own advantages and challenges and all are still in the experimental phase.

3.4. Source of HSCs used in clinical trials

A significant number of CD34 positive cells, ranging from 1 to $10x10^6$ CD34+ cells/kg (Table 5), can be collected from patients following bone marrow mobilization with G-CSF. However, some patients mobilize poorly which results in an insufficient number of CD34 cells being collected. These patients would therefore forfeit participation in a clinical trial (Candotti et al. 2012; Kang et al. 2010). However, Plerixafor can be provided with G-CSF to assist with bone marrow mobilization (To et al. 2015). A hematopoietic stem cell (HSC) transplant generally requires $4x10^6$ CD34+ cells/kg (Heimfeld, 2003). The most common method currently used to isolate HSCs is magnetic bead isolation of CD34+ cells. The CD34 cell surface marker is only a proxy for HSPCs (A and B) and is not indicative of the engraftment capacity of the cells (Genovese et al., 2014). The isolated CD34+ cells are heterogeneous, containing a large number of committed cells, progenitors and short-term stem cells and a small proportion of long-term engrafting cells (Gao et al. 2001). This very primitive bone marrow isolated HSC population can vary greatly between patients which affects the success of long term engraftment. This variation in long-term engraftment capacity of CD34 isolates is clearly demonstrated by secondary transplantation of immunodeficient mice. The percentage of human CD45 chimerism in secondary recipient mice (mice that received the injection of bone marrow from the primary mice) at week 17 was observed by Wiekmeijer et al. (2014) to be between 30% and 80% (Wiekmeijer et al., 2014) and by Cai et al. (2011) to be between 55% and 70% (Cai et al., 2011). By including additional markers such as CD133, CD38, CD45RA, CD49f and CD90 (Wiekmeijer et al., 2014) or by performing assays such as the side population assay (Brushnahan et al., 2011), an estimate of the size of the true primitive HSC population can be obtained.

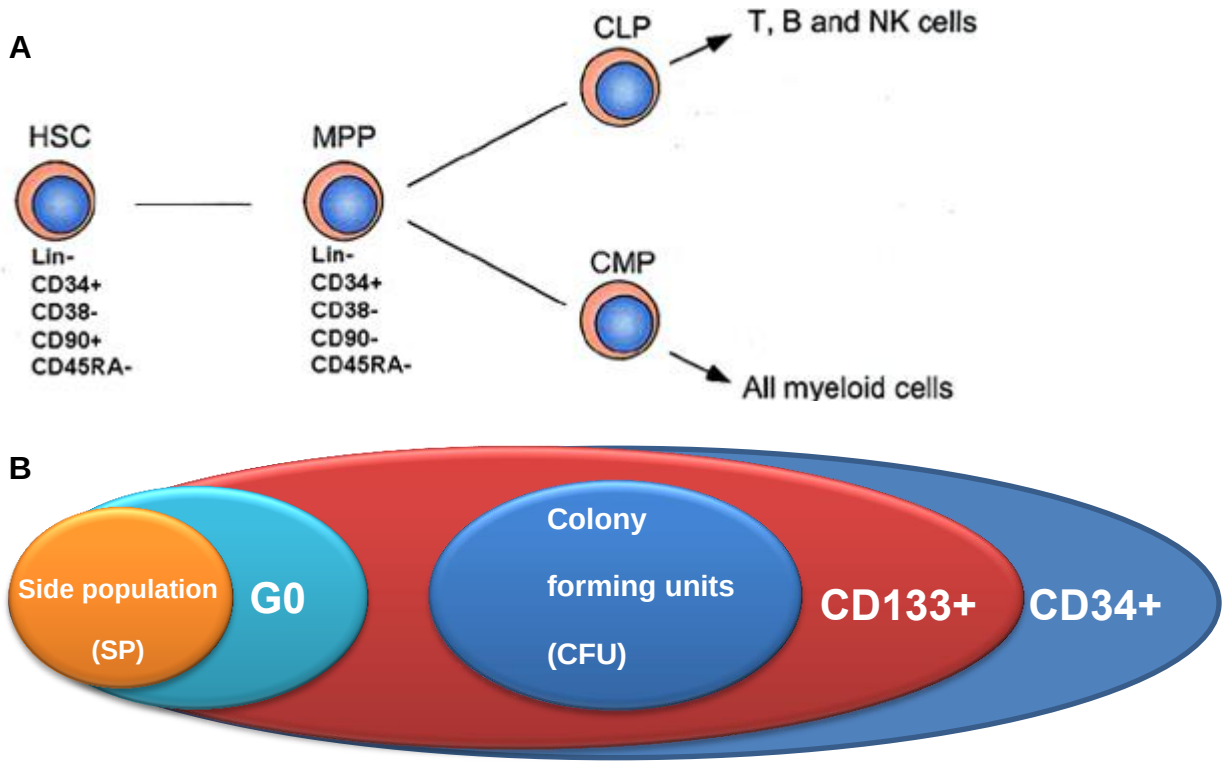

Figure 4: Hematopoiesis of HSCs into differentiated cells. **(A)** The CD34+ cell surface marker in a characteristic of HSPCs. **(B)** The heterogeneous population of cells within the CD34 cell population.

The side population assay makes use of the characteristic of certain stem cell types to efflux toxins and dyes better than differentiated cells, which allows these stem cells to be quantified using flow cytometry. Interestingly, it has been found that a proportion of cells that are CD34- do have long-term engrafting properties and are even more primitive than the CD34+ cells (Engelhardt and Lu 2002; Zanjani et al. 2003). Detailed analysis of the extent of the long-term HSC population present in a particular patient, could help to determine whether the patient should be excluded from the trial because of a small primitive HSC population that would not provide sufficient long-term engraftment (Genovese et al., 2014). It would thus be beneficial to rather do negative selection with lineage positive markers which will result in the removal of the differentiated cells which results in both the CD34+ cells stem cells and the primitive CD34- stem cells being isolated (Mizokami et al., 2009). It has been reported that the primitive CD34- cells cannot migrate to the bone marrow because they express low levels of CXCR4 which will not contribute to engraftment (Sonoda, 2008). However, CXCR4 expression can be induced in primitive CD34- cells during transduction by cytokine stimulation (Kollet et al., 2002) which should enable them to “home” (Kahn et al. 2015). This may contribute to having more transduced primitive HSCs in the sample for infusion. Bone marrow mobilized peripheral blood provides the highest number of CD34+ cells (Greinix & Worel, 2009), but with the use of techniques to expand HSCs without differentiation other sources of HSCs can also be used, such as umbilical cord blood derived HSCs (Boitano et al., 2010; Chaurasia, Gajzer, Schaniel, Souza, & Hoffman, 2014) (discussed in the “Engraftment of transduced cells” section, page 60).

3.5. Viruses used to transduce human cells

The leukemia that developed in the four out of ten patients that took part in a clinical trial performed in 2003, developed because the γ-retrovirus used inserted near the LMO2 oncogene (Hacein-bey-abina et al., 2008a). The γ-retrovirus vectors are one of many virusal vectors that have been used in clinical trials including vectors with varying genotoxicities such as those constructed from the backbone of the human foamy virus, murine Moloney leukemia retrovirus (MML), γ-retrovirus, adenovirus and lentivirus. The clinical trials mentioned in Table 5 show that γ-retroviruses are unsuited for HSC genetic engineering due to their poor efficiency and reduced safety. Self-inactivating lentivirus and γ-retrovirus vectors have been developed to reduce the risk of activating or deactivating oncogenes by ensuring a limited number of inserted gene copies and preventing interference of endogenous gene regulation (Baum, 2008).

Lentivirus vectors have several advantages over previously used virus vectors and are becoming the vector of choice (Throm et al., 2009). Various envelope proteins can be used with lentiviruses including the envelope glycoprotein (VSV-G), which allow lentiviral vectors to transduce a range of cell types. Lentiviruses can also transduce non-dividing cells (Naldini et al. 1996). The packaging capacity of lentivirus vectors is relatively large (>10kb transgene cassette), and the integration of the transgenes is stable with long term expression. Lentiviruses are associated with low immunogenicity and tend to integrate within thymine rich coding regions but do not favour integration near known protooncogenes (Naldini et al., 1996). Lentiviral vectors can be derived from HIV, feline immunodeficiency virus (FIV) (Kang et al. 2002), simian immunodeficiency virus (SIV) (Mangeot et al., 2000), bovine immunodeficiency virus (BIV)(Olsen, 2003), and equine infectious anemia virus (EIAV)(Olsen, 2003). HIV derived lentiviral vectors are most

widely investigated and used in clinical trials (Table 5 and Table 6). The vector system has been progressively changed to prevent replication competent recombination (RCR) by reducing the number of virion genes and separating them into 3 (2nd generation) or 4 plasmids (3rd generation) (Salmon & Trono, 2007). Non-primate vector systems have also been investigated such as FIV which has the potential to be used for the transduction of HSCs due to its ability to transduce non-dividing cells (Kohn et al. 1999). Alternative envelope glycoproteins such as Ross River Virus (RRV) have also been investigated to improve transduction rates; however, the RRV glycoproteins have only been demonstrated to improve the transduction of liver cells as compared to VSV-G (Kang et al. 2002). The incorporation of the polypurine tract/central termination sequence (PPT/CTS) dramatically increased the transduction efficiencies by playing a crucial role in nuclear import of the preintegration complex (Scherr et al., 2002; Sirven, Ravet, Charneau, Coulombel, & Gue, 2001). During initiation of HIV-1 reverse transcription of the plus-strand DNA at the cPPT and termination at the CTS, a three-stranded DNA structure is formed, called the HIV-1 central DNA flap. This DNA flap acts as a cis-active determinant of HIV-1 genome nuclear import enabling replication of non-dividing cells (Zennou et al., 2000).

Lentivirus vectors therefore do not require cell division to deliver their genetic cargo into the host cell genome (Barrette, Douglas, Seidel, & Bodine, 2014). Most current protocols, including those used for clinical trials, use a combination of cytokines thatimprove lentivector transduction by inducing cell division or by promoting stem cell preservation (Table 5) (Logan, Lutzko, and Kohn 2002). As a result, the percentage of transduced cells is often above 10-20% and HSCs keep their stem cell properties prior to transplantation (Barrette et al., 2014). This rate of transduction is generally 100-fold

higher compared to what is achieved with gamma-retroviruses (Table 5), which provides sufficient numbers of corrected cells to achieve normal or near-normal cellular functions without requiring further amplification (Blaese et al., 2015). Also, since all current lentivectors have self-inactivating LTRs, the risk of host gene activation by virus insertion is virtually absent. The only risk that can be imagined would be the disruption of an anti-oncogene by vector insertion leading to unwanted proliferation due to a 2-fold decrease of this specific oncogene (the chance of disruption of the second allele of this oncogene is virtually zero) (Knight et al., 2012). Aberrant gene expression by deregulated splicing has been reported for patients participating in clinical trials using lentivirus vectors (Knight et al., 2012; Moiani et al., 2012). The truncated cellular transcripts caused by a proto-oncogene that was spliced to an acceptor site within the vector resulted in dominant clonal expansion (Cavazzana-Calvo et al., 2010). Methods that reduce the risk of aberrant splicing have been described but still need proper optimization (Knight et al., 2012). Novel lentivirus vector backbones further include the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), rev response element (RRE), tat-independent SIN and the Rous sarcoma promoter elements to increase safety and transduction efficiency (Table 6)(Salmon & Trono, 2007). The number and orientation of transgenes used in constructs as well as promoter choice were also found to affect virus titer and transduction rates (Salmon & Trono, 2007).

Novel lentivirus vectors thus overcome previously experienced limitations; however, vector design is not trivial and proper optimization is needed (Salmon & Trono, 2007). Alpharetrovirus vectors have the potential to further improve safety by having the most neutral integration profile among retroviruses as evidenced by their integration profile that does not favor certain areas. Also, they do not produce aberrantly spliced transcripts,

containing cellular exons fused to alpharetroviral sequences, as in the case of lentivirus vectors (Kaufmann et al., 2013; Suerth, Maetzig, Galla, Baum, & Schambach, 2010). The SIN alpharetroviral vector system described by Kufmann et al. (2013) has demonstrated its potential as a safe platform for the future treatment of hematologic disorders with gene therapies. This is a novel vector system and still needs to be properly investigated.

Table 6 Transduction experiments of CD34+ cells with lentivirus vectors.

Vector name	RNAi promoter(s)	Transfection	Transduction conditions (MOI, Exposure time, cytokine combination)	Transduction	Transduction efficiencies	Animal engraftment efficiencies	References
RtatpEGFP	-	CaPO4	MOI 20, 2x24h, 300ng/ml SCF, 300ng/ml Flt-3L, 10ng/ml IL-3, 10ng/ml IL-6	Protamine sulfate	59%	-	(Evans et al. 1999)
pHR'-CMV-GFP	-	CaPO4	MOI 1000, 25h, 25ng/ml SCF, 5ng/ml IL-3, 16.5ng/ml IL-6	Retronectin	45.5%, 12.4%	-	(Case et al., 1999)
TRIPΔU3-EF1α	-	CaPO4	MOI 1000, 24h, 100ng/ml SCF, 100ng/ml Flt-3L, 60ng/ml IL-3, 10ng/ml TPO	Comparison	45%, 44%	-	(Sirven et al., 2001)
-	-	CaPO4	MOI 10/50, 2x16h , 100ng/ml SCF, 100ng/ml Flt-3L, 100ng/ml IL-3, 200ng/ml IL-6, 100ng/ml TPO	Retronectin, Protamine sulfate, spinoculation, deoxynucleoside triphosphate	42, 36%	40.8, 32.3%	(Scherr et al., 2002)
pHIV	U6, VA1	CaPO4	MOI 20, 10ng/ml SCF,10ng/ml IL-3, 10ng/ml IL-6	Fibronectin	15%		(M. Li et al., 2003)
pHIV-7-GFP	U6	CaPO4	MOI 10-20, 2X 24h , 100ng/ml SCF, 100ng/ml Flt-3L, 20ng/ml IL-3, 20ng/ml IL-6, 10ng/ml TPO	Polybrene	>90%	3-53%	(Banerjea et al., 2003)
HIV-1ΔENΔV3		CaPO4	MOI 50, 3h, 100ng/ml SCF, 50ng/ml IL-6, 50ng/ml IL-3	-	20%	-	(Gruber et al. 2003)
pHIV-7-GFP	U6, VA1	CaPo4	MOI 40, 24h, 100ng/ml SCF, 100ng/ml Flt-3L, 20ng/ml IL-3, 20ng/ml IL-6, 10ng/ml TPO	Retronectin, pre-stimulation	<90%	-	(M. Li et al., 2005)
pHIV	eIF2α	Lipofection	MOI 1, 2X 24h, 100ng/ml SCF,10ng/ml IL-3, 10ng/ml IL-6	Polybrene, expanded, stroma cell co-culture	82%	-	(Dimitrova et al. 2005; Sutton et al. 1998)
pRSC-SMPGW	U6	CaPO4	MOI 100, 18h, 50ng/ml SCF, 50ng/ml G-SCF, 50ng/ml Flt-3L	Retronectin, sodium pyrovate, protamine sulfate	6-50%	<20%	(Trobridge et al., 2009)
FV-SMPGW	H1, U6	CaPO4/PEI	MOI 2, 24h24h, 100ng/ml SCF, 100ng/ml G-SCF, 100ng/ml Flt-3L, 100ng/ml IL-3, 100ng/ml IL-6, 20ng/ml TPO	Retronectin, pre-stimulation	5-50%, <80% selection	8.2%	(Kiem et al. 2010)
pCLC-20	-	CaPO4	MOI 1000, 24h, 100ng/ml SCF, 100ng/ml Flt-3L, 100ng/ml TPO	Comparison	<40%	-	(Hanawa et al., 2002; Uchida et al., 2011)
CCLc-MNDU3-x-PGK-EGFP	U6	Lipofection	MOI 10, 3h, 50ng/ml SCF, 50ng/ml TPO, 50ng/ml Flt-3L	Protamine sulfate	-	17.5%	(Anderson et al., 2009; Walker et al., 2012)
pNLT2ΔenvBLB	NL4-3 LTR	PEI	8h, StemSpan CC110	Pre-stimulation, Polybrene, spinoculation	30%	5%	(Hauber et al. 2013)

Methods used in transfection and transduction procedures are compared. The transduction and engraftment success is compared.

3.6. Virus particle production

Clinical scale production of lentivirus vectors is challenging because no stable producer cell lines exist. Therefore, a transient transfection process is followed to produce clinical-grade vectors (Throm et al., 2009). The transduction of 10^6/ml HSCs at an MOI of 100 requires a final concentration of 10^8/ml lentivirus particles. The initial number of CD34+ cells that was transduced in the listed clinical trials was between 1.0 and 11.3 x 10^6 cells (Table 6) which required 1.0 to 11.3 x 10^9 virus particles per patient at an MOI of 100 (http://lentilab.unige.ch/). Third generation lentiviral vector particles are produced by co-transfecting the gene transfer vector (e.g. pCLX-UBI-GFP, pLVTHM), a packaging system (e.g. pCMV-dR8.91, pCMV-dR8.74, psPAX2) and an envelope plasmid (e.g. pMD2G, pCAG-VSVG) containing the virion packaging elements into cells that produce the virus particles (Dull et al., 1998), http://lentilab.unige.ch/). The HEK 293T cell line is most commonly used for produceing virus vectors due to the ease of handling, high transfection rate obtained and high production of viral particles. By producing lentivectors using transient transfection where the viral genome is spread over 3 to 4 different plasmids, the risk of possible recombination activity is minimized (Laakso and Sutton 2006). The most frequently used transfection method is calcium chloride transfection (Table 6). It is inexpensive, very sensitive to minor buffer changes but requires a large amount of plasmid DNA (Dull et al., 1998). Obtaining such large amounts of plasmid DNA limits the practicality because it is very expensive to use maxi-prep kits for the isolation of plasmids from bacterial cultures. Other transfection techniques include the use of electroporation, Lipofectamine, Fugene6 or the Neon transfection system which is more expensive but uses much less plasmid DNA (Hahn, Hagstrom, Corporation, &

Rd, 2006)(Moore et al. 2010). Polyethyleneimine (PEI), however, is a cost-effective polyplex-forming compound with high transduction efficiency and low toxicity (Ehrhardt et al., 2006). The use of such reagents makes the testing of many constructs and the production of large amounts of virus particles easier because midi-preps rather than maxi-preps can be used for plasmid isolation. Efficient production of high quality lentivirus stocks is critical as a larger quantity of virus particles (high titer) is needed to transduce a sufficient number of HSCs as compared to cell lines (Table 6). To obtain a large quantity of virus particles in a small volume a vector concentration step is required. This can be done with ultracentrifugation at $5x10^5$ g-force or with the use of polyethylene glycol 6000 precipitation (Kutner, Zhang, and Reiser 2009).

3.7. Transduction efficiency

A major limitation in the process is the ability to obtain large numbers of transduced CD34+ cells (Petrillo et al., 2014). The number of positively transduced CD34+ cells that is used ranges between 2 and $4.5x10^6$ cells/kg which on average is $1.84x10^8$ positively transduced cells per (62kg) person (Table 5). The short term engraftment from such cells is very low ranging between 0.01 and 4% of total bone marrow cells (Biffi et al., 2013; Kang et al., 2010), and the results from such low engraftment therapies are difficult to interpret. The transduction procedure is generally performed under normal cell culture conditions with the addition of cytokines, with or without human albumin (Table 5). Only with consecutive transduction using an MOI of 100 can 90% transduction be obtained (Aiuti et al. 2013; Biffi et al. 2013). Consecutive transductions and high MOI increase the number of inserted copies of transgenes which increases the risk of disrupting endogenous gene function (Kustikova et al., 2003).

Successful clinical application of HSC-based gene therapy has been hampered by low transduction rates (Tisdale et al. 2000). The ideal situation would be to use very high quality vector preps which would transduce the maximum percentage of cells with the lowest possible MOI, resulting in practically only transduced cells engrafting. Many attempts have been made to improve transduction efficiency of primitive HSCs. However, attempts to determine whether the transduced cells have maintained their engraftment potential have largely failed due to the *in vitro* surrogate progenitor assays such as the use of the CD34 cellular marker which does not accurately represent the cell fraction capable of repopulating the human hematopoietic system (Schilz et al. 1998). A CD34 cellular population can contain no

HSCs such as in peripheral blood. Transduction efficiency is sensitive to many variables including cell source, the presence of serum, polycations used, content and quality of virus stock and transduction method (Moore and MacKenzie 1999). Transduction of non-dividing cells has been improved by increasing the deoxynucleotide concentration in the transduction medium (Ravot, Comolli, Lori, & Lisziewicz, 2002). It has also been reported that the cytokine combination, concentration and priming used with transduction play an important role in the transduction efficiency of lentivirus vectors as well as affecting the ability of CD34+ cells to engraft post-transduction (Uchida et al., 2011). The cytokine combination generally used in clinical trials includes stem cell factor (SCF), thrombopoietin (TPO), IL-3 and Flt-3L with concentrations in the range of 10-300 ng/ml (Table 5). This can influence transduction efficiency by causing pre-stimulation. Pre-stimulation of CD34+ cells with cytokines has been used in some studies to activate normally quiescent cells in the cell cycle (Moore and MacKenzie 1999), because the transgene integration of HIV occurs mainly when cells are at the G2/M phase (Davy and Doorbar 2007; Zhao and Elder 2005). Even though it is possible for lentivirus vectors to transduce cells in the quiescent cell cycle phase, efficiency is lower than when cycling cells are transduced (Uchida et al., 2011) (Liu et al. 2009). Uchida et al. (2011) reported that a 24h prestimulation in serum free medium containing SCF, TPO and Flt3 followed by a single 24h transduction provided optimal transduction of engrafting CD34+ cells (Uchida et al., 2011). However, Liu et al. (2009) demonstrated the importance of an extended post transduction culture period (Liu et al. 2009). The viral accessory protein R (Vpr) plays a key role in permitting virus core entry into the nucleus (He, Choe, Walker, Marzio, & Morgan, 1995), but it has also been found to increase the duration of the G2/M cell cycle phase which favours

transgene integration (Gummuluru and Emerman 1999; He et al. 1995). However, to improve the safety of lentiviral vectors, many viral genes have been removed including the viral protein R (*Vpr*) gene (Zhang et al. 2006). With the removal of the Vpr gene the integration efficiency of the lentivirus is significantly decreased (Brenner and Malech 2003a), but by artificially arresting the cells in the G2M phase with genistein or nocodazole, transduction rates can be improved. Zang *et al.* (2006) demonstrated that with nocodazole (a protein kinase inhibitor (PTK) which interferes with the polymerization of microtubules) and genistein, a 2 fold increase in transduction efficiency of CD34+ cells can be obtained (Zhang et al. 2006). Genistein works in a similar manner to nocodazole but also induces the expression of Cyclophilin A (CypA), a host cellular peptidyl-prolyl isomerise (PPIase) protein that forms a complex with the HIV-1 capsid (CA) necessary for early HIV-1 infection (Zhang et al. 2006). Cyclosporin A (CsA), an immunosuppressive compound, has been used on its own to improve lentivirus transduction by its binding to CypA. The binding of CsA to CypA inhibit the Ca2+- dependent phosphatise calcineurin and thereby activates pro-inflammatory cytokines such as IL-2 as well as disrupt the CypA-CA interaction to increase viral integration (Sokolskaja & Luban, 2006). Rapamycin (Rapa), structurally related to (CsA), binds to and inhibits FKBP12, another host PPIase, to exert immunosuppression and improve early LV infection independent of viral capsid blocking. It has been demonstrated that Rapa and CsA can provide a 2 and 3 fold increase in lentivirus transduction and also increase the VCN/cell 2 and 4 fold respectively (Petrillo et al. 2014; Wang et al. 2014)

Before gene transfer can occur, transduction is dependent on the interaction of the retrovirus particle with the target cell. This occurs through the binding of the

envelope protein to cell surface markers. For this binding to take place, retrovirus particles, with a half-life of 5-8 h at 37°C, need to come into close proximity with target cells by Brownian motion. Of note, vector particles have a traverse capacity of 480-610µm in a static solution (Chuck et al., 1996); however, the majority of virus particles beyond 480-610µm will not reach a cell's surface to bind in this time (Chuck, Clarke, and Palsson 1996). Various retrovirus transduction strategies have been used to overcome this physiochemical limitation including flow-through transduction, centrifugal transduction and the use of fibronectin to bring virus particles in close proximity with the surface of cells (Chuck, Clarke, and Palsson 1996; Moore and MacKenzie 1999). With flow through transduction, virus particles are actively flowed over cells by mechanical mixing or stirring to increase virus-cell interactions (Hutchings et al., 1998). However, due to micro-hydrodynamic limitations in bulk fluid agitation, virus encounters with cells are still dependent on Brownian motion. Target cells are rather seeded on porous surfaces and virus is flowed through the layer of cells (Chuck, Clarke, and Palsson 1996; Moore and MacKenzie 1999). Virus particles are hereby brought close to the target cells independent of Brownian motion to increase gene transfer rates by an order of magnitude (Chuck, Clarke, and Palsson 1996). A continuous perfusion system has been developed for non-adherent CD34+ cells which improved transduction efficiencies from 4% to 39% with overnight transduction at a MOI of 10 (Hutchings et al., 1998) and 100% transduction was obtained with a 7 day transduction process (Eipers, Krauss, Emerson, Todd, & Clarke, 1995). Centrifugal transduction or "spinoculation" has been used to promote virus uptake by increasing virus-cell association and decreasing the rate of dissociation. Transduction efficiencies using spinoculation significantly improved transduction of CFU-GM reaching >70%; this was found to be

directly correlated with time of centrifugation and centrifugal force and indirectly correlated to cell number (Schilz et al. 1998). The addition of polycations such as protamine sulphate and polybrene has been used in some cases (Cavazzana-Calvo et al., 2010); this has been reported to provide an additional increment in transduction efficiency (Goerner et al. 1999; Moore and MacKenzie 1999).

It has been demonstrated that retroviral gene transfer can be enhanced by performing transduction procedures on plates coated with fibronectin. The fibronectin molecule binds via the cell-binding heparin and CS1 domains to β1-integrins, VLA4, VLA5, proteoglycans of hematopoietic progenitor cells and to retrovirus particles via the heparin domain, bringing the virus particles and cells into close proximity (Hanenburg et al., 1997; Mohri, Katoh, Iwamatsu, & Okubo, 1996; Verfaillie, Benis, Lida, McGlave, & McCarthy, 1994). The recombinant fibronectin fragment CH-296 (or Retronectin) enhances the transduction of CD34+ cells better than full length fibronectin (Chono, Yoshioka, Ueno, & Kato, 2001). Several clinical trials have used Retronectin to obtain large percentages of transduced CD34+ cells, but only a few used Rectronectin after prestimulation even though Hanenburg *et al.* reported that prestimulation is essential to obtain efficient gene transfer (Hanenburg et al., 1997). The large percentages of transduced CD34+ cells obtained with Retronectin engrafted well; however, sustained long-term chimerism diminished considerably (Marina Cavazzana-Calvo et al. 2010; Kang et al. 2010). This could be explained by the fact that Retronectin does not enhance the transduction of primitive hematopoietic stem cells (Moore and MacKenzie 1999). In a study performed by Larochelle et al., (2014) more than 80% of primary colony forming units were transduced; however, less than 3% of cells capable of repopulating severe combined

immunodeficient mice, also known as SCID-repopulating cells, were found to be transduced (Larochelle et al., 2014). Similar results were obtained by Hanenburg *et al.* (1997) with up to 68% transduction of CD34+/CD38+ cells and up to 17% transduction of CD34+CD38- cells with prestimulated CD34+ cells and preloaded Retronectin (Hanenburg et al., 1997).

The rate of transduction of long-term HSCs is more important than the generally mentioned transduction rate observed with CD34+ cells because the long-term HSCs witll provide the long-term therapeutic effect. Of further importance is the insert copy number per cell observed with the particular transduction strategy. In order to achieve this, combining transduction strategies might be the answer. Schilz *et al.* (1998) obtained 98% transduction of CD34+ cells and up to 56% transgene positive engraftment of NOD-SCID mice with a transduction procedure that includes serum free pre-stimulation, the addition of protamine, spinoculation and culturing in Retronectin coated plates (Schilz et al. 1998). The transgene copy number per cell is not indicated in this study and results are only shown for gibbon ape leukemia virus (GALV)-pseudotyped murine retrovirus vectors. The same approach will likely improve transduction with VSV-G pseudotyped lentivirus vectors which will greatly benefit clinical gene therapy, but further testing is needed.

Although various methods have been used to improve the transduction of virus vectors the standard lentivirus transduction procedure is performed by culturing cells in the presence of lentivirus particles under normal culture conditions which include 20% O_2 (Ye et al., 2008). Hematopoietic stem cells, however, undergo spontaneous differentiation at 20% O_2 (Briones et al., 1999; Ivanovic, 2009) and the maintenance

of a primitive population capable of long term engraftment is not favoured. This is due to the fact that a proportion of positively transduced cells would commit to a cell linage during transduction and thus lose the ability to engraft (Briones et al., 1999). Much attention has been directed at developing vector strategies for the treatment of diseases mentioned in Table 5. However, attempts to improve the engraftment potential of genetically manipulated HSCs are lacking.

3.8. Engraftment of transduced cells

Most clinical trials make use of CD34+ cells where only a fraction of the cells are transduced (Table 5). The implication is that the non-transduced cells will also engraft with the result that the final proportion of transduced cells in the patient will be very small (Table 5). The engraftment rates observed for transduced CD34+ cells in clinical trials are much lower compared to what is reported in animal models (Table 6). The outcome may be that the anticipated therapeutic effect of the genetically manipulated cells is not observed even if the vector constructs work.

3.8.1. Culturing

Conditions that favour the maintenance of primitive stem cell populations can be implemented in the transduction procedure and the transduced cells expand thereafter while maintaining their primitive characteristics. Attempts have been made to mimic the bone marrow niche and thereby promote the expansion of primitive HSCs. One important characteristic investigated is hypoxia. It has been found to promote quiescence and maintain the primitive characteristics. The addition of molecules such as Stemregenin 1 (Boitano et al., 2010), valproic acid (Chaurasia et al., 2014) and prostaglandin E2 (Takizawa, Schanz, & Manz, 2011) to HSC cultures not only favours stem cell maintenance but significantly expands NOD-SCID repopulating cells. Stemregenin 1 antagonizes AHR which activates pluripotency genes (Boitano et al., 2010). Valproic acid is a histone deacetylase inhibitor (HDACI) which upregulates pluripotency genes (Chaurasia et al., 2014). Prostaglandin E2 up regulates CXCR-4 and induce proliferation (Takizawa et al., 2011). Combining the developments in basic science mentioned above and applying them to the clinical

setting may positively influence the success rate of clinical trials.

3.8.2. Selection

A pure population of cells containing the transgene can be obtained prior to transplantation by fluorescence activated cell sorting (FACS) with the use of fluorescent markers such as enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP). It is, however, not desirable to express exogenous proteins in humans as it might initiate an immune response and cause cellular abnormalities. Endogenous alternatives have previously been investigated which showed potential for the use of clinical selection markers. *In vitro* selection genes such as $DHFR_{L22Y}$ which provide resistance to the antifolate drug trimethotrexate (TMTX) (Evans et al., 2001; Liebert et al., 2000), and $\alpha 1_{Q118R/N129D}$ that provides resistance to ouabain, a selective Na^+/K^+-ATPase inhibitor (Evans et al., 2001; Liebert et al., 2000; Treschow et al., 2007), can also be used, resulting in death of the non-transduced cells. This greatly increases the size of the final transduced cell population. *In vivo* selection of transduced HSCs can also be used to improve engraftment success. A clinical trial (NCT01769911) used chemotherapy resistant genes called O^6-benzylguanine (O6BG) and bis-chloroethylnitrosourea (BCNU) to select gene modified cells, which resulted in an engraftment level of above 10% as reflected in the peripheral blood.

3.8.3. Conditioning

A further limitation is that successful HSC transplantation is partially dependent on cytoreduction of the patient's bone marrow resulting in the creation of a "niche" prior

to infusion. Chemotherapy or irradiation are given to patients to eradicate disease, prior to the infusion of HSCs, such as for example with acute myeloid leukaemia (Ciurea & Andersson, 2009). The immune system can be suppressed depending on the regimen, which reduces the possibility of the host rejecting the graft but increases susceptibility to infection. A conditioning therapy widely used for HSCT as an alternative to total body irradiation includes Busulfan (Bu) (Ciurea & Andersson, 2009). The advantages of using Busulfan include controlled administration to obtain an absolute dose, and combined with Cyclophosphamide (Bu/Cy), Fludarabine (Bu/Flu) and Melphalan (Bu/Mel) can more specifically target myeloid or lymphoid lineages (Ciurea & Andersson, 2009). Busulfan is relatively inexpensive and toxicities are much lower (Copelan et al. 2000). However, while engraftment rate improves in patients with increasing doses of Busulfan, the risk of side effects also increases (Biffi et al. 2013; Kuramoto et al. 2004). Full myeloablation with a dose of 20mg/kg body weight favours engraftment; however, the risk of mortality is high. Selective advantage of the gene-modified cells plays an important role in selecting a conditioning regimen and finding a balance between *in vivo* selection of cells and pre-transplant conditioning (Watts, Adair, and Kiem 2012). In the treatment of SCID, HSCs containing the corrected gene have a selective advantage over mutant cells which allows a therapeutic effect to be obtained with a small number of manipulated cells without providing a conditioning regimen (Aiuti & Roncarolo, 2009; Fischer et al., 2010). However, a small number of gene-corrected cells failed to provide a therapeutic effect in the treatment of Fanconi anemia and Gaucher's disease as they had no selective advantage over non-treated cells and no conditioning regimen was provided (Liu et al. 1999; Sidransky, LaMarca, and Ginns 2007). Providing a preparative regime might not be necessary if the gene-corrected cells have a

selective advantage; however, in cases where there is no selective advantage, conditioning is likely to be required (Watts, Adair, and Kiem 2012).

3.9. Achieving success

The twenty years of developing gene therapy has not come easily; however, the bumpy road of overcoming the obstacles has come to a culmination. Two studies from the same group have demonstrated how novel vector constructs and genetic manipulation procedures can be applied to achieve efficient correction of genetic diseases with low risk profiles. Biffi et al. (2013) treated nine patients with MLD and presented the follow up results of three patients. Autologous bone marrow CD34+ cells from three presymptomatic late infantile-MLD patients were transduced with a third generation lentivirus vector containing the arylsulfatase A (ARSA) cDNA driven by the PGK promoter. Isolated cells were pre-stimulated for 24h in retronectin-coated bags in CellGro SCGM medium containing 60 ng/ml IL-3, 100 ng/ml TPO, 300 ng/ml SCF and 300 ng/ml Flt3-L. Two rounds of transduction were conducted at an MOI of 100 and a period of 14h each and a concentration of $2x10^6$ to $10x10^6$ cells/ml were infused back into the patients after receiving a myeloablative regimen using Busulfan. A transduction efficiency of 90 to 97% with stable engraftment of 45% to 80% transduced cells was obtained to provide above normal ARSA expression. A VCN of 2.5 to 4.4 was obtained with no aberrant clonal behaviour and a clear therapeutic benefit was observed as the disease did not progress in any of the patients (Biffi et al. 2013)(Table 5). Aiuti et al. (2013) treated three patients with WAS with a third generation lentivirus vectors that contained a functional WAS gene driven

by a WAS promoter and all three patients displayed alleviated symptoms. A similar protocol as was used by Biffi et al. (2013) was followed to obtain 90% transduction efficiency with a 25 to 50% stable long-term engraftment of gene-corrected HSCs after receiving reduced-intensity myeloablative conditioning regimen. An average VCN of 2.6 was obtained and no long-term evidence of clonal expansion was observed which is an indication that the vector system used is safe (Aiuti et al. 2013) (Table 5).

3.10. Conclusion

The genetic manipulation of hematopoietic stem cells has been used to treat many patients in registered clinical trials with promising results. Recent developments in basic science can be incorporated into current clinical trial procedures to provide effective and safe therapies. Gene therapy has come a long way and has the potential to be provided as a routine medical therapy for many haematological diseases with the incorporation of recent developments.

Chapter 4:

In vitro analysis of techniques used to expand hematopoietic stem cells

4.1. Introduction

The identification of culture conditions that promote the expansion of primitive HSCs and HPCs (collectively referred to as HSPCs) has the potential to significantly improve hematopoietic stem cell transplantation. The culturing of HSPCs generally leads to differentiation and the loss of stem cell characteristics after 2 to 3 weeks because the culture conditions are very different from the conditions the cells experience *in vivo* (Chaurasia et al. 2014; Cheng et al. 2000; Mikkola and Orkin 2006). One of the challenges of culturing HSPCs related to use in transplantation is to maintain them in a primitive state that is capable of long-term engraftment. Mimicking the bone marrow niche has been shown to be effective in improving *in vitro* culture conditions by limiting differentiation and promoting quiescence which is beneficial for maintaining the primitive state (Cheng et al. 2000; Guitart et al. 2010). Long-term hematopoietic stem cells (LT-HSCs), which reside at the endosteal niche of the bone marrow where the oxygen concentration is below 3%, can either be in a quiescent cell cycle state (G0) or a cycling state (G1/S/G2/M phase) (Takubo, 2012). The LT-HSCs can be stimulated to cycle and migrate to the central vein to differentiate. However, various factors present in the bone marrow contribute to maintaining the cells in a state of quiescence. These include cytokines, components of the hematopoietic niche and hypoxia (Cheng et al. 2000; Cipolleschi, Sbarba, and Olivotto 1993; Guitart et al. 2010; Hermitte et al. 2006; A. Wilson and Trumpp 2006). Conditions that promote the expansion of HSPCs have been identified but conditions that favour the cycling state of LT-HSCs without causing differentiation need to be improved (Huang et al. 2012; Mikkola and Orkin 2006).

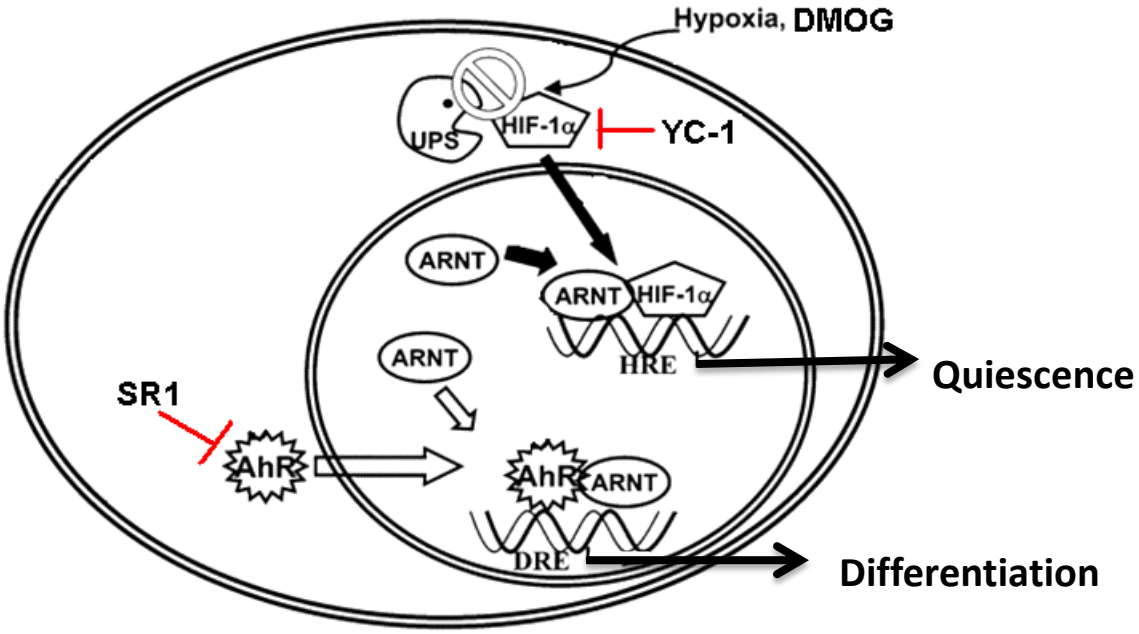

Figure 5: Regulation of the aryl hydrocarbon receptor nuclear translocator. The binding of ARNT to HIF-1 allows it to bind to the HRE promoter elements and activate gene expression that promotes cellular quiescence. The binding of ARNT to AhR allows it to bind to the DRE promoter elements and activate gene expression that promotes cellular differentiation.

Under conditions of hypoxia, hypoxia-inducible factor (HIF) forms a complex with Aryl hydrocarbon receptor nuclear translocator (ARNT) which binds to the hypoxia response elements (HRE) in the promoter regions of various genes (Takubo, 2012). Genes containing HRE transcription elements contribute significantly to maintaining cells in a quiescent state (Takubo, 2012). ARNT can complex with the aryl hydrocarbon receptor (AhR) to induce genes that promote differentiation (Boitano et al., 2010). ARNT/AhR protein complexes bind to dioxin response element (DRE) promoter elements to directly regulate genes including HES-1, c-Myc and C/EBP, which promote cell differentiation (Lindsey & Papoutsakis, 2012). PU.1-, β-catenin-, CXCR4-, and Stat5-dependent hematopoietic processes are also affected by AhR regulation (Singh et al. 2009) (Figure 5). There are strong indications that the regulation mediated by AhR is cell and differentiation-stage specific, which makes it difficult to characterize (Dever & Opanashuk, 2012). ARNT thus plays a key role in the regulation of hematopoiesis via AhR and HIF-1α and needs to be further investigated as a possible drug target. The binding of AhR to ARNT can be suppressed with Stemregenin 1 (SR1), an antagonist of AhR, which limits differentiation (Mulero-Navarro et al., 2006). It has been shown that SR1 induces cord blood-derived CD34+ cell expansion and CFU formation and also improves the engraftment capacity of these cells by suppressing differentiation (Boitano et al., 2010).

Bone marrow, adult G-CSF mobilized peripheral blood and cord blood samples used in hematopoietic transplants are heterogeneous in nature and contain various cell sub-populations including HSCs. There are *in vitro* assays that provide information on candidate HSCs (Gordon, 1993). The functional effects of SR1 as well as

conditions that increase HIF-1α and a combination of the two were explored in cultured CD34+ cells using SR1 and/or DMOG (which mimics hypoxia by stabilizing HIF-1α) in various assays. CD34 and CD133 cell surface molecules, that diminish with differentiation, were used to quantify a sub-population of HSPCs that includes further sub-populations at various stages of differentiation (Figure 4A) (Ivan et al., 2001; Takubo, 2012). The colony forming cell (CFC) assay was used to directly quantify cells in the HSPC population of primary cultures that are committed progenitors. This assay is also used to indirectly quantify the maintenance/expansion of pre-CFC during primary cultures that are not committed but give rise to committed progenitor cells during secondary cultures (Gothot, Pyatt, McMahel, Rice, & Srour, 1998). Side population (SP) cells, that we and others consider to be a surrogate marker of HSCs, were used to identify the minor primitive HSC subset. This technique is based on the capacity of primitive HSCs to efflux drugs such as Hoechst 33342 or Vybrant® DyeCycle™ Violet (VDC) more efficiently than most cells in a sample (Arai & Suda, 2008).

Our objective was to determine how the culturing of CD34+ cells under conditions that influence ARNT complex formation affects the expansion of HSPCs. Previous work has established that conditions that stabilized HIF-1α promote quiescence and conditions that antagonize AhR suppress differentiation. We hypothesized that the use of culture conditions that stabilize HIF-1α and antagonize the AhR protein in combination would promote the expansion of the primitive HSC population.

4.2. Materials and methods

4.2.1. Isolation of CD34+ cells

Fresh umbilical cord blood units (<24 hrs old) were obtained from the Etablissement Français du Sang (EFS) in Bordeaux (France) and cultured in Bordeaux, or the Steve Biko Academic Hospital and Netcare Femina Hospital, both in Pretoria (South Africa) and cultured in Pretoria. $CD34^+$ cells were isolated using the indirect $CD34^+$ MicroBead kit (Miltenyi Biotech, #130-046-703) according to the manufacturer's instructions. The cells were passed through isolation columns (#130-042-401) to obtain > 80% purity.

4.2.2. Primary liquid culture

CD34+ cells were seeded at a density of $2{,}3x10^5$ cells/ml and cultured under normoxic (5% CO_2; 20% O_2) or hypoxic conditions (5% CO_2; 1% O_2) in Dulbecco's Modified Eagle Medium (DMEM) containing 1% penicillin/streptomycin (PS) and 10% fetal bovine serum (FBS). The serum free expansion medium Stemline™ II was used as an alternative with 1% PS. The cytokines used were either 10 ng/ml hIL-3 or a mixture of hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), and TPO (20 ng/ml). Dimethylsulfoxide (DMSO), DMOG, YC-1 (an inhibitor of HIF-1α) and/or SR1 were added to the culture medium. DMSO was used as a vehicle control in the 1% O_2 and 20% O_2 conditions. DMOG (Sigma, USA, #D3695) was used at concentrations ranging from 0.1 to 2.5mM to determine the concentration that efficiently arrests cells in the G0 cell cycle phase without causing cellulartoxicity. The concentration selected for further experiments was 0.1mM. The same was done for YC-1, which is an inhibitor of HIF-1α (Sigma, USA, #Y102), with concentrations

ranging from 0.25 to 20 µM; 0.5µM was selected for further experiments. A concentration of 0.75 µM SR1 (Biovision, USA, #1967-1) was tested for toxicity and was deemed to be non-toxic: this concentration was used for further experiments as previously described (Boitano et al., 2010).

4.2.3. Cell viability and cell cycle analysis

Cell viability was assessed on days 0, 2, and 7 using flow cytometry. Samples were stained with 7-Aminoactinomycin (7-AAD) and absolute counts were obtained through the addition of Flowcount^{TM} Fluorospheres (Beckman Coulter, Miami, USA). The quantification of viable cells, counted on day 7, was used to indicate the rate of cell proliferation that occurred during culture. Cells were stained with anti-CD34 (PC7-conjugated mouse antibody, Ref. #21691, Beckman Coulter, Miami, USA) and anti-CD133 antibodies (PE-conjugated mouse antibody, Beckman Coulter, Miami, USA). Cell cycle analysis was done on days 0, 2, and 7 of culture. Approximately 100,000 cells were fixed with 0.5% paraformaldehyde (PFA), permeabilized with 0.05% Saponin (Sigma, USA), and stained with an anti-human Ki67 antibody (PE-conjugated mouse antibody, Ref: 556027, BD Biosciences, New Jersey, USA) and the DNA stain, TOPRO III. The cells were analyzed using a Canto II flow cytometer (BD Biosciences).

4.2.4. Western blot assays

Proteins were isolated from 1x10^6 cells for each treatment condition on day 7 using cell extraction buffer (FNN0011, Life Technologies, Invitrogen) and quantified using the Bradford assay (B6916, Sigma Aldrich, St. Louis, USA). The Invitrogen iBlot® system was used according to the manufacturer's instructions. Twenty micrograms

of protein from each sample were separated on a NuPAGE® Novex® 4-12% Bis-Tris Gel (Life Technologies, Invitrogen) and transferred to Nitrocellulose membranes using the iBlot® Gel Transfer Device and iBlot® Gel Transfer stacks (Life Technologies, Invitrogen). HIF-1α, AhR, β-actin and GAPDH proteins were identified using anti-HIF-1α (NB100-479, Novus Biologicals®, Littleton, USA), anti-AhR (NB100-2289, Novus Biologicals®, Littleton, USA) and anti-β-actin (A2066, Invitrogen®) antibodies generated in a rabbit host. The iBlot® Western Detection Chromogenic Kit, which uses an anti-rabbit Alk-Phosphatase secondary antibody (Life Technologies, Invitrogen), was used to visualize protein bands.

4.2.5. Colony forming assays

Colony assays were used to analyze the colony forming unit (CFU) capacity of each treated sample. One hundred cells from each sample on days 0 and 7 were plated in 250 µl Stem-α1D (Stem Alpha, St. Genis L'Argentière, France, http://www.stemalpha.fr) and colonies were counted 12-16 days after the cells were plated. The number of colonies counted was divided by 100 to obtain the proportion of cells capable of forming CFUs. The total number of CFUs in each sample was calculated by multiplying the proportion of cells capable of forming CFUs by the total number of cells in the sample.

4.2.6. Side population analysis

Side population analysis was done on days 0 and 7. The cells ($3x10^5$ cells/tube) were divided into two tubes, centrifuged at 200 g, and the supernatant was removed. The cells in the first tube were resuspended in 150 µl DMEM+ (Dulbecco's modified

Eagle medium, Invitrogen), 2% (v/v) FBS, 10 mM HEPES (Sigma, USA) and 5 µM VDC Violet stain (Life Technologies, Invitrogen). The cells in the second tube were resuspended in DMEM+ containing 50 µM verapamil (#B7431, Life technologies, California, USA). Both tubes were incubated at 37°C for 2 hours after which the cells were placed on ice and washed with 2 ml cold HBSS+ (Hank's balanced salt solution, Invitrogen, California, USA), 2% (v/v) FBS and 10 mM HEPES (Sigma) and stained with anti-CD34 and anti-CD38 (PC5-conjugated mouse antibody, Beckman Coulter) antibodies together with 7-AAD or annexin V-FITC kit (Beckman Coulter, Miami, USA). The cells were then washed with 1 ml HBSS+ and analyzed by flow cytometry (Gallios flow cytometer, Beckman Coulter, USA). The cells were kept below 4°C after incubation. Live cell selection was based on 7-AAD negative staining and the side population was measured using a FL9 (620/30BP) versus FL10 (550/40 BP) scatter plot. The region that selects for cells that efflux VDC violet dye was set up according to the histogram generated from the untreated verapamil control. After the initial setup, the same gate was maintained for all treatments to allow for comparison of the data.

4.2.7. Secondary liquid culture

After the cells had been cultured for 7 days (primary liquid culture), each sample was washed with PBS and $2{,}3x10^5$ cells were plated in 1ml secondary liquid culture medium composed of DMEM, 10% FBS, 1% Penicillin/Streptomycin, hSCF (100 ng /ml) (Life Technologies, Invitrogen), hIL-3 (3 ng/ml) (Life Technologies, Invitrogen), hTPO (20 ng /ml) (Life Technologies, Invitrogen) and hG-CSF (100 ng /ml) (Life Technologies, Invitrogen). Every second day, cells were resuspended to

obtain a homogenous culture and 50 µl cell suspension was removed for the purposes of determining cell count and measuring the proliferative capacity of the treated cells as shown in Figure 6. On days 6, 13 and 19 the cells were washed with PBS and replated at 10% of the total cell count in 1 ml secondary liquid culture medium. To quantify the number of CFU in each treated sample, 100 cells from days 2, 4, and 6; 400 cells from days 8, 11, and 13; and 800 cells from every second day after day 13 were plated in 250 µl Stem-α1D and colonies were counted 12 to 16 days after the cells were plated (Figure 6, page 85).

4.2.8. Statistical Analysis

Data are given as mean ± S.D. or median of replicate experiments as indicated. Column comparisons are done by using Welch's t-test for unpaired data using GraphPad Prism 5.04. Statistical significance is indicated on the graphs as follows: (*) $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.0005$ and (****) $p < 0.00005$.

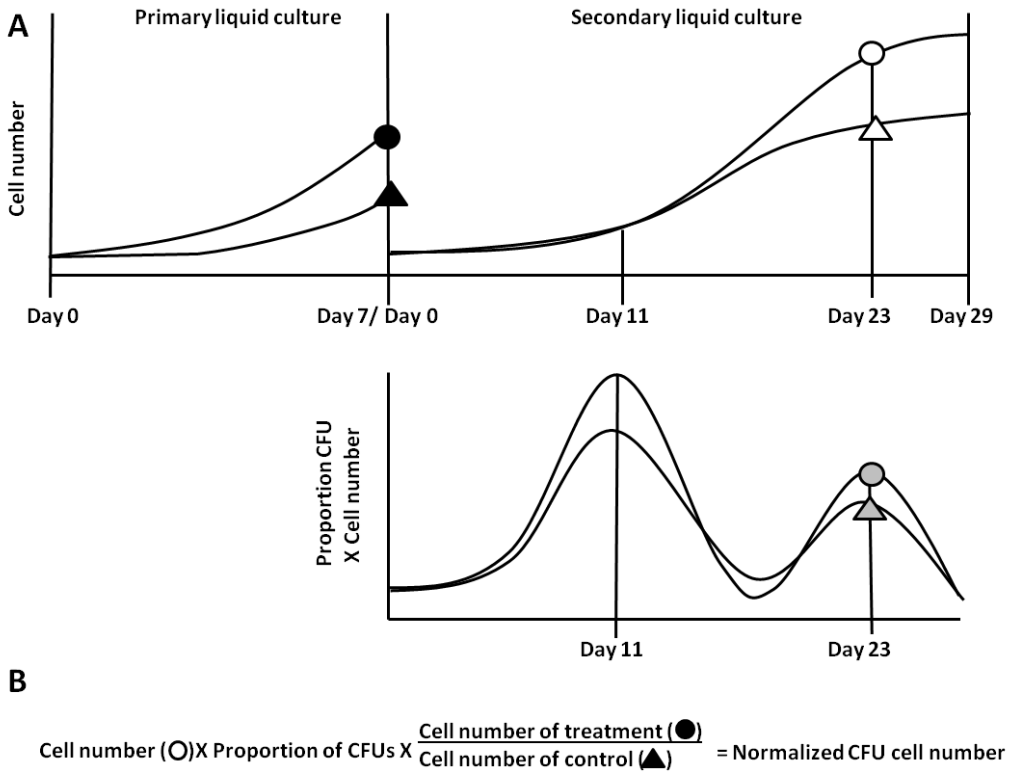

Figure 6. Experimental procedure explained. **(A):** Freshly isolated CD34+ cells were seeded in the presence of various compounds (primary liquid culture). Circles represent controls, while triangles are treated cultures. On day 7, the proportion of each sub-population was determined (Figure 8C); this was multiplied by the cell number (Figure 8A) to obtain the derived number of cells in the sub-populations (Figure 10A-E). The cell number obtained for the treatment condition was divided by that of the control condition to obtain the normalization coefficient. The cells were also washed and plated in the mentioned culture medium without any addition of compounds (secondary liquid culture). Cells were counted every 2 days and plated in semisolid medium to determine the proportion of cells capable of forming CFUs. The number of cells capable of forming CFUs was derived by multiplying the cell count obtained at day 23 as indicated in Figure 11A with the proportion of cells capable of forming CFUs (Figure 11B). This derived CFU cell number was then multiplied by the normalization coefficient to obtain the normalized CFU cell number shown in Figure 11C. **(B):** The equation used to calculate the normalized CFU cell number. The cell number obtained at a specific time point in the secondary liquid culture is multiplied with the proportion of cells that are capable of forming colonies. This value is then multiplied by the fold increase of proliferation in the primary liquid culture compared to the control.

4.3. Results

4.3.1. Establishing culture conditions and drug dosages that maintain > 80% viable cells on day 7

Culturing cells in 20% O_2 and a mixture of cytokines (as detailed in the Materials and Methods section) for 7 days provided a SP percentage of 1.02 ± 0.30 (n=7) and a 13.02 fold (n=7) increase in cell number compared to day 0 (Figure 7, page 87). Cultures containing only hIL-3 gave a SP percentage of 0.33 ± 0.08 (n=3) and the cell number on day 7 was 4.92 fold (n=3) higher than on day 0. Cultures containing hIL-3 (3 ng/ml), hSCF (100 ng/ml) and TPO (20 ng/ml) gave a SP percentage of 1.39 ± 0.40 (n=4) and a 3.02 fold (n=4) increase in cell number on day 7 compared to day 0. Cultures expanded in Stemline II™ with the mixture of cytokines, which does not contain bovine elements and is thus more appropriate for clinical use, provided a 65 fold increase in cell number with a SP percentage of 1.32± 0.26 (n=4) (Figure 7). The Stemline II™ medium was not used for further experiments as the contents of the medium, which are not disclosed, could affect the cellular processed being analyzed. All further experiments were performed with the cytokine cocktail using DMEM. Cell viability was > 80% on day 7 in all experimental conditions except where indicated.

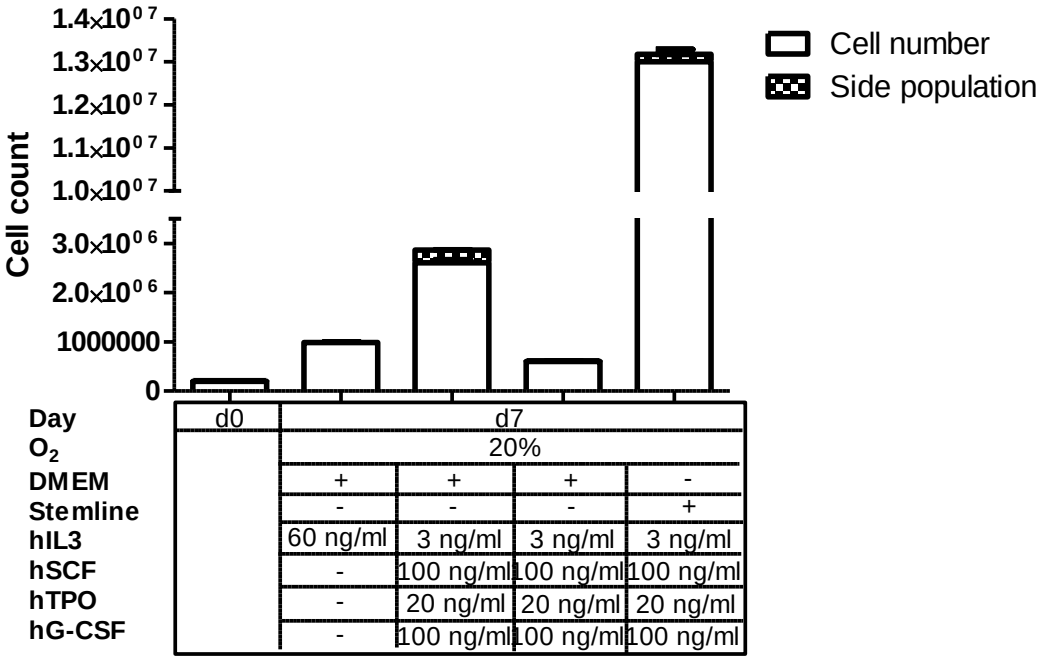

Figure 7. Proliferation and side population. The proliferation of CD34+ cells treated with cell culture conditions in initial experiment is shown as well as the number of cells determined that have side population characteristics.

4.3.2. Establishing conditions that promote quiescence

Western blot revealed that the expression of HIF-1α in cells from the 20% O_2 sample (CD34+ exposed to the mixture of cytokines, 20% O_2 and DMSO) was absent and AhR expression was high (Figure 8, page 89). The 1% O_2 sample (mixture of cytokines, 1% O_2 and DMSO) effectively stabilized HIF-1α (Figure 8) as previously documented (Ivanovic, 2009). A concentration of 0.1mM DMOG, which is much lower than the commonly used toxic concentration of 1mM (Asikainen et al., 2005), was selected for further experiments as it gives > 80% viable cells and a similar level of HIF-1α and AhR expression as the 1% O_2 sample (Figure 8). The 1% O_2/YC-1 sample was included as a control because YC-1 is known to cause HIF-1α degradation as previously documented (Figure 8)(Kim, Yeo, Chun, & Park, 2006). The concentration selected for YC-1 was 0.5μM, which gives > 80% viable cells. A condition referred to as 1% O_2/DMOG showed that the addition of DMOG to 1% O_2 does not affect the stabilization of HIF-1α (Figure 8). The expression of HIF-1α and AhR in the 20% O_2/SR1 sample was not affected by SR1 (Figure 8)(Boitano et al., 2010). A concentration of 0.75μM was selected for SR1 which gives > 80% viable cells. HIF-1α expression was inhibited and AhR was unchanged in the sample containing both DMOG and SR1, referred to as 20% O_2/DMOG/SR1 (Figure 8).

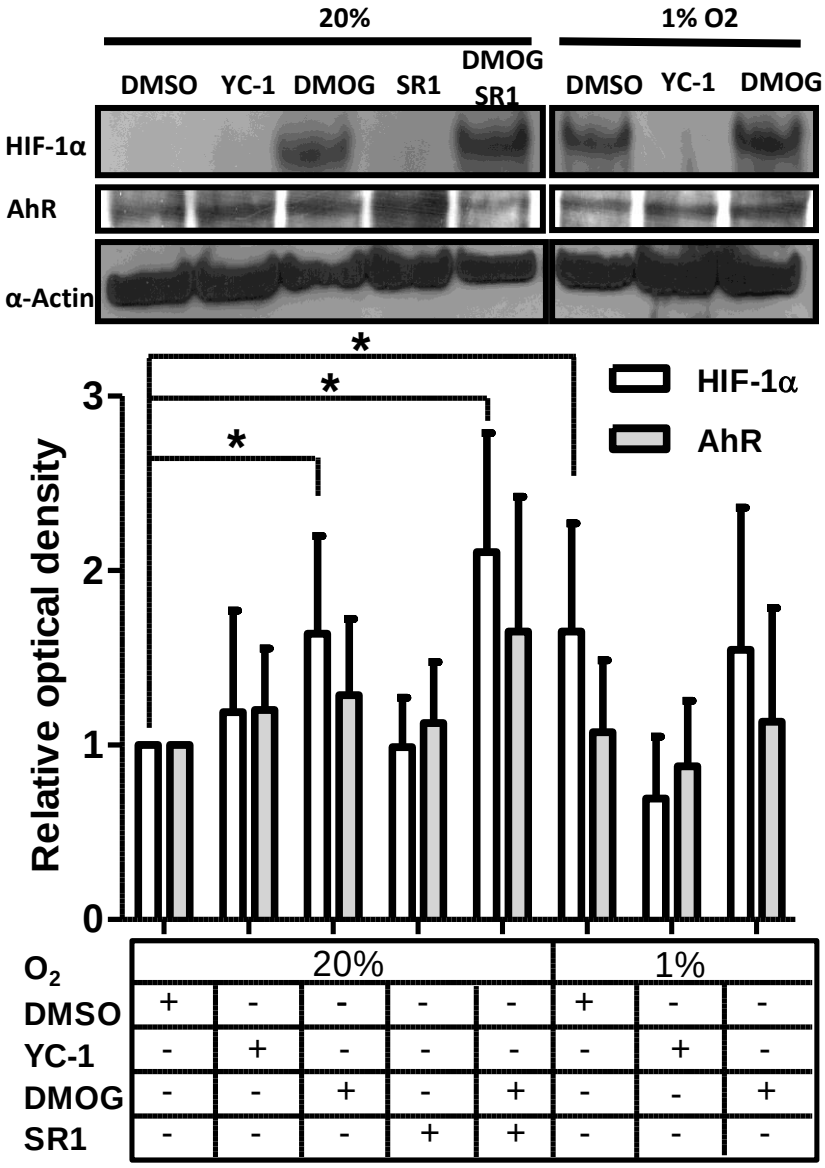

Figure 8. Establishing conditions that promote quiescence. HIF-1α, AhR and β-Actin proteins visualized by Western blot and densitometric quantification of HIF-1α and AhR proteins (normalized to β-Actin). The number of replicates for all conditions is 4. Statistical significance is indicated in the graph as described on page 85.

4.3.3. Effect of O_2, DMOG, SR-1 and YC-1 on cell viability and cell cycle

Cell proliferation and viability were determined on freshly isolated CD34+ cord blood cells cultured for 7 days under the various conditions selected (Figure 9A). When grown in the presence of 1% O_2, 20% O_2/DMOG or 20% O_2/DMOG/SR1, proliferation was significantly reduced when compared to the 20% O_2 control (Figure 5A). No significant change was observed with cells grown in the presence of 20% O_2/YC-1 or 20% O_2/SR1. However, lower proliferation and a higher mortality were observed with 1% O_2/YC-1 (46.6% dead cells) compared to 1% O_2 (18.6% dead cells) (Figure 9A). Cell cycle analysis performed on days 2 and 7 of the various culture conditions, revealed major variations in proliferation between the different conditions (Figure 9B). The percentage of G_0 cells observed in each sample at day 2 was inversely correlated with total cell counts. Figure 9B shows that after 2 days, the population of cells in the G_0 phase decreased rapidly in samples cultured under normoxic conditions, namely 20% O_2, 20% O_2/YC-1 and 20% O_2/SR1. On the other hand, the % of G_0 cells in samples cultured under hypoxic or hypoxia-mimicking conditions, namely 1% O_2, 20% O_2/DMOG and 20% O_2/SR1 remained significantly higher (Figure 9B). A smaller G_0 population was observed in the 1% O_2/YC-1 sample compared to 1% O_2. Similar results were observed on day 7 where the 20% O_2/DMOG and 1% O_2 conditions had significantly higher percentages of cells in the G0 phase ($p<0.05$, Figure 10). However, no significant differences were observed between the absolute numbers of cells in the G0 phase (Figure 12D).

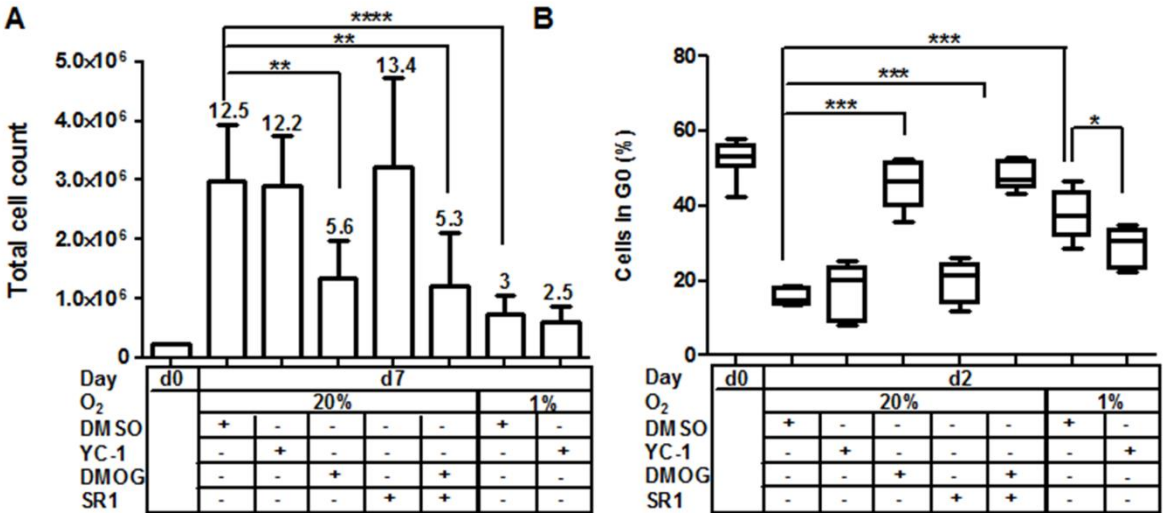

Figure 9. Proliferation and G0 cell cycle percentages of CD34+ cord blood cells. (**A**)**:** CD34+ cord blood cells were cultured for 7 days under the various conditions shown. Cells were counted on day 7 and cell viability was determined using Trypan blue. (**B**)**:** The percentage of cells in G0 on day 2 of primary culture. Eight experiments were performed with a sample number per condition that varied between 3 and 8.

CD34+	91.12	69.74	62.76	78.57	82.43	84.26	88.36	87.06
CD133+	35.40	28.03	27.4	24.13	33.8	23.63	24.16	26.67
CFU	32.83	11.5	13	21.83	24.83	25.5	19	11
G0	52.63	3.6	3.48	8.4	5.65	9.43	11.92	9.45
SP	5.68	1.02	1.26	1.43	2.61	3.56	1.48	0.52

Day	d0	d7						
O_2		20%			-		1%	
DMSO		+	-	-	-	-	+	-
YC-1		-	+	-	-	-	-	+
DMOG		-	-	+	-	+	-	-
SR1		-	-	-	+	+	-	-

Figure 10. Sub-population percentages. The percentages obtained for the CD34, CD133, CFU, G0 and side population (SP) subpopulations are provided. Eight experiments were performed with a sample number per condition that varied between 3 and 8.

4.3.4. Effect of O_2, DMOG, SR-1 and YC-1 on progenitor expansion and CD34+ and SP cell phenotype

The proportion of cells in the CD34+ sub-populations of each condition was determined (Figure 10) and was used to calculate absolute cell numbers (Figure 12A-E). A selection of histograms is shown in Figure 11 to demonstrate the effect of the treatment conditions on HSC subpopulations. Compared to day 0, the proportion of CD34+ cells did not change after 7 days of culture in 1% O_2 while this decreased by 30% in 20% O_2 (Zini, Salati, Bianchi, Ferrari, & Manfredini, 2008). Differentiation that occurred in the 20% O_2 sample is also evident in the small proportional increase of the primitive SP and G_0 populations compared to a significant increase observed in the less primitive CD34, CD34+/CD133+ (Figure 12A and B) and CFU populations (Figure 12C). Even with higher levels of differentiation, the 20% O_2 sample had higher cell numbers in the primitive populations than the 1% O_2 sample which did not increase from day 0 (Figure 12D and E).

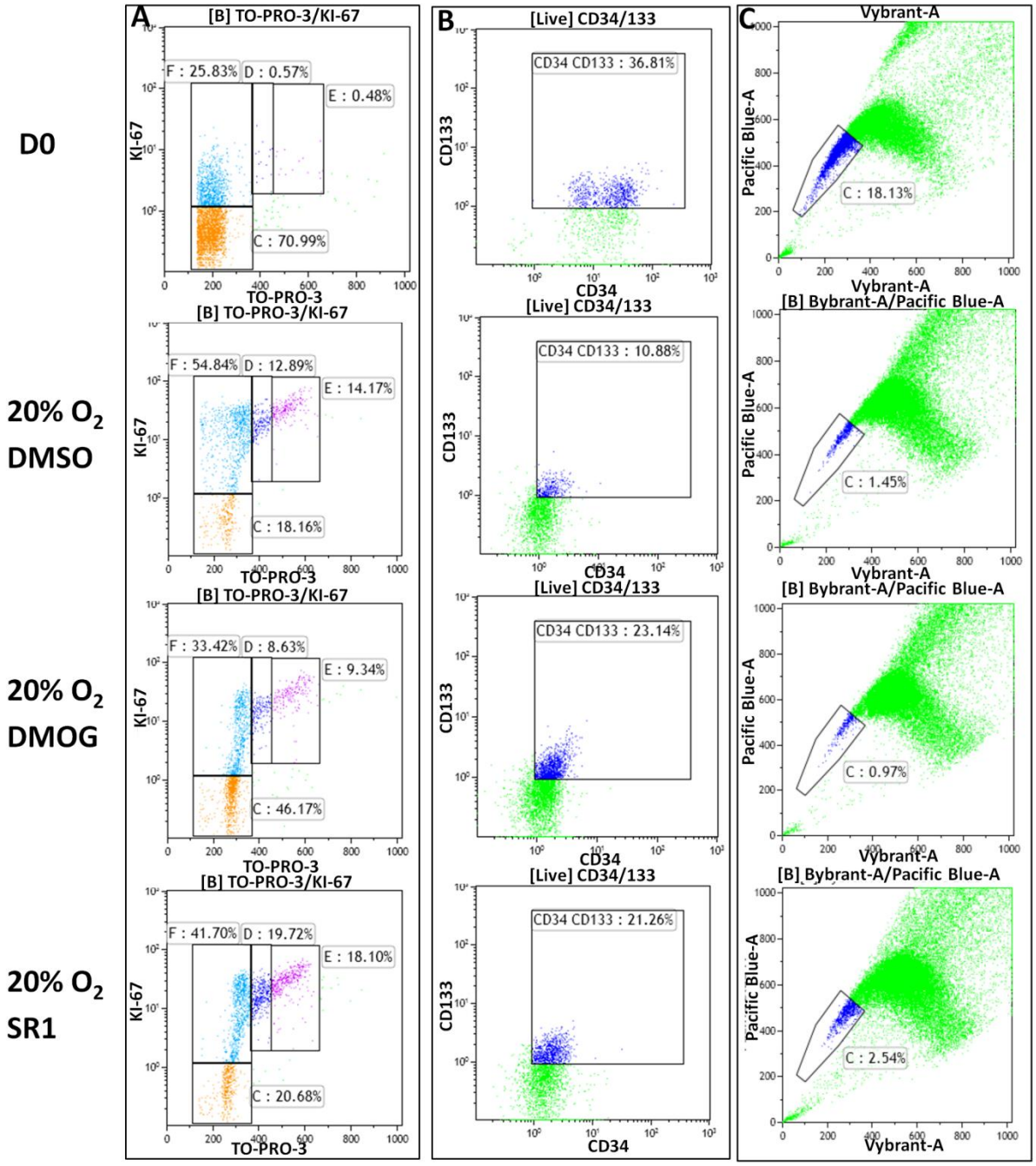

Figure 11. Selection of FACS scatter plots. (A): Cell cycle analysis of cells cultured with the indicated compounds at day 3. Selected histograms indicate the proportions of cells that are in the G0 phase shown (B): The proportions of cells that are both CD34 and CD133 positive at day 7 are shown in indicated boxes of selected histograms (C): The proportion of cells of the indicated samples that is capable of effluxing VDC violet dye at day 7 and which is indicative of the side population, is shown in the indicated boxes of selected histograms.

The 20% O_2/DMOG sample proliferated at a slow rate, similar to the 1% O_2 sample (Figure 9, page 91) and the sizes of the cell sub-populations (Figure 12A-E) were also comparable. Figure 12 indicates that the total number of cells in all the cell sub-populations is greater in the presence of 20% O_2/DMOG than 1% O_2. The inhibition of HIF-1α by YC-1 in the 1% O_2/YC-1 sample reduced cell quiescence (Figure 9B) and increased cell differentiation (Figure 12A-E). The cell numbers in all the sub-populations of the 1% O_2/YC-1 sample were reduced as expected when compared to the 1% O_2 sample (Figure 12, page 96).

The addition of SR1 to cells cultured at 20% O_2 caused no significant change in cell proliferation (Figure 9A). SR1 did however cause all the cell sub-populations to increase proportionally. The proportion of cells with SP characteristics decreased from day 0 to day 7 in all samples, but the decrease was significantly less in the presence of 20% O_2/SR1 and 20% O_2/DMOG/SR1 when compared to 20% O_2 (Figure 10). Proliferation thus occurred mainly in the SP (2.3 fold more) (Figure 12D). Cells cultured in 20% O_2 with DMOG and SR1 gave similar results regarding cell cycle and cell proliferation as treatment with DMOG alone (Figure 10 and Figure 12A). However, a significantly larger SP population was observed, which is similar to treatment with SR1 alone (Figure 12).

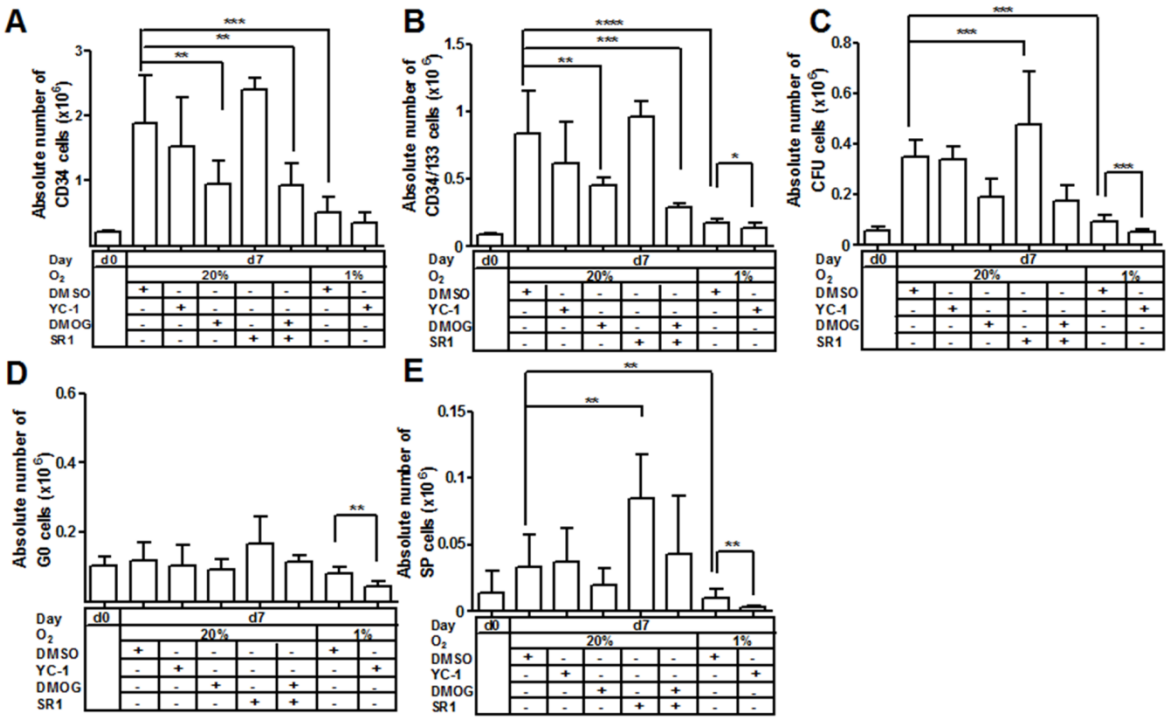

Figure 12. Cell numbers obtained in the CD34+, CD34+/CD133+, CFU, G0 and SP sub populations. CD34+ cord blood cells were cultured for 7 days under various conditions as shown. (**A**)**:** The proportion of CD34 positive cells was multiplied by the total number of cells in solution. The sample number is 8. (**B**)**:** The proportion of total CD34+/CD133+ cells was multiplied by the total number of cells in solution. The sample number is 3. (**C**)**:** The proportion of total colony forming units relating to the number of cells plated was multiplied by the total number of cells in solution. The sample number is 8. (**D**)**:** The proportion of cells in G_0 phase was multiplied by the total number of cells in solution. The sample number is 7. (**E**)**:** The proportion of cells with side population characteristics was multiplied by the total number of cells in solution. Eight experiments were performed with a sample number per condition that varied between 3 and 8. Statistical significance is indicated in the graph as described on page 85.

4.3.5. Effect of O_2, DMOG, SR-1 and YC-1 on the proliferation rate of the cells

Cells from the primary cultures were washed and initiated in secondary cultures and the proliferation rates of cells in secondary cultures are shown in Figure 13. The proliferation rates of samples treated with 20% O_2 and 20% O_2/YC-1 were initially high, but were surpassed by cells treated with 20% O_2/SR1 and 1% O_2/SR1 at approximately day 15 (Figure 13). This indicates that more cells in the 20% O_2 and 20% O_2/YC-1 samples were initially in a cycling state which gave rise to greater proliferation at the early time points. The proliferation of the 20% O_2/DMOG and 20% O_2/DMOG/SR1 samples was similar until approximately day 19, whereafter proliferation in 20% O_2/DMOG/SR1 surpassed proliferation in 20% O_2/DMOG (Figure 13). This difference indicates that even though the proliferation rate during primary liquid culture was similar, the cells in the 20% O_2/DMOG/SR1 sample were more primitive as they provided prolonged proliferation. The fact that the total cell proliferation of the 20% O_2/DMOG, 20% O_2/SR1, 20% O_2/DMOG/SR1 and 1% O_2 samples was significantly greater at day 29 when compared to cells grown in the presence of 20% O_2 (Figure 13) supports our data above that shows that cells used at the start of this assay were more primitive. On the other hand, the 1% O_2/YC-1 sample had significantly less proliferation compared to the 1% O_2 sample.

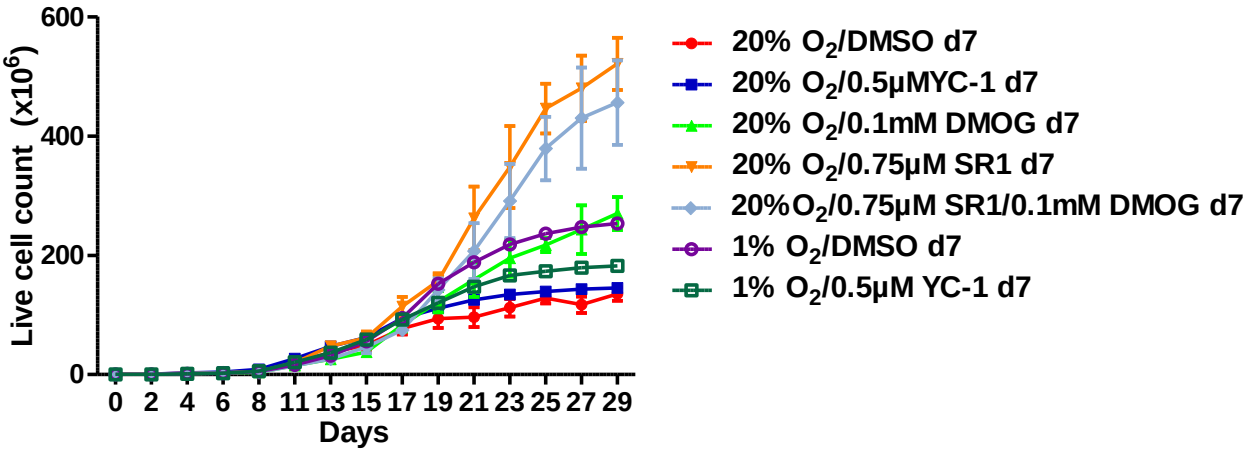

Figure 13. Proliferation assay of secondary cultures. Proliferation of cells in secondary liquid culture (LC2). The live cell counts of every second day until day 29 are represented on the graph. Eight experiments were performed with a sample number per condition that varied between 3 and 6.

4.3.6. Effect of O_2, DMOG, SR-1 and YC-1 on the ability of the cells to form long-term colony forming units

Cells were removed from the secondary culture every second day and placed into semisolid medium to allow colony formation. The number of colonies observed was used to create a pre-CFC profile. The first peak analyzed at day 11 provides indirect information on the “stemness” of the committed progenitors present at day 7 of primary liquid culture (Figure 14A). The second peak analyzed at day 23 provides information on the long-term colony forming units (LT-CFUs) (Figure 14A). The first and second peaks of the 20% O_2/DMOG, 20% O_2/SR1, 20% O_2/DMOG/SR1 and 1% O_2 samples were statistically higher than the 20% O_2 sample, indicating that more committed progenitors and pre-CFCs were present.

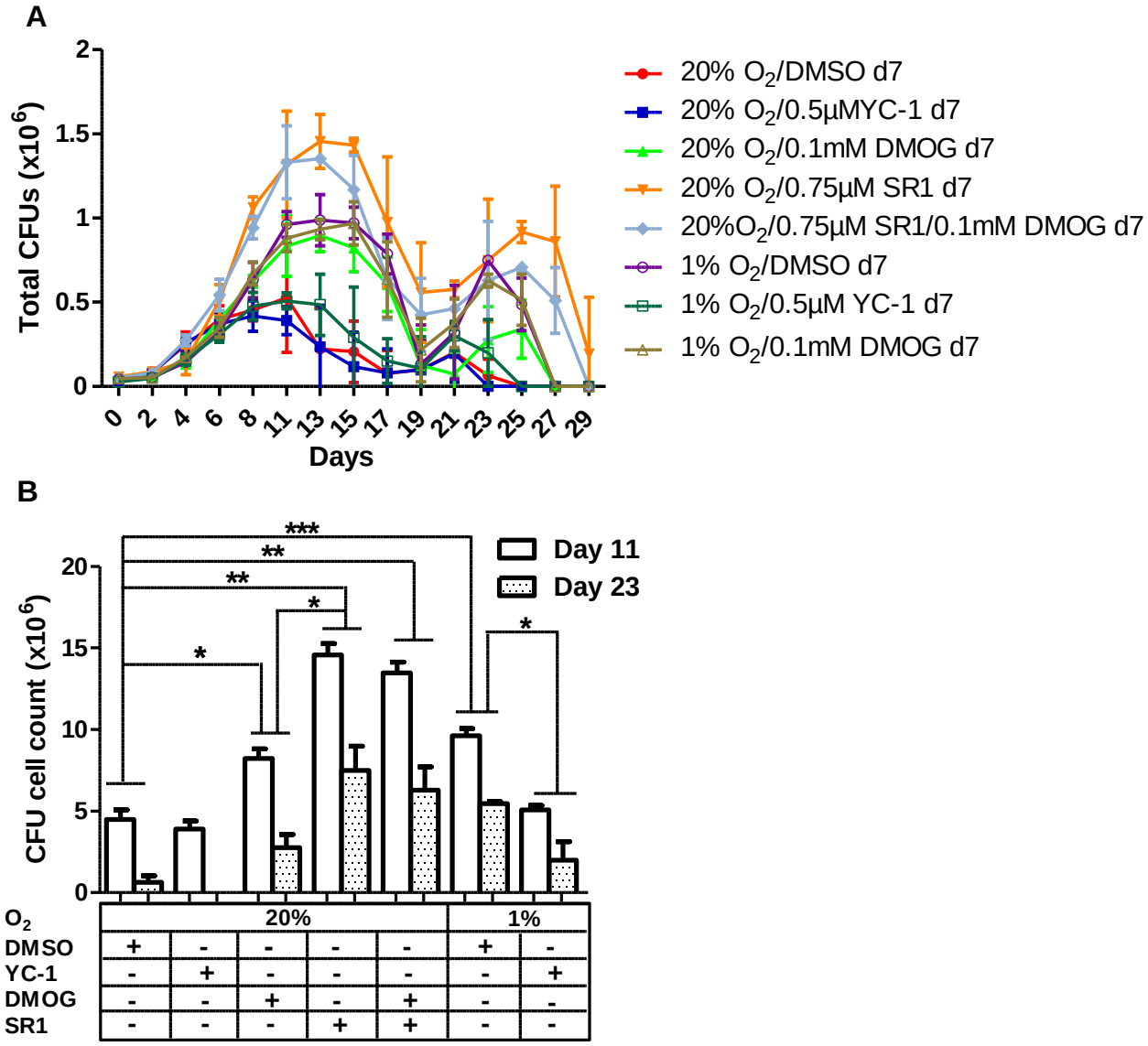

Figure 14. Pre-CFC assay of secondary cultures. (**A**): The numbers of CFUs of every second day until day 29 are represented on the graph. (**B**)**:** The number of CFUs on day eleven and twenty three are presented for each sample. Eight experiments were performed with a sample number per condition that varied between 3 and 6. Statistical significance is indicated in the graph as described on page 85.

4.3.7. Effect of O_2, DMOG, SR-1 and YC-1 on the ability of the cells to form long-term colony forming units (normalized)

The absolute CFU cell numbers obtained by normalizing the pre-CFC data shown in Figure 14, page 100 to the proliferation obtained in primary culture (Figure 9, page 91) are shown in Figure 15A and B. Figure 15A reveals the magnitude of the difference in the differentiation capacity of progenitors in the 20% O_2/SR1 sample. The total LT-CFU cell number obtained for the 20% O_2/SR1 sample is 13.9 fold higher than for the 20% O_2 sample (Figure 15B, page 102).

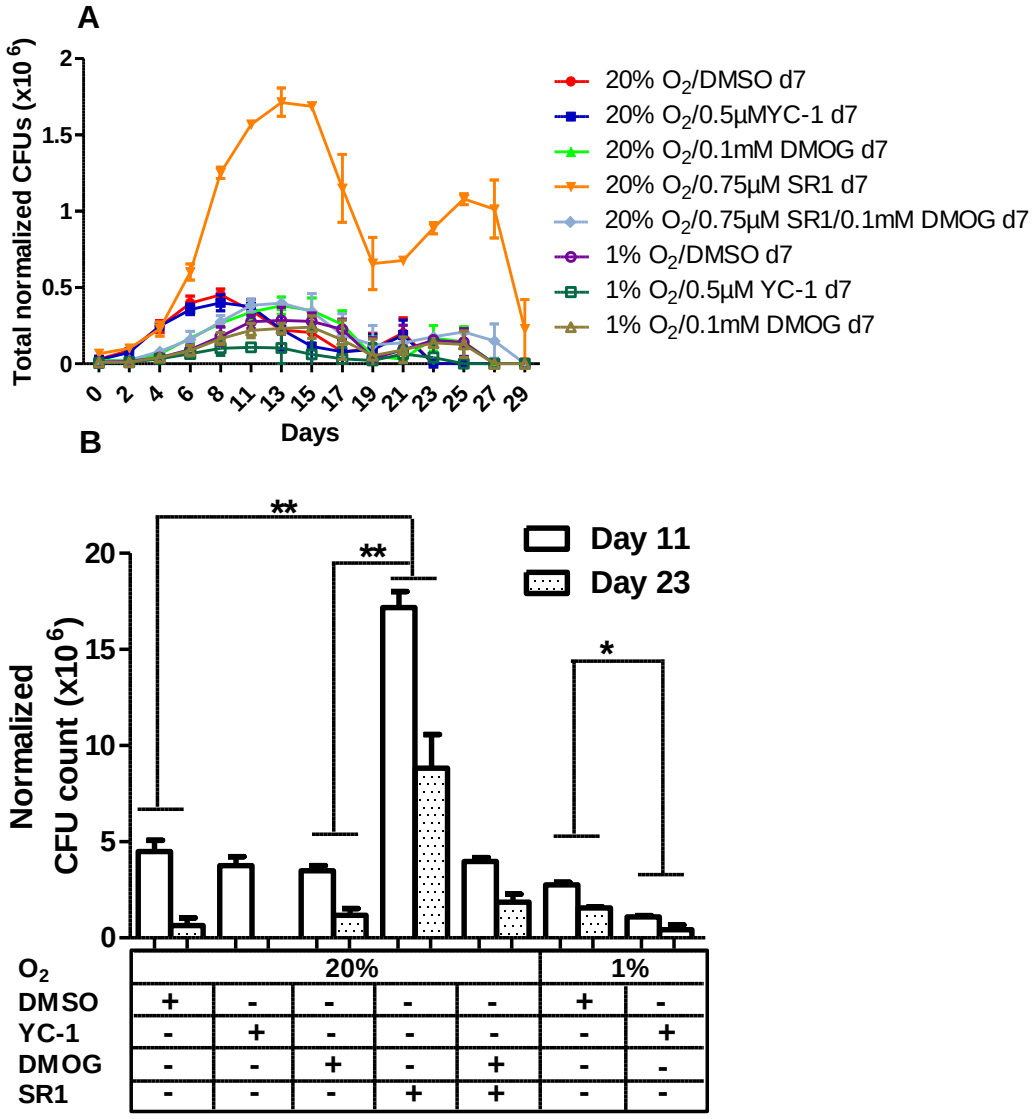

Figure 15. Pre-CFC assay of secondary cultures (normalized). (**A**): The normalized numbers of CFUs of every second day until day 29 are represented on the graph. (**B**): The normalized CFU cell number on day eleven and twenty three are presented for each sample. Eight experiments were performed with a sample number per condition that varied between 3 and 6. Statistical significance is indicated in the graph as described on page 85.

4.4. Discussion

The CD34+ population in umbilical cord blood can easily be isolated and cultured, but is functionally heterogeneous, containing many progenitor cells but only a small proportion of stem cells (Forristal, Winkler, Nowlan, Barbier, & Walkinshaw, 2013). The latter are important for long-term engraftment following transplantation and when expanded *in vitro* (Allison, Leugers, Pronold, Zant, & Laurel, 2004). Previous studies have found that the ARNT transcription factor plays an important role in the regulation of hematopoiesis (Lindsey & Papoutsakis, 2012; Shimba, Hayashi, Ohno, & Tezuka, 2003). In order to further investigate how ARNT complex formation can benefit the expansion of the CD34+ sub-populations, cord blood CD34+ cells were cultured under selected conditions. Hypoxia (Ivan et al., 2001; Takubo et al., 2010) has a dose related effect on promoting cellular quiescence that in turn promotes the maintenance of HSPCs (Cheng et al. 2000; Guitart et al. 2010) by affecting cell proliferation, self-renewal and differentiation (Ivanovic, Belloc, & Faucher, 2002; Ivanovic, 2009). However, 1% O_2 is not suitable for expanding HPCs due to the suppression of cell proliferation which results in a low final number of HPCs after culture.

Similar results are obtained for cultures treated with a low concentration of DMOG (0.1 µM) which is a mimic of 1% O_2. A minimal loss of the CD34 marker in the 20% O_2/DMOG samples suggests that since there is no direct relationship between CD34 expression and maintenance of self-renewal, DMOG slows down the differentiation process that occurs at 20% O_2 by stabilizing HIF-1α. The 20% O_2/DMOG sample proliferated at a slow rate, similar to the 1% O_2 sample and the sizes of the cell sub-

populations were also comparable. This demonstrates that the addition of 0.1mM DMOG to the culture medium at 20% O_2 can be used to obtain a level of differentiation inhibition almost similar to a hypoxic environment of 1% O_2. The total number of cells in all the cell sub-populations is greater in the presence of 20% O_2/DMOG than 1% O_2, which makes 20% O_2/DMOG a better condition to expand HPCs. The 1% O_2/YC-1 condition was used as a negative control to suppress HIF-1α/ARNT complex formation (Li et al. 2008). The inhibition of HIF-1α by YC-1 in the 1% O_2/YC-1 sample reduced cell quiescence and increased cell differentiation. The cell numbers in all the sub-populations of the 1% O_2/YC-1 sample were reduced as expected when compared to the 1% O_2 sample. These results support the observation that HIF-1α plays an important role in hematopoiesis.

The ARNT protein can also complex with AhR to promote cell differentiation which is unfavorable for expanding cells (Boitano et al., 2010). It is known that HIF-1α is degraded and AhR binds ARNT in cells cultured under normoxic conditions (20% O_2) (Boitano et al., 2010; Ivanovic, 2009), proliferate at a high rate but only maintain a small proportion of quiescent cells. However, even though the percentage of primitive and progenitor cells is low, the total/absolute number is significantly higher than in samples cultured under hypoxia.

To determine if the expansion of primitive HPCs can be improved by limiting ARNT/AhR complex formation, CD34+ cord blood cells were cultured with SR1, an antagonist of AhR (Boitano et al., 2010). Isolated CD34+ cells cultured in 20% O_2 with SR1, where it is known that both HIF-1α and AhR are suppressed (Boitano et al., 2010; Ivanovic, 2009), proliferate at a high rate. We demonstrate that this occurs

mainly in the SP cell compartment which suggests that the expansion of the primitive HPC population is favored.

Cells cultured in both DMOG (Ivanovic, 2009) and SR1 (Boitano et al., 2010) proliferate at a similar rate to cultures containing DMOG alone. However, the primitive stem cell population is larger than when cells are cultured in DMOG alone. Culturing cells in DMOG or hypoxia with SR1 is useful to obtain a high percentage of primitive progenitor cells. The hypothesis that the use of culture conditions that stabilize HIF-1α as well as antagonize the AhR protein would promote the expansion of the primitive HSC population is thus accepted.

4.5. Conclusion

The use of SR1 at 20% O_2 proved to be the best condition for the expansion of HPSCs into larger cell numbers as has been established before; however, the use of SR1 at 1% O2 resulted in an increase in the proportion of primitive HSCs in the sample. This proportion is 2.83 fold higher than that 20% SR1 condition as used in previous studies. It will in the future be necessary to confirm the effectiveness and safety of cells treated with SR1. We have demonstrated the value of comparing the HPC sub-populations of treated samples in terms of proportion and absolute cell number.

Chapter 5:

In vivo analysis of techniques used to expand hematopoietic stem cells

5.1. Introduction

The transplantation of genetically modified hematopoietic cells for patients that need gene therapy due to hematopoietic disorders or the transplantation of non-modified hematopoietic cells for patients that have received chemotherapy is not a trivial matter (Burnett et al. 2012; Weissman and Shizuru 2008). The reason is that the hematopoietic system is complex and serves many functions including the transport of oxygen, wound healing and the protection against pathogens through the immune system (Gordon, 1993). Patients that have hematopoietic disorders or patients that have received chemotherapy need their hematopoietic system replaced with a fully functional and healthy system. The hematopoietic product provided to patients thus needs to contain/produce all the components of the hematopoietic system (Hofmeister et al. 2007; Williams and Gress 2008). The transfusion of peripheral blood products into patients only provides short term (ST) benefit. This is due to the inability of PB to migrate to the bone marrow compartments and engraft, as it lacks HSCs (Rosa et al. 1999, Rossig and Brenner, 2004). Bone marrow isolates however do have the ability to engraft, but take a few weeks to produce enough immune cells that can provide an immune response (Takubo, 2012). Bone marrow transplant patients are thus susceptible to pathogenic infection in the first few weeks and need antibiotics or the co-transplantation of bone marrow with PB to prevent infection (Coomes, Hubbard, and Moore 2011; Engelhard et al. 2009).

The limited number of HSCs obtained from bone marrow isolates can cause transplantation failure (Candotti et al. 2012; Kang et al. 2010). Cord blood units contain a large proportion of HSCs; however, the HSC numbers are very low and the cells tend to take much longer to produce a functional immune system (Hofmeister et

al., 2007; Seggewiss & Einselle, 2010). Various factors influence the success of a hematopoietic transplantation including the origin of the cells, the source, the age and the cell number. The ability to expand primitive HSCs *in vitro* without differentiation is very important due to the fact that culturing HSCs *in vitro* that leads to differentiation (Chaurasia et al. 2014; Cheng et al. 2000; Mikkola and Orkin 2006). Various techniques have been investigated to achieve this and the inhibition of the AhR protein with the use of SR1, in combination with 20% O_2, has been proven to provide a drastic benefit (Boitano et al., 2010). The use of 20% O_2, however, is not physiological and facilitates an abnormal rate of cell cycling. The oxygen concentration of the endogenous niche where the primitive HSCs reside, ranges between 0.1% and 5% O_2 which stabilizes HIF-1α (Cheng et al. 2000; Cipolleschi, Sbarba, and Olivotto 1993; Guitart et al. 2010; Hermitte et al. 2006; A. Wilson and Trumpp 2006). This in turn maintains HSCs ability to self-renew and differentiate into all the differentiation lineages in a non-skewed manner. Oxygen concentrations higher than 10% O_2 activate genes which play a major role in the commitment of HSCs to differentiation lineages and the migration thereof into the blood circulation (Singh et al. 2009).

We hypothesized that that the use of culture conditions that stabilize HIF-1α and antagonize the AhR protein would promote the engraftment capacity of the LT-HSC population with low total cell numbers. Currently, the only test to identify self-renewing pluripotent long-term human HSCs after culturing is serial xenografting of immune deficient mice. NOD SCID gamma mice (NSG, NOD.Cg-Prkdc<scid> Il2rg<tm1Wjl>/SzJ)(Brehm et al., 2015) were used to determine the short-term and long-term engraftment capacity of the primitive HSC population expanded with SR1

at 20% O_2 or SR1 at 1%O_2. Differentiation lineages were analyzed to determine the effect of the treatments on the differentiation capacity of the cells.

5.2. Materials and methods

5.2.1. Isolation of CD34+ cells

$CD34^+$ cells were isolated from fresh umbilical cord blood units (<24 hrs old) obtained from Netcare Femina Hospital in Pretoria (South Africa) using the indirect $CD34^+$ MicroBead kit (Miltenyi Biotech, #130-046-703), according to the manufacturer's instructions. A cell purity of > 80% was used i.e. in which >80 of the cells were CD34 positive.

5.2.2. Liquid culture

CD34+ cells were seeded at a density of $2{,}3x10^5$ cells/ml and cultured under normoxic (5% CO_2; 20% O_2) or hypoxic conditions (5% CO_2; 1% O_2) in Dulbecco's Modified Eagle *Medium* (DMEM) containing 1% penicillin/streptomycin (PS), 10% fetal bovine serum (FBS). A cytokine mixture of hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), and TPO (20 ng/ml) was used. DMSO was used as a vehicle control in the 1% O_2 and 20% O_2 conditions. A concentration of 0.75 µM SR1 (Biovision, USA, #1967-1) was used for experiments as previously described (Boitano et al., 2010).

5.2.3. Engraftment assays

NOD SCID gamma (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) mice (NSG mice) obtained from Jackson laboratories (Bar Harbor, Maine, USA) were used in this study. Mice were maintained in micro-isolator cages under specific-pathogen-free conditions and fed with autoclaved food and water. Only female mice were used in these experiments.

Ethics approval was obtained from the Animal Ethics Committee of the University of Pretoria. Busulfan (50mg/kg, Sigma Aldrich, Munich, Germany), dissolved in DMSO and diluted with PBS, was administered to 5 to 8 week old female mice by split intraperitoneal injections with a 24h delay. Either freshly isolated or cultured CD34+ cells were injected 48h after the initial Busulfan injection by i.v. tail vein injection at cell numbers ranging from 300 to $1x10^4$cells as indicated. Alternatively, their expansion equivalent was injected. Bone marrow was collected at 8 weeks after transplantation of CD34+ cells. Red blood cell lysis was performed on the isolated cells using an ammonium chloride solution and the remaining cells were labeled with specific antibodies and immune phenotyped by flow cytometry (Gallios, Beckman Coulter). Bone marrow cells obtained from these mice (primary engraftment) were injected at 50% of isolated volume into a second set of mice (secondary engraftment) which were sacrificed at 8 weeks post transplantation. Limiting dilution analysis was done according to Hu and Smyth 2009 (Hu & Smyth, 2009).

5.2.4. Differentiation assays

Peripheral blood and spleens were collected at 8 weeks after transplantation of CD34+ cells. Red blood cell lysis was performed on the isolated cells using an ammonium chloride solution and the remaining cells were labeled with specific antibodies and immune phenotyped by flow cytometry (Gallios, Beckman Coulter).

5.2.5. Statistical Analysis

Data are given as mean ± S.D. or median of replicate experiments as indicated. Column comparisons are done by using Welch's t-test for unpaired data using GraphPad Prism 5.04. Statistical significance is indicated on the graphs as follows: (*) $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.0005$ and (****) $p < 0.00005$.

5.3. Results

5.3.1. Effect of O_2 and SR-1 on cells capable of short-term engraftment

The short-term engraftment capacity of freshly isolated CD34+ cells and cells expanded for 7 days under various conditions is portrayed by the percentage human CD45+ cells related to mouse CD45+ cells (Figure 16A). The cell numbers transplanted and the expansion equivalent cell numbers are shown in Table 7. The positively engrafted (>0.5%) mice in the primary engraftment studies are shown in Table 7 and the limiting dilution graphs are shown in Figure 16B. An incremental increase in engraftment was observed in mice that received $3x10^2$, $1x10^3$, $3x10^3$ or 10^4 freshly isolated cells. Similar engraftment levels were observed in mice that received the expansion equivalent cell numbers cultured at 20% O_2. Significantly higher engraftment was observed in mice that received an expansion equivalent of $1x10^3$ cells expanded at 20% O_2/SR1 and 1% O_2/SR1 compared to those that received a expansion equivalent of $1x10^3$ cells expanded at 20% O_2. The short-term SCID repopulating cells (ST-SRC) and long-term SCID repopulating cell (long-term SRC) frequencies, which refer to the frequency of obtaining mice in a transplanted cohort with 95% confidence that demonstrate the positive identification of human CD45+ cells in the blood 8 weeks after transplantation and more than eight weeks after transplantation respectively, are shown in Table 8. The mice that received an expansion equivalent of $3x10^2$ cells from the 1% O_2/SR1 culture showed significantly higher engraftment compared to the 20% O_2/SR1 culture.

5.3.2. Effect of O_2 and SR-1 on differentiation capacity of treated cells

The capacity of the engrafted cells to differentiate into the various hematopoietic lineages is shown in Figure 17A and B. Figure 17A demonstrates that no significant differences in the differentiation capacity can be observed in the peripheral blood between the expanded and fresh CD34+ cells. Similarly, Figure 17B demonstrates that no significant differences in the differentiation capacity can be observed in the spleen between the expanded and fresh CD34+ cells.

5.3.3. Effect of O_2 and SR-1 on cells capable of long-term engraftment

Positively engrafted (>0.5%) mice in the secondary engraftment studies are shown in Table 7 and the graphs of the limiting dilution analysis are shown in Figure 16C. A limiting dilution analysis refers to an assay in which groups of mice are injected with cells that decrease in number in a linear manner for each group. This is done to determine at what dilution i.e. cell number; the cells no longer provide positive engraftment and thereby the SRC is determined. The LT-SRC frequencies obtained with the 95% confidence intervals are shown in Table 8. The LT-SRC frequency increased with the 20% O_2 condition from 1/3771 to 1/2265 while the 1% O_2 condition only increased to 1/3487 whereas the 10%/SR1 condition gave a frequency of 1/890. This is a substantial increase in LT-SRCs obtained with a cell dose after expansion of 13430 cells. The 1% O_2/SR1 condition provided a similar LT-SRC frequency of 1/990 which was however obtained with a dose after expansion of 3770 cells (Table 7).

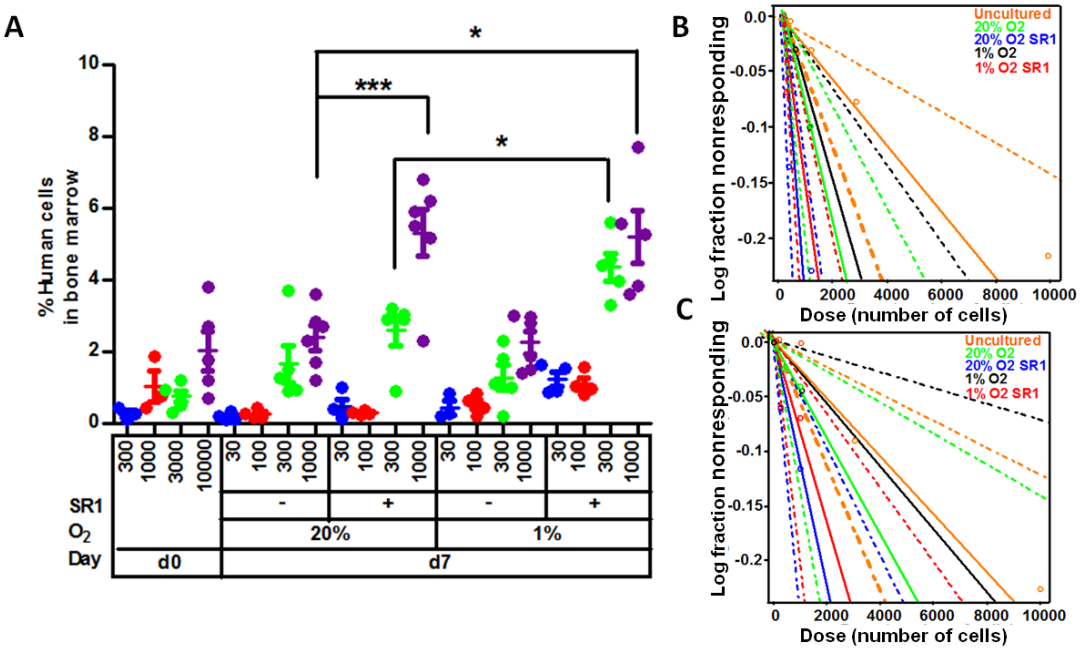

Figure 16. Engraftment of expanded CD34+ cells. (**A**): Human CD45+ cell chimerism obtained in NSG mice with primary engrafted CD34+ cells. Freshly isolated human CD34+ cells were transplanted at 300, 1000, 3000 and 10000 cells. The expansion equivalent of 30, 100, 3000 and 10 000 cells was also transplanted. The percentage human CD45 cells to mouse CD45 cells in the femurs of the mice at week 8 is shown. (**B**): This graph indicates the linear dilution analysis of non-responding cells that did not engraft. Uncultured as well as 7 day expanded cells were transplanted separately into NSG mice and analyzed after 8 weeks to reveal the extent of primary engraftment. (**C**): This graph indicates the non-responding cells that did not engraft when cells, obtained from the femurs of mice of the primary engraftment, were transplanted into NSG mice and analyzed after 8 weeks to reveal the extent of secondary engraftment. Statistical significance is indicated in the graph as described on page 85.

Table 7 Primary and secondary engraftment of treated HSCs

Conditions	Cell dose transplanted		Primary transplant	Secondary transplant
Uncultured	300		0/4	0/4
	1000		1/4	0/4
	3000		3/5	3/5
	10000		5/5	5/5
Control	Fraction of 7 day culture			
	Equivalent starting dose	Dose after expansion	Primary transplant	Secondary transplant
	30	374.4	0/4	0/4
	100	1248.0	0/4	0/4
	300	3744.0	2/5	1/5
	1000	12480.0	4/6	2/6
SR1	30	402.9	0/4	0/4
	100	1343.0	0/4	0/4
	300	4029.0	4/5	2/5
	1000	13430.0	6/6	4/6
1% O2	30	91.2	0/4	0/4
	100	304.0	0/4	0/4
	300	912.0	1/5	0/5
	1000	3040.0	4/6	2/6
1% O2 SR1	30	111.3	0/4	0/4
	100	371.0	0/4	0/4
	300	1113.0	3/5	2/5
	1000	3710.0	5/6	3/5

The cell doses injected into marrow ablated mice are indicated. The cell dose of the uncultured cells is provided as well as the equivalent cell dose which refers to the number of cells injected had they not been expanded. The dose after expansion refers to the number of cells injected after 7 days of expansion. The primary transplant column refers to the number of mice out of the injected primary engraftment mice that demonstrated the presence of >0.5% human CD45 cells in the blood. The secondary transplant column refers to the number of mice out of the injected secondary engraftment mice that demonstrated the presence of >0.5% human CD45 cells in the blood.

Table 8 Short-term and long-term engraftment of treated HSCs

Culture condition	ST-SRC frequency per starting cells	95% Confidence interval	ST-SRC frequency per total cells
Uncultured	1/3098	1/1430 - 1/6690	64
20% O2 DMSO	1/889	1/389 - 1/2032	3374
20% O2 SR1	1/284	1/134 – 1/601	11355
1% O2 DMSO	1/1114	1/461 - 1/2687	1095
1% O2 SR1	1/537	1/253 -1/1138	2274
Culture condition	LT-SRC frequency per starting cells	95% Confidence interval	LT-SRC frequency per total cells
Uncultured	1/3771	1/1749 -1/8134	53
20% O2 DMSO	1/2265	1/724 - 1/7086	1324
20% O2 SR1	1/890	1/390 - 1/2033	3623
1% O2 DMSO	1/3487	1/886 - 1/13725	349
1% O2 SR1	1/992	1/403 – 1/2444	1237

The ST-SRC and LT-SRC frequency per starting cells columns refer to the frequency of obtaining a cell in a number of cells in a sample that is capable of contributing to short-term or long-term engraftment. The 95% confidence intervals are provided and the frequencies per total cells in uncultured and expanded samples are indicated.

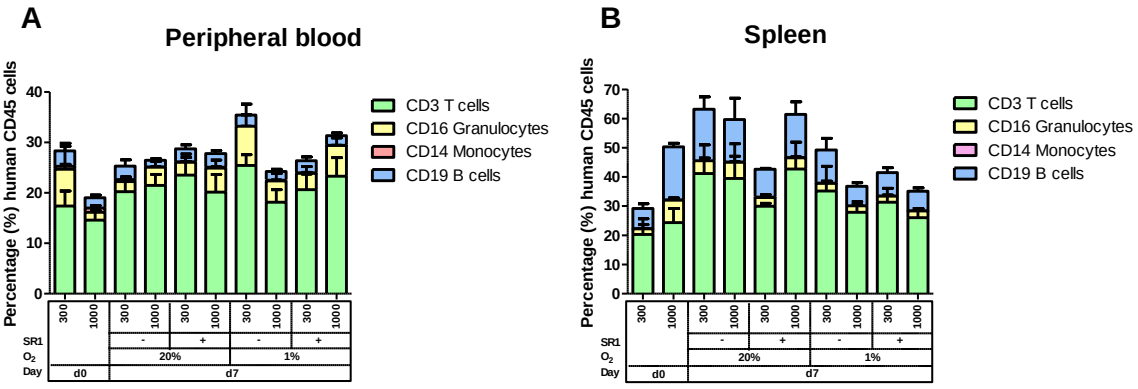

Figure 17. *In vivo* differentiation capacities of engrafted cells. (**A**): Percentages of T cells, granulocytes, monocytes and B cells in the human CD45 cell population of the peripheral blood of mice that received fresh or cultured cells, are shown. (**B**): Percentages of T cells, granulocytes, monocytes and B cells in the human CD45 cell population of the spleen of mice that received fresh or cultured cells, are shown.

5.4. Discussion

The characteristics of the various HSC sub-populations in a donor sample could influence the decision regarding hematopoietic transplantations. For example, a donor sample with a high CD34 cell count is generally used for a transplantation; however, if it is known that the CD34 cells contain a small population of cells that are SP positive it would preferable not be used as it would not provide long-term engraftment. The percentage of HPCs, determined with the CFU assay, could affect the short-term engraftment capacity of a transplant. *In vitro* expansion techniques differ regarding the influence on these sub-populations, favoring some above others. To provide short-term and long-term therapeutic benefit an adequate number of LT-SRC is needed that can facilitate the long-term therapeutic effect as well as ST-SRC together with progenitor cells that can generate immune cells shortly after transplantation (Burnett, Zaia, and Rossi 2012; Penn and Mangi 2008; Takubo 2012).

The effect of hypoxia, SR1 and the combination of the two on various hematopoietic sub-populations has been investigated *in vitro* as shown in Chapter 4. The data provide valuable information on changes in the progenitor populations and candidate stem cells. However, the techniques used to quantify the candidate stem cells must be confirmed *in vivo*. This was done with *in vivo* engraftment of NSG mice.

The *in vitro* data demonstrated that the use of an AhR antagonist in combination with 20% O_2 significantly increases the absolute number of cells with SP characteristics which can include ST-SRCs and LT-SRCs. The pre-CFU assay indicated that the ST-CFU ability was significantly increased which corresponds to the short-term engraftment ability. The increased LT-CFU ability was also confirmed with the long-

term engraftment observed with the secondary engraftment results. A 4.23 fold increase in LT-SRC frequency was obtained with a cell dose after expansion of 13430 cells.

The stabilization of HIF-1α did not increase the absolute number of cells with SP characteristics or cells with ST and LT-CFU ability. The lack of improved expansion of HSCs was also observed in short-term and long-term engraftment which was similar to the 20% O_2 condition.

The stabilization of HIF-1α together with the use of an AhR antagonist significantly increased the proportion of cells that have SP characteristic as well as increased the proportion of cells that have ST and LT-CFU abilities. However, the absolute number of cells was similar to the condition where only HIF-1α was stabilized. The engraftment data support this finding where the short-term and long-term (LT-SRC frequency of 3.8) engraftment was similar to the condition where HIF-1α was not stabilized and an AhR antagonist was used (LT-SRC frequency of 4.23). This was, however, obtained with a much lower cell dose after expansion of 3770 cells. It thus indicated that this condition increased the capacity of the cells to engraft and repopulate the bone marrow but did not increase the number of these cells during culturing. The hypothesis that the use of a culturing condition that stabilizes HIF-1α and antagonizes the AhR protein would promote the engraftment capacity of the LT-HSC population was thus accepted.

5.5. Conclusion

It has previously been established that the use of SR1 in culture conditions with 20% O_2 significantly increases engraftment in NSG mice. The present study has shown that the increased engraftment observed is due to an increase in the size of the primitive cell population as evidenced by the increased number of SP cells. This increase is mainly due to the increase of the total cell population. This study has further established that the culturing of isolated CD34+ cells with hypoxia and SR1 is useful for improving the long-term engraftment capacity of primitive HSCs without increasing the cell number. This condition favors the expansion of the primitive HSC (SR-HSC and LT-HSC) population without causing the expansion of the total cell population. We believe that our results will impact on the translation of *in vitro*/*in vivo* findings to the clinic.

Chapter 6

In vitro analysis of techniques used to transduce hematopoietic stem cells

6.1. Introduction

Lentiviral vectors were developed in the 1990's and since then various improvements have been made to ensure that their use in human cell transduction systems is safe (Naldini et al. 1996). Self-inactivating (SIN) vectors have had the viral promoter and enhancer removed from the integrated cassette, which reduces the genotoxic potential caused by the random insertion of the DNA fragment into the host cellular genome (Baum, 2008). The removal of viral elements such as the viral accessory protein R (VpR) increases safety but compromises the integration efficiency of the virus (Brenner and Malech 2003b; Gummuluru and Emerman 1999). The VpR plays a role in permitting virus core entry into the nucleus, by arresting cells in the G2/M phase of the cell cycle. This role of VpR is absent from SIN lentivirus vectors. Although both dividing and non-dividing (quiescent) cells can be transduced, the transduction of HSCs for therapeutic purposes faces several challenges (Ye et al. 2008). Transduction of HSCs requires a significantly greater number of lentiviral particles compared to other cell types to obtain similar proportions of positively transduced cells (Arafat et al. 2000; Barrette et al. 2014; Steven et al. 2002). This is mainly due to the fact that the majority of freshly isolated HSCs that are quiescent and the cellular components used by the lentivirus vector to integrate into the genome of the cell is not present or is present at low concentrations. The virus particle to cell number ratio needed to transduce an acceptable number of cells can be problematic for clinical applications (Steven et al. 2002).

The number of naïve CD34+ cells used for hematopoietic stem cell transplantation of an adult, which contains both HSCs and HPCs,is usually $5x10^6$ cells/kg body weight (Jillella & Ustun, 2004). The fact that the prescribed number of cells cannot always

be obtained from a patient is a limiting factor. A further limitation is to obtain an acceptable number of positively transduced CD34+ cells which ranges between 2 and $4.5x10^6$ cells/kg as documented in completed clinical trials (DiGiusto et al., 2010; Ott et al., 2006). To obtain 50% positively transduced CD34+ cells with lentiviral vectors *in vitro,* a multiplicity of infection (MOI) of 55 was documented to be sufficient (Cavazzana-Calvo et al., 2010). However, the calculated number of virus particles needed to obtain this transduction efficiency is $2x10^8$. The large amount of plasmid needed to produce this number of virus particles for a clinical trial is a labor intensive procedure and this makes it impractical. An excessively high MOI such as 55 increases the occurrence of multiple insertions in single cells which increases the risk of insertional mutagenesis (Montini et al., 2009). It was found that one in 138 γ-retrovirus vector inserts occurred in oncogenic loci, compared to one in 2224 for lentivirus vectors (Aiuti et al. 2013). The transduction efficiency of HSCs is particularly low compared to other cell types and is affected by many variables (Moore et al. 2010). Previous studies have investigated ways to improve the efficiency, which include the use of genistein and nocodazole to artificially induce G2/M cell cycle arrest (Zhang et al. 2006), adjusting the cytokine combination used in the medium, the use of pre-stimulation and the addition of polycations (Moore and MacKenzie, 1999).

Transduction occurs once the lentivirus particles come into contact with the target cells. The HSCs settle to the bottom of the transduction plate, and the lentivirus particles traverse the static medium to reach the cells before they die. Lentivirus particles can only reach the cells through Brownian movement, which allow particles to move 480-610 µm in their 10-16h lifespan (Chuck et al. 1996). The majority of

virus particles in the upper part of the culture medium are thus residual as they will not reach a cell before they become inactive (Figure 18) (Chuck et al. 1996).

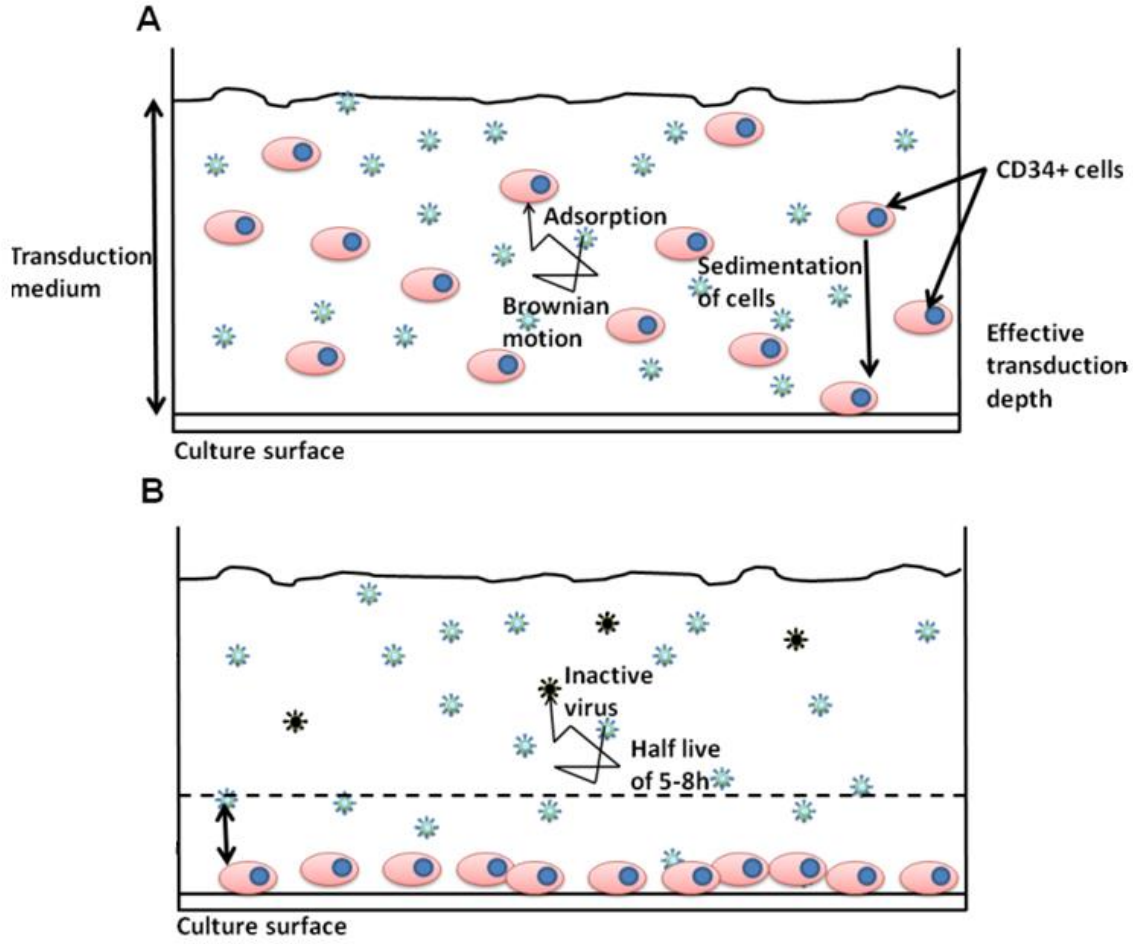

Figure 18. Absorption of lentivirus vector particles by CD34+ cells (Adapted from Chuck et al., 1996). **(A)** Mixture of cells at the initiation of the transduction process. **(B)** Settling out of cells during the transduction process.

Transduction methods used to overcome this limitation by promoting virus uptake include flow through transduction, centrifugal transduction or 'spinoculation' and the use of Retronectin coated plates (Moore et al. 2010; Schilz et al. 1998). These methods have been used successfully to increase the transduction of CD34+ cells. However, poor long-term engraftment of these cells has been reported (Chuck et al. 1996; Hutchings et al. 1998; Larochelle et al. 2014). Hanenberg *et al.* (Hanenburg et al., 1997) found that by combining the use of Retronectin with pre-stimulation methods, transduction of the CD34+CD38+ and CD34+CD38- cell populations can be significantly improved. It was also found that by combining the use of Retronectin with pre-stimulation, polycations and spinoculation, up to 56% SRCs could be obtained (Schilz et al. 1998). The above mentioned transduction methods were performed at 20% O_2 (Ye et al. 2008), which causes spontaneous differentiation of HSCs (Ivanovic, 2009). Maintenance of primitive HSCs capable of long-term engraftment was not favored by the above mentioned techniques (Briones et al., 1999). This means that a proportion of positively transduced cells commit to a cell lineage during transduction and thus lose the ability to engraft. It is thus of interest to investigate methods that could potentially improve the transduction as well as the maintenance or expansion of long-term HSCs. The efficiency of genistein and spinoculation in the transduction of primitive HSCs was investigated in the current study. The maintenance of HSCs in culture can be partially achieved with the use of DMOG which stabilizes HIF-1α and thereby mimics a hypoxic environment (Guitart et al., 2010; van Harmelen et al., 2006). Stemregenin 1, an antagonist of the AhR, favors the proliferation of HSCs with limited differentiation (Boitano et al., 2010). The ability of these reagents to improve the transduction of primitive HSCs and/or result in their expansion during the transduction process was investigated.

6.2. Materials and methods

6.2.1. Isolation of CD34+ cells

Umbilical cord blood units (<24 hrs old) were obtained from Netcare Femina Hospital, Pretoria (South Africa). Ficoll-Paque™ density gradient centrifugation was used to isolate mononuclear cells, from which $CD34^+$ cells were isolated using the indirect $CD34^+$ MicroBead kit (Miltenyi Biotech, #130-046-703), according to the manufacturer's instructions. A CD34+ cell purity of >80% was obtained by passing the cells through the isolation columns twice (#130-042-401). Fifty thousand cells were analyzed by flow cytometry using an anti-CD34 antibody (PC7-conjugated mouse antibody, Ref. #21691, Beckman Coulter, Miami, USA) to determine CD34+ cell purity.

6.2.2. Production of virus particles

Lentivirus particles were produced via transient transfection of HEK-293T cells. HEK-293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 1% penicillin/streptomycin (PS), 10% fetal bovine serum (FBS),cryo-preserved with 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. Three wells of a six-well plate were seeded with $1{,}2x10^5$ HEK-293T cells each and cultured for 18-24 hours. The cultures were transfected at ± 90% confluence using TransIT®-2020 transfection reagent (Mirus, Madison, USA) according to the manufacturer's guidelines. The plasmid DNA used for the transfection was 0.8 µg of the envelope vector (pMD2.VSV-G), 0.5 µg of the pSPAX2 packaging plasmid and 1.2 µg of the gene transfer vector. The gene transfer vector used to produce virus particles was either pLVTH (Appnecix A. 2 C.) that contains the enhanced green fluorescent

protein (EGFP) gene or TRC2-pLKO-puro (Appendix A 2 C, obtained from Sigma, USA) that contains the puromycin resistance gene and an anti-CCR5 shRNA (Dull et al., 1998). The supernatant was removed and replaced with fresh medium 24 hours post vector-transfection. The supernatant was collected 48 hours post vector-transduction, filtered using a 0.45µM filter and concentrated by ultra-centrifugation at 50 000 g-force (Anderson et al. 2007b). The titre of the virus in the concentrated supernatant was determined by transducing $1x10^4$ HEK-293T cells with a serial dilution of the virus sample (1.25, 2.5, 5, 10 µl).

6.2.3. Transduction

Transduction medium contained Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% BIT9500 (Stem Cell Technologies, Vancouver, BC, Canada), 100 ng/ml SCF, 100 ng/ml G-CSF, 100 ng/ml Flt3 ligand, 10 ng/ml TPO (PeproTech, Rocky Hill, NJ, USA), and 20 ng/ml IL-3 (R&D Systems, Minneapolis, MN, USA). $5x10^5$ freshly isolated CD34+ cells were transduced in 50 µl transduction medium with virus particles to an equivalent MOI of 5 and exposed to various conditions. The toxicity of genistein was tested using 30 and 60 µg/µl and 30 µg/µl was selected for further experiments on the basis of cell viability which was >80%. Spinoculation was performed by centrifuging the CD34+ cells in the presence of the virus particles at 800 g and 32°C for 2 hours. 0.1 mM DMOG and 0.75 µM SR1 were added to the cultures. After 18-24h, all the cell samples were washed twice with PBS and cultured in transduction medium. Puromycin (0.5 µM) was added to the cultures transduced with the puro-resistance virus particles, 24 hours after transduction. Transduction success was quantified after four days by flow cytometry for the EGFP

virus particles, and by live cell count for puro-resistance virus particles.

6.2.4. Analysis of transduction efficiency

Positively transduced cells were analyzed seven days after transduction. Samples were stained with 7-Aminoactinomycin (7-AAD) and absolute counts were obtained through the addition of Flowcount^{TM} Fluorospheres (Beckman Coulter, Miami, USA). Live cells, counted on day 7, were used to indicate the rate of cell proliferation that occurred during culturing. Cells were stained with an anti-CD34 (PC7-conjugated mouse) antibody. Positively transduced cells were quantified by analyzing EGFP for the LVTH vectors, and viable cells for the TRC2-pLKO-puro vector.

6.2.5. Colony-forming unit (CFU) assay

The CFU capacity of each treated sample was determined. One hundred cells from each sample on day 7 were plated in 250 µl Stem-α1D (Stem Alpha, St. Genis L'Argentière, France, http://www.stemalpha.fr) and colonies were counted 12 to 16 days after the cells were plated. The total number of CFUs in each sample was calculated by multiplying the proportion of cells capable of forming CFUs by the total number of cells in the sample.

6.2.6. Side population (SP) discrimination assay

On day 7 after transduction, the proportion of cells in each sample that had SP characteristics was determined. 3 x 10^5 cells were removed from each sample and divided into a control tube and an SP tube, centrifuged at 200 g, and the supernatant removed. The cells in the SP tube were resuspended in 150 µl DMEM+ (Invitrogen,

California, USA), 2% (v/v) FBS, 10 mM HEPES (Sigma, USA) and 5 µM VDC Violet stain (Life Technologies, Invitrogen, USA). The cells in the control tube were resuspended in DMEM+ containing 50 µM verapamil (Life technologies, California, USA). Both tubes were incubated for 2 hours at 37°C after which the cells were washed with 2 ml cold HBSS+ (Hank's balanced salt solution (Invitrogen, California, USA), 2% (v/v) FBS and 10 mM HEPES (Sigma)) and stained with 7-AAD, anti-CD34 and anti-CD38 (PC5-conjugated mouse antibody, Beckman Coulter) antibodies. The cells from both tubes were washed with 1 ml HBSS+ and analyzed by flow cytometry (Gallios flow cytometer, Beckman Coulter, USA). The cells were kept on ice after incubation. The cell population that stained negative for 7-AAD was selected to determine the SP using a FL9 (620/30 BP) versus FL10 (550/40 BP) scatter plot. The histogram generated from the verapamil control tube was used to set up the SP region that selects for the cells that efflux VDC violet dye. The same gate was maintained for all treatments to allow for comparison of data.

6.2.7. Statistical analysis

Data is presented as mean ± standard deviation for replicate experiments. Welch's t-test was used to determine statistical significance for unpaired data using GraphPad Prism 5.04. Statistical significance is indicated on the graphs as follows: (*) $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.0005$ and (****) $p < 0.00005$.

6.3. Results

6.3.1. Establishing the effect of selected conditions on the transduction and proliferation of CD34+ cells

Lentivirus particles that transduce the EGFP gene were produced by transient transfection of HEK-297T cells and the average titer obtained was $2x10^6$/µl, as determined using HEK-293T cells. Freshly isolated CD34+ cells collected at a purity >80% were transduced with the virus particles at an MOI of 5 or as otherwise indicated using the techniques described above. Flow cytometric analysis of the control condition (0.1% DMSO), seven days after transduction, indicated a 6-fold increase in the mean total number of cells and a transduction rate of 9.26% (Figure 19A). The fold increase in the total number of cells for the treatment conditions was not significantly different from control conditions, except for the DMOG condition in which there was a 3.2 fold average decrease due to inhibited proliferation of the cells. The average transduction rate obtained with SR1, genistein and spinoculation was 20.3%, 17.1% and 18.3%, respectively (Figure 19A). Skatterplots of treated samples analyzed for EGFP expression are shown in Figure 19A. Only one colony of EGFP positive cells can be observed for the non-transduced control condition (MOI = 5). The mean fluorescence intensity (MFI) change of the GFP+ colony compared to the EGFP negative cells (non-transduced control condition) is 22.83 (Figure 19B). The MFI change for the treatment conditions was between 18.9 and 25.3 (Figure 19B) which is similar to the transduced control condition and indicative of a similar VCN The MFI change for the EGFP positive colony of the control condition exposed to a MOI of 40 was 40.69, which is indicative of a high VCN.

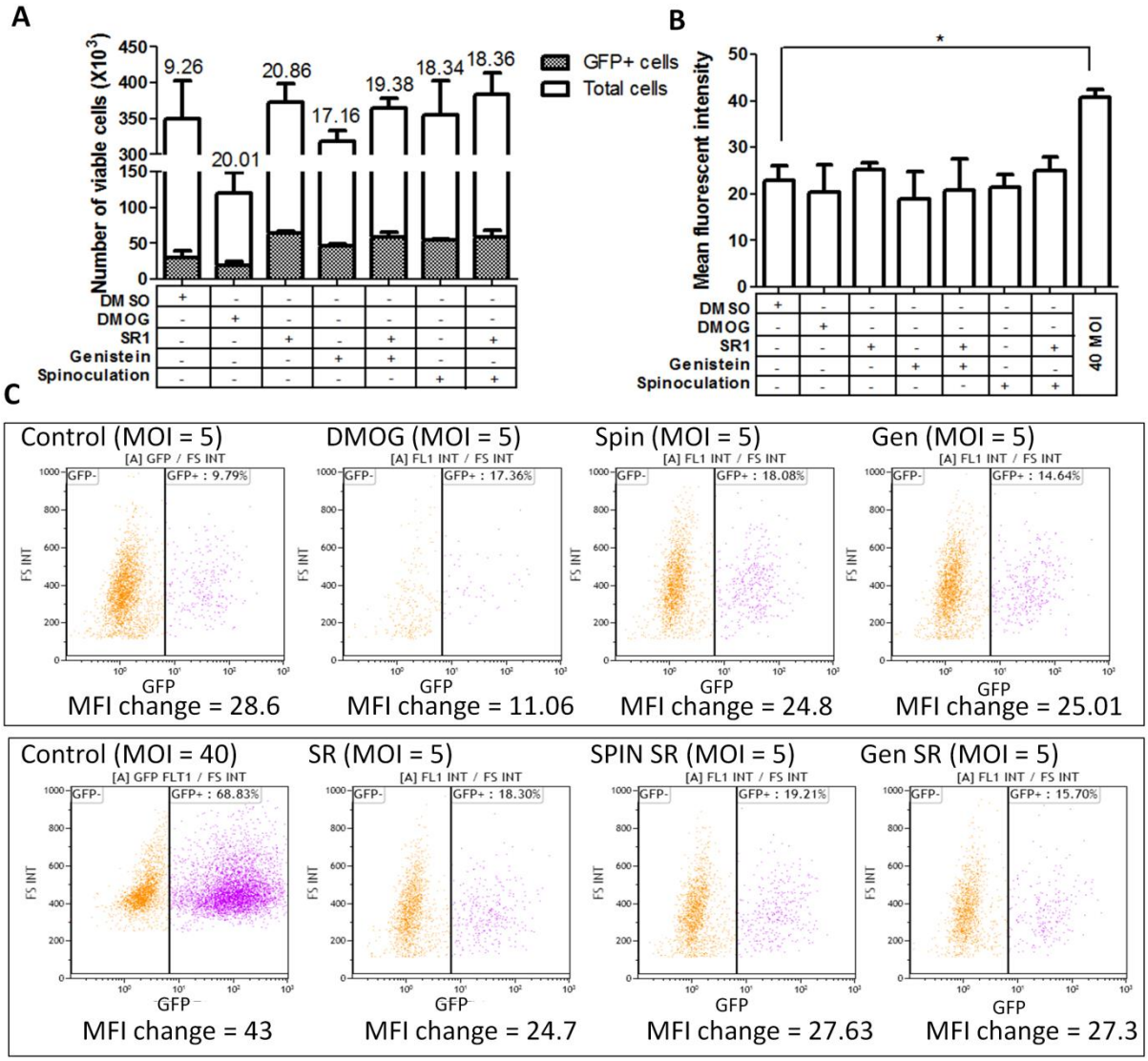

Figure 19. Transduction of freshly isolated CD34+ cells. **(A)** The total cell number and the number of positively transduced EGFP positive cells are shown. The transduction rates are indicated at the top of the bars. **(B)** The change in the mean fluorescent intensity of the GFP positive cells related to the EGFP negative cells is shown. **(C)** Selected scatterplots of the treatments are shown indicating the percentage of GFP cells and the mean fluorescence intensity. The change in MFI is indicated. Sample number is 3. Statistical significance is indicated in the graph as described on page 85. The MOI used is 5 for the treatment conditions and 40 for the control condition (as indicated).

6.3.2. Establishing the effect of the selected conditions on the transduction rate of HPCs capable of forming CFUs

To determine whether the HPCs within the CD34 cellular population were successfully transduced and whether their proliferation was affected, cells from each sample were plated in medium that allowed them to form CFUs. The number of cells in each sample that were capable of forming colonies larger than 50 cells was calculated and is shown in Figure 20. The total number of CFUs observed for the DMOG (20.6), genistein (19.6) and spinoculation (21.6) samples was similar to the control (16). However, for the samples treated with SR1 a greater number of CFUs (27.3) were observed compared to the control. The colonies expressing EGFP were counted to calculate the percentage of positively transduced cells. The fold increase in the percentage of EGFP positive colonies of the samples containing genistein or treated with spinoculation compared to the control sample was 2.1 and 2.3 and in combination with SR1 it was 2.5 and 2.6, respectively (Figure 20).

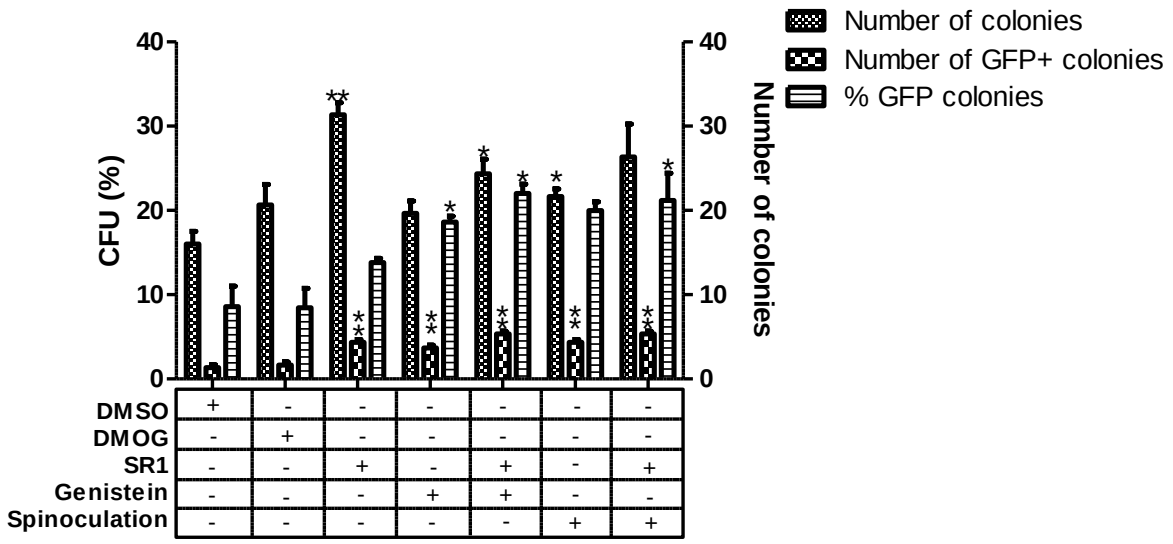

Figure 20. Positively transduced CFUs. The calculated number of total CFUs and GFP+ colonies for each sample is shown. The percentages of positively transduced CFUs are indicated by the third column of each group. Statistically significant differences are indicated at the top of the columns for treatment samples compared to the control. Statistical significance is indicated in the graph as described on page 85.

6.3.3. Establishing the effect of the selected conditions on the transduction rate of SP cells

The SP discrimination assay that identifies a candidate primitive HSC population was performed for each sample. This assay is based on the fact that stem cells efflux drugs and harmful molecules more than differentiated cells in order to prevent DNA damage. The gating strategy followed to obtain the desired data is illustrated in selected histograms in Figure 21A. The total number of cells in the SP of the genistein-treated sample was 1.6-fold less than the control, and it was 5-fold more in the SR1 sample (Figure 21B). The SP size of the other conditions was similar to the control (Figure 21B). The average calculated number of cells in the SP, positive for EGFP, was 575 for the control condition, 1721 for DMOG, 484 for genistein and 1441 for spinoculation (Figure 21C). An average number of 2844 SP cells in the SR1 sample was EGFP positive; in combination with genistein and spinoculation it was 1078 and 3943, respectively (Figure 21C).

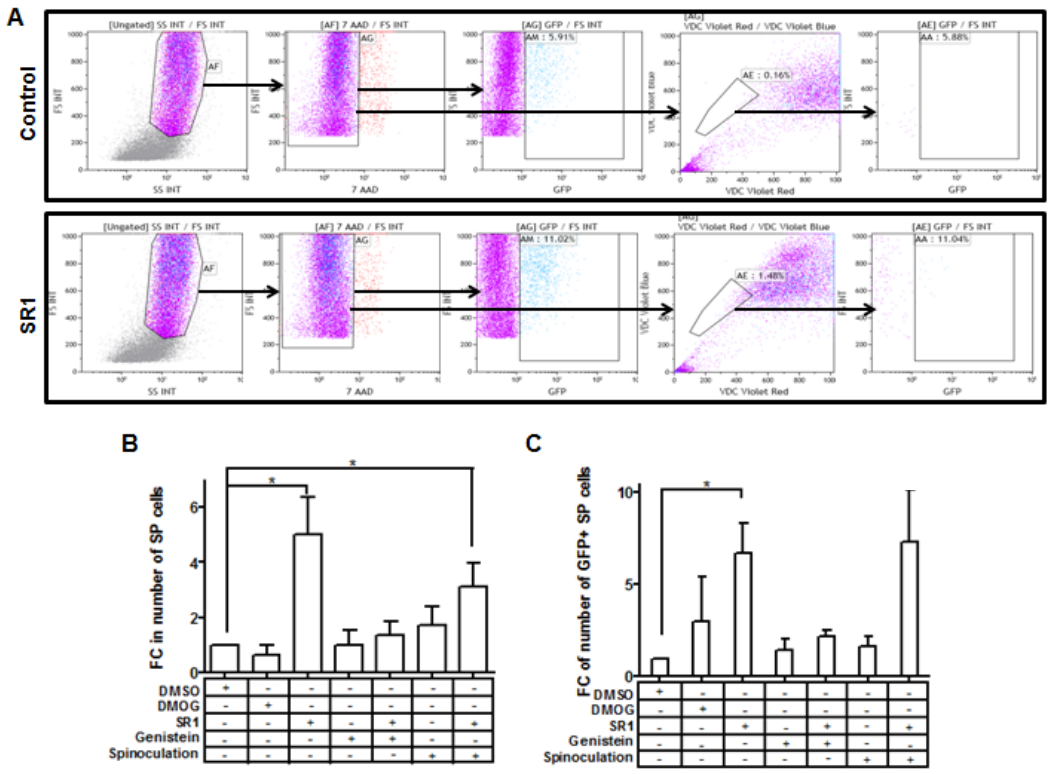

Figure 21. Positively transduced SP cells. **(A)** Selected histograms of the gating strategy used to determine the numbers of cells in each sample that are EGFP positive and have SP characteristics are shown for the control and SR1. The forward scatter (FC) and fluorescence of cells are indicated. **(B)** The fold change (FC) in the total number of cells with SP characteristics is shown for each treatment condition. Sample number is 4. **(C)** The fold change (FC) in the total number of GFP+ cells with SP cells is shown for each treatment condition. Sample number is 4. Statistical significance is indicated in the graph at the top of the columns as described on page 85.

6.3.4. Establishing the effect of the selected conditions on cells transduced with the TRC2-pLKO-puro virus

The EGFP marker can be used to select for positively transduced cells. As an alternative, we used a lentiviral vector that could be used in a clinical trial setting to provide HIV resistance to HSCs. This vector expresses the puromycin resistance gene and an anti-CCR5 shRNA. The percentage of HEK-293T cells that survive puromycin treatment was used to calculate the transduction efficiency. Freshly isolated CD34+ cells were transduced as mentioned in section 6.3.2. The quantification of viable cells and CFU assays were performed on day 7 and produced similar results to those obtained with the EGFP vectors (Figure 22A). The average number of cells that survived puromycin treatment seven days post transduction for the control and DMOG-treated sample was $4{,}4x10^4$ and $8{,}1x10^4$, respectively. The average number of cells that survived for the other treatments was +/- $1{,}0x10^5$. The proportion of cells in the control condition that survived the puromycin treatment and was capable of forming CFUs was 1%; however, for the genistein and SR1 conditions it was +/- 4% (Figure 22B). For the SR1 and genistein combination and the spinoculation samples it was +/- 6%. Eight percent of the cells treated with spinoculation and SR1, that survived the puromycin treatment, were able to form CFUs (Figure 22B).

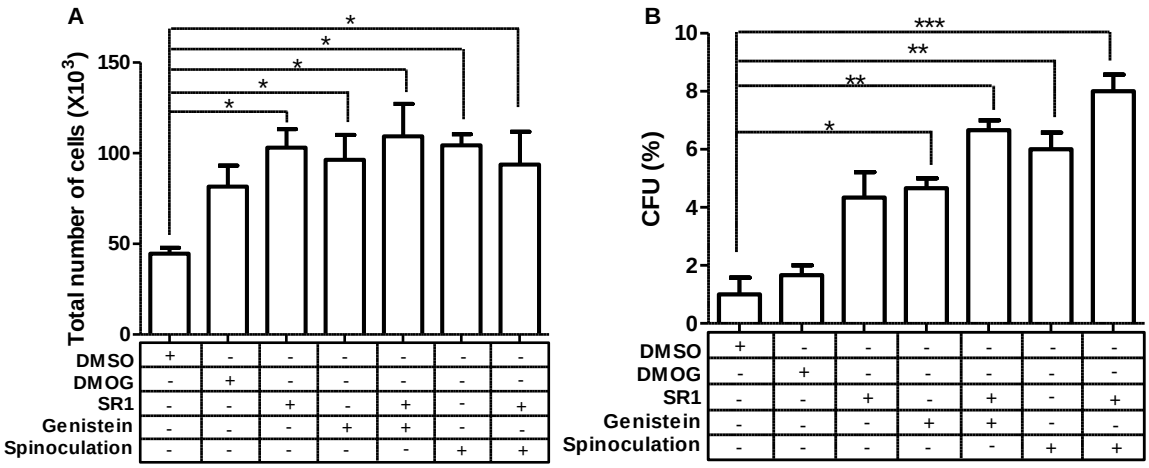

Figure 22. Transduction of cells with puromycin resistance viral particles. **(A)** The total number of viable cells that survived 7 days post transduction treatment of puromycin is shown for each condition. **(B)** The percentage of cells capable of forming CFUs is shown for each condition. Statistical significance is indicated above the columns. Sample number is 3. Statistical significance is indicated in the graph as as described on page 85.

6.4. Discussion

One of the major challenges in engineering genetically manipulated HSPCs for clinical transplantation is to obtain enough positively transduced primitive cells (Glimm et al., 2014). The MOI of vector particles used correlates directly with the number of transduced cells, and also with the vector copy number (VCN) per cell (Charrier et al., 2011). Even though SIN-lentivirus vectors are safer than γ-retrovirus vectors, a higher VCN increases the risk of insertional mutagenesis and must be minimized (Aiuti et al. 2013; Montini et al. 2009). The techniques used to transduce HSPCs with lentivirus vectors significantly affect transduction efficiencies and can be critical for obtaining therapeutic effects in clinical trials (Tisdale et al. 2000). The ideal would be to use a transduction technique with a minimal number of virus particles to obtain a high percentage of transduced cells with one VCN per cell (Dropulic, 2005).

In this study we investigated whether previously used and novel transduction techniques might provide higher transduction rates without increasing VCN per cell. It is important that the technique investigated does not compromise the size of the primitive HSC populations in the sample, as this will negatively affect engraftment efficiency (Glimm et al. 2014; Schilz et al. 1998). Two transduction conditions previously described to improve lentivirus transduction, and two conditions previously described to improve HSPC preservation were analyzed in this study to determine their effect on the transduction efficiency of HSPCs. These conditions included the use of genistein (Zhang et al. 2006), spinoculation (Schilz et al. 1998), DMOG and SR1 (Boitano et al., 2010). We also investigated whether the combined use of SR1 and genistein or SR1 and spinoculation might improve transduction efficiency as well as the expansion of the transduced HSPCs.

Zhang et al., 2006 reported that genistein increases the transduction of various cell types (Zhang et al. 2006). However, data regarding the transduction of CD34+ cells is limited. Zhang et al., 2006 treated the cells with a high concentration of lentivirus particles (MOI=100) and only the number of EGFP-positive colonies was indicated. Two concentrations of genistein were tested with no indication of how cell viability was affected. One of the objectives of our study was to further investigate the effect of genistein on HSPC transduction with an MOI of 5. The results of this study indicate that 60 µM of genistein caused significant cell death and 30 µM caused less than 20% cell death. Treatment with 30 µM genistein caused a 2-fold increase in both transduced CFUs and total cells. The transduction experiments where a clinically relevant vector TRC2-pLKO-puro, a lentivirus vector that expresses anti-CCR5 shRNA was used, demonstrates that SR1, genistein and spinoculation can be used for transducing CD34+ cells.

The data obtained in this study using lentivirus vectors that express EGFP or puromycin, confirms previous findings that genistein and spinoculation improve the transduction efficiency of CD34+ cells. The data also reveal that the treatment conditions increase the transduction of HSCs without jeopardizing the size of the HSPC sub-populations. It has been documented that the short-term engraftment of transduced HSCs in clinical trials is less than 4% and that long-term engraftment is less than 1%, which is very low (Capotondo et al. 2007; Kang et al. 2010; Kohn et al. 1999). This could be a major limitation for gene therapy, where good long-term engraftment is essential. The reason for the low long-term engraftment rate could be due to the engraftment potential of the cells that was lost with the transduction

procedure (Glimm et al., 2014) or the transduction of the primitive HSCs that was less effective compared to the main population. This is demonstrated in Figure 21Bby the observation that the transduced HSC population of the control condition, represented as GFP+ SP, was very small on day 7 after transduction. The addition of SR1 to the transduction medium resulted in a 5-fold increase in positively transduced cells which had SP characteristics. Even though DMOG lowered cell proliferation, the number of transduced SP cells was 3-fold greater than the control condition. Although genistein improved the transduction of total CD34+ cells and CFUs, it did not favor the improved transduction of primitive HSCs. Spinoculation on the other hand provided a 2.5-fold increase in transduced SP cells and in combination with SR1 resulted in a 6.8-fold increase. This indicates that SR1 can be added to other transduction methods to further improve the transduction of primitive HSCs.

We demonstrate that the increase in transduction efficiency observed with the transduction methods tested does not increase the VCN per cell, as shown by the MFI change that is similar to the control.

6.5. Conclusion

Genistein and spinoculation improve the transduction efficiency of HSPCs without jeopardizing the size of HSPC sub-populations or increasing VCN. Stemregenin 1 promotes the maintenance of candidate HSCs during transduction and increases the total number of transduced HSPCs, but more importantly increases the number of positively transduced SP cells and short-term engrafting cells. Stemregenin 1 has the potential to benefit various transduction methods used in basic research and in clinical trials. It will be important to elucidate the long-term engraftment potential of these cells in immuno-compromised mouse models and clinical trials.

Chapter 7

In vivo analysis of techniques used to transduce hematopoietic stem cells

7.1. Introduction

The engraftment capacity of transduced hematopoietic stem cell isolates plays a significant role in the success of gene therapy procedures (Watts et al., 2012). Factors that affect the engraftment capacity include the number of transduced cells as well as the capacity of the cells to engraft (Müller et al., 2008). This is even more important in gene therapy trials where the transgene does not provide the cells with a selective advantage over surrounding cells (Aiuti and Roncarolo 2009; Fischer, Hacein-Bey-Abina, and Cavazzana-Calvo 2010; Liu et al. 1999; Sidransky, LaMarca, and Ginns 2007). Transduction protocols used in clinical trials make use of conditions that include 20% $O_{2;}$ however, the O_2 in the endogenous niche of the HSCs is lower than 3% $O_{2.}$ The routine use of 20% O_2 activates genes that initiate differentiation which in turn causes the cells to lose their ability to engraft (Cheng et al. 2000; Cipolleschi, Sbarba, and Olivotto 1993; Guitart et al. 2010; Hermitte et al. 2006; Wilson and Trumpp 2006). Although cytokine mixtures, Retronectin and lipofection have been used to improve transduction (Chono et al., 2001; Larochelle et al., 2014), few attempts have focused on improving stem cell preservation or expansion during the transduction process (Moore and MacKenzie 1999). Proposed protocols include a 24 hour prestimulation period, a split transduction period of 48 hours and an expansion period of 72 hours (Hanenburg et al., 1997; Mohri et al., 1996; Verfaillie et al., 1994). This is a long period for the cells to be in culture during which they tend to lose their engraftment capacity. We hypothesize that the use of an AhR antagonist such as SR1 in the transduction medium during the transduction process will limit the expression of differentiation genes and thereby improve the engraftment capacity of the transduced cells.

The current gold standard test to identify long term (LT) human HSCs after culturing is xenografting of immune deficient mice. NOD SCID gamma mice (NSG, NOD.Cg-Prkdc<scid> Il2rg<tm1Wjl>/SzJ) were used to determine the short-term and long-term engraftment capacity of CD34+ cells isolated from umbilical cord blood and transduced at 20% O_2 with or without SR1. A lentivirus construct that makes use of EGFP as a marker was used to quantify the hematopoietic cells engrafted in the bone marrow of these mice.

7.2. Materials and methods

7.2.1. Isolation of CD34+ cells

$CD34^+$ cells were isolated from umbilical cord blood units (<24 hrs old) obtained from Netcare Femina Hospital, Pretoria (South Africa) using the indirect $CD34^+$ MicroBead kit (Miltenyi Biotech, #130-046-703). A CD34+ cell purity of >80% was obtained by passing the cells through the isolation columns (cat# 130-042-401) twice.

7.2.2. Transduction

Transduction medium contained Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% BIT9500 (Stem Cell Technologies, Vancouver, BC, Canada), 100 ng/ml SCF, 100 ng/ml G-CSF, 100 ng/ml Flt3 ligand, 10 ng/ml TPO (PeproTech, Rocky Hill, NJ, USA), and 20 ng/ml IL-3 (R&D Systems, Minneapolis, MN, USA). $5x10^4$ freshly isolated CD34+ cells were transduced in 50 µl transduction medium with virus particles to an equivalent MOI of 5 and exposed to 20% O_2 and 20% O_2 with SR1. 0.75 µM SR1 was added to selected cultures. After 18-24h, the cells were washed twice with PBS and cultured in transduction medium.

7.2.3. Engraftment assays

NOD SCID gamma mice (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) obtained from Jackson laboratories (Bar Harbor, Maine, USA) were used in this study. Mice were maintained in micro-isolator cages under specific-pathogen-free conditions and fed with autoclaved food and water. Only female mice were used in these experiments. Ethics approval was obtained from the Animal Ethics Committee of the University of

Pretoria. Busulfan (50mg/kg, Sigma Aldrich, Munich, Germany) dissolved in DMSO and diluted with PBS was administered to 5 to 8 week old female mice by a split intraperitoneal injection with a 24h delay. Freshly isolated and transduced CD34+ cells were injected 48h after the initial Busulfan injection by i.v. tail vein injection at cell numbers ranging from $3x10^3$ to $1x10^4$ or alternatively, the expansion equivalent of the transduced cells was injected. Bone marrow, peripheral blood and spleens were collected at 8 weeks after transplantation of CD34+ cells (primary engraftment). The level of chimerism was determined by staining bone marrow cells with anti-human CD45 and anti-mouse CD45 antibodies and Vybrant® DyeCyclin Ruby™ (Thermofisher, USA) and analyzing the cells by flow cytometry (Gallios, Beckman Coulter). Fifty percent of the isolated volumes was injected into a second set of mice (secondary engraftment) which were sacrificed at 8 weeks post transplantation for the collection of bone marrow, spleen and peripheral blood. Limiting dilution analysis was done to determine the occurrence of positively engrafted EGFP cells in the bone marrow samples according to Hu and Smyth 2009 (Hu & Smyth, 2009).

7.2.4. Differentiation assays

Peripheral blood and spleens were collected at 8 weeks after transplantation of CD34+ cells. Red blood cell lysis was performed on the isolated cells using an ammonium chloride solution and the remaining cells were labelled with specific antibodies and Vybrant® DyeCyclin Ruby™ (Thermofisher, USA) and immune phenotyped by flow cytometry (Gallios, Beckman Coulter).

7.2.5. Statistical analysis

Data is presented as mean ± standard deviation for replicate experiments. Welch's t-test was used to determine statistical significance for unpaired data using GraphPad Prism 5.04. Statistical significance is indicated on the graphs as follows: (*) $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.0005$ and (****) $p < 0.00005$.

7.3. Results

7.3.1. Establishing the effect of SR1 on the capability of transduced cells to engraft

The short-term engraftment capacity of freshly isolated CD34+ cells and cells transduced with EGFP expressing lentivirus particles in a 7 day protocol under 20% O_2 and 20% O_2/SR1 is portrayed by the percentage human CD45+ cells (Figure 23). The cell dose of uncultured CD34+ umbilical cord blood cells as well as the equivalent starting dose and dose after expansion are indicated in Table 9. An incremental increase in engraftment was observed in mice that received $3x10^2$, $1x10^3$, $3x10^3$ or 10^4 freshly isolated cells. The engraftment levels of the cells transduced under 20% O_2 and 20% O_2/SR1 compared to the same number of uncultured cells are significantly higher due to the expansion that took place during culturing. The percentage of EGFP positive CD45 cells, obtained in mice transplanted with an equivalent number of cells which were transduced in the presence of SR1, is significantly higher than in mice transplanted with the same number of cells transduced without SR1. This was observed for all the tested cell doses (Figure 23).

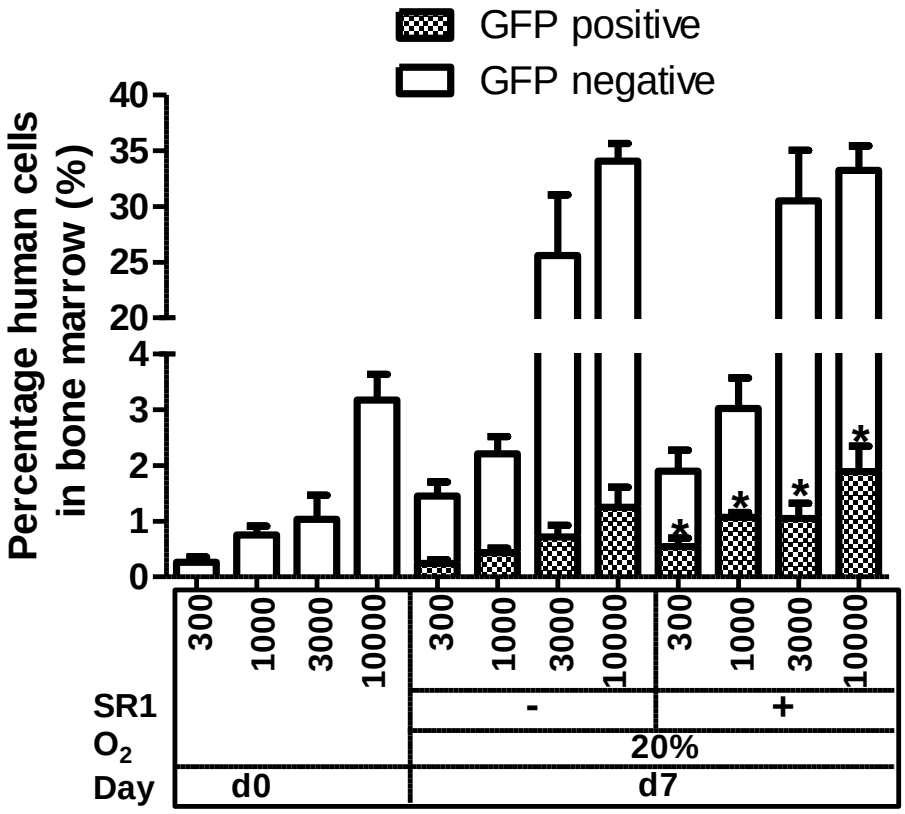

Figure 23. Engraftment of hematopoietic stem cells transduced with lentivirus particles expressing EGFP. The percentage EGFP+ and EGFP- human CD45+ cells compared with mouse CD45+ cells is indicated for mice transplanted with treated and untreated cells. The number of mice in each group is 5. The first four columns indicate mice transplanted with 300 to 10 000 freshly isolated untransduced cells, and the other columns indicate mice transplanted with 300 to 10 000 cells transduced with EFGP expressing lentivirus vectors in the absence or presence of SR1 as indicated. Statistical significance is indicated in the graph as described on page 85

Table 9 Cell dose of transduced HSCs and rate of GFP positive engraftment

Conditions	Cell dose transplanted		Primary transplant	Secondary transplant
Uncultured	300		0/4	0/4
	1000		0/4	0/4
	3000		2/5	1/5
	10000		4/5	2/5
Control	Fraction of 7 day culture		Primary transplant	Secondary transplant
	Equivalent starting dose	Dose after expansion		
	300	3363	0/5	0/5
	1000	11210	2/5	0/5
	3000	33630	3/5	1/4
	10000	112100	4/5	2/5
SR1	300	3246	3/5	0/5
	1000	10820	4/5	1/5
	3000	32460	5/5	2/3
	10000	108200	5/5	3/3

The cell doses injected into ablated mice are indicated. The cell doses of the uncultured cells are provided as well as the equivalent cell dose which refers to number of cells injected if the cells had not been expanded. The dose after expansion refers to the number of cells injected after 7 days of expansion. The primary transplant column refers to the number of mice out of the injected primary engraftment mice that demonstrated the presence of >0.5% GFP positive human CD45 cells in the blood. The secondary transplant column refers to the number of mice out of the injected secondary engraftment mice that demonstrated the presence of >0.5% human GFP positive CD45 cells in the blood.

7.3.1. Establishing the effect of SR-1 on the ST-SRC and LT-SRC frequency

Mice in the primary and secondary engraftment studies that had a chimerism (hCD45%) of >0.5% were accepted as positively engrafted and are shown in Table 9. The limiting dilution graphs of the non-responding mice are shown in Figure 24A and B. Significantly higher engraftment success of EGFP positive cells was observed in the bone marrow of mice that received CD34+ cells that were transduced in 20% O_2 SR1 compared to cells that were transduced only in 20% O_2 (Table 9). The number of short-term SRC obtained and their 95% confidence intervals are shown in Table 10. The short-term SRC frequency of the EGFP positive cells obtained from the transduction process where only 20% O_2 was used is calculated to be one in every 1961 total cells. However, the short-term SRC frequency of the EGFP positive cells obtained from the transduction process where 20% O_2 and SR1 was used is calculated to be one in every 475 total cells (Table 10).

The long-term SRC frequency of the EGFP positive cells obtained from the secondary engraftment experiments indicates that one in every 6957 cells in the total cell population in the 20% O_2 condition were EGFP long-term SRC cells. However, the long-term SRC frequency of the EGFP positive cells obtained from the transduction process where 20% O_2 and SR1 were used is calculated to be one in every 3324 total cells (Table 10).

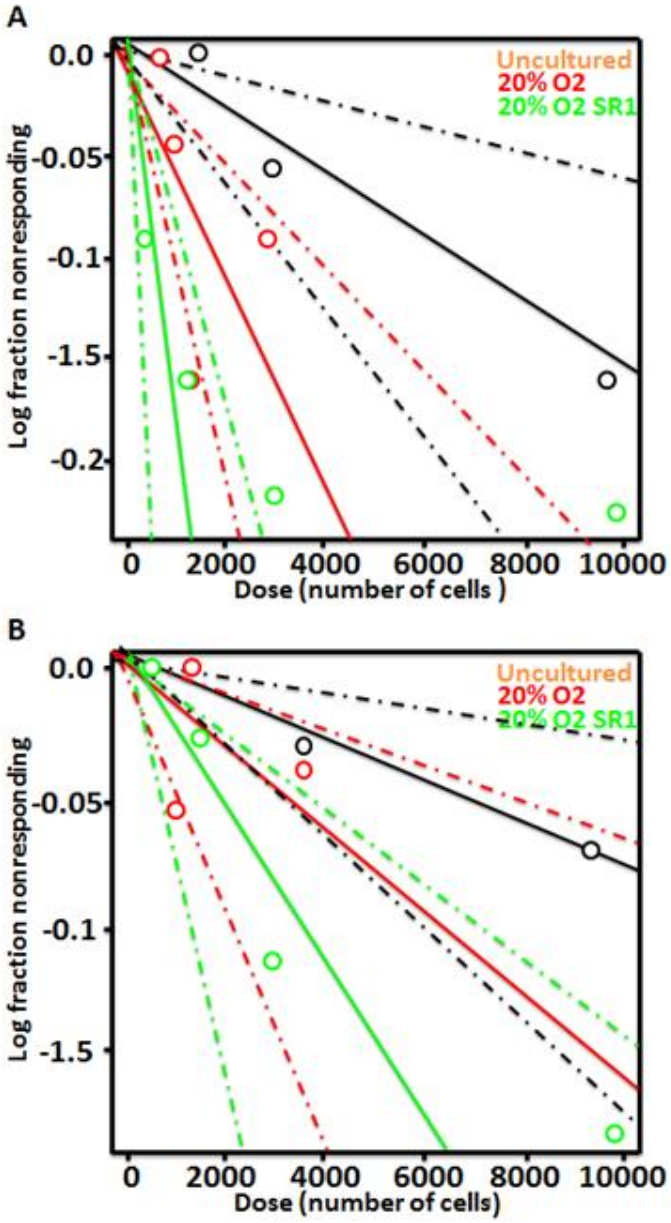

Figure 24. Limited dilution assay of GFP positive engraftment. (**A**): Linear dilution analysis of non-responding GFP positive cells that did not engraft. Uncultured as well as transduced cells were transplanted separately into NSG mice and analyzed after 8 weeks to reveal the extent of primary engraftment. (**B**): Non-responding cells that did not engraft when cells, obtained from the femurs of mice of the primary engraftment, were transplanted into NSG mice and analyzed after 8 weeks to reveal the extent of secondary engraftment of GFP positive cells.

Table 10 Short-term and long-term engraftment of transduced HSCs

Culture condition	ST-SRC frequency per starting cells	95% Confidence interval	ST-SRC frequency per total cells
Uncultured	1/7071	1/3091 - 1/16178	7
20% O2 DMSO	1/1961	1/974 - 1/3945	25
20% O2 SR1	1/475	1/209 - 1/1079	105
Culture condition	LT-SRC frequency per starting cells	95% Confidence interval	LT-SRC frequency per total cells
Uncultured	1/1967	1/6171-1/606157	25
20% O2 DMSO	1/6957	1/2213-1/21871	7
20% O2 SR1	1/3324	1/1359-1/8134	15

The ST-SRC and LT-SRC frequency per starting cells column refers to the frequency of obtaining a cell in a number of cells in a sample that is capable of contributing to short-term or long-term engraftment respectively. The 95% confidence intervals are provided and the frequencies per total cells in uncultured and expanded samples are indicated.

7.3.2. Effect of O_2 and SR-1 on the differentiation capacity of treated cells

The capacity of the transduced cells that engrafted to differentiate into the various hematopoietic lineages is shown in Figure 25A and B. Figure 25A demonstrates that the only significant difference in the differentiation capacity observed in the peripheral blood between mice that received cells expanded in 20% O_2 and cells expanded in 20%O_2/SR1 is the increased number of CD3 T cells in the 20%O_2/SR1 mice. The percentage of GFP positive CD14 and CD3 cells in the peripheral blood of mice that received cells transduced with 20%O_2/SR1 was significantly higher than mice that received cells transduced with 20%O_2. Similarly, Figure 25B demonstrates that no significant differences in the differentiation capacity can be observed in the spleen between mice that received cells expanded in 20% O_2 and mice that received cells expanded in 20%O_2/SR1. No significant difference can be observed in the percentage GFP positive human cells in the spleen.

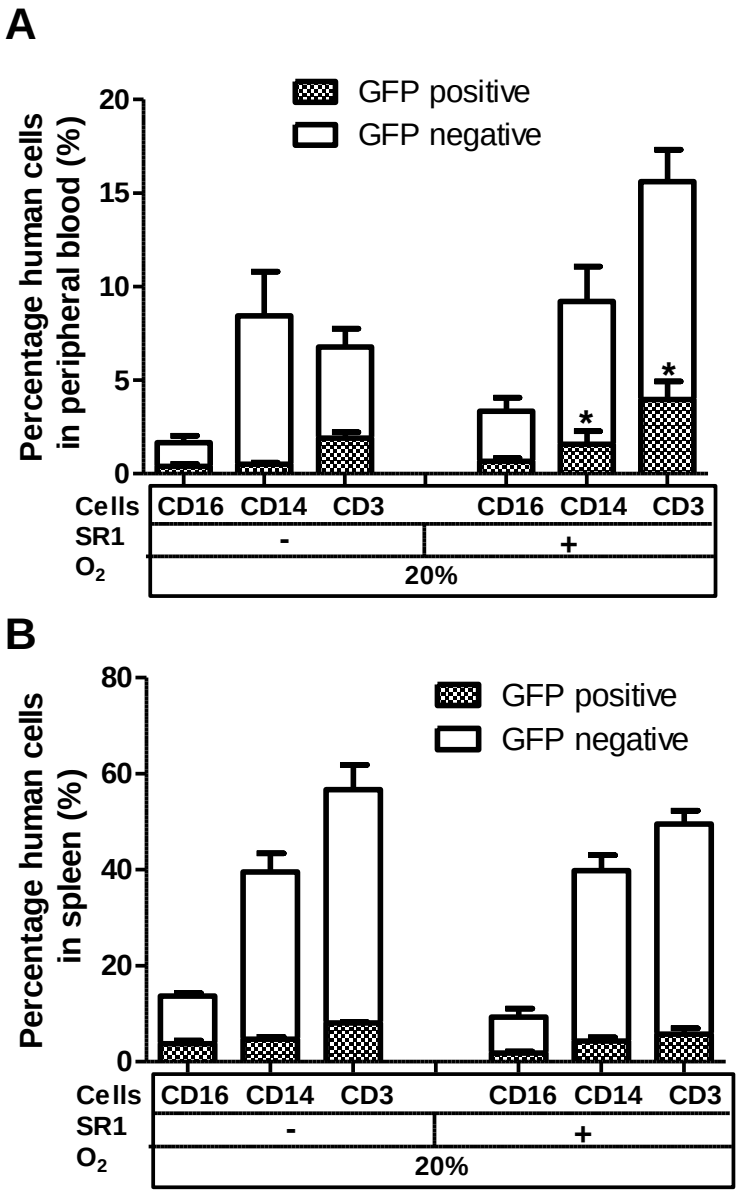

Figure 25. In vivo differentiated cells. (**A**): Percentages of T cells, granulocytes, monocytes and B cells in the human CD45 cell population of the peripheral blood are shown. (**B**): Percentages of T cells, granulocytes, monocytes and B cells in the human CD45 cell population of the spleen are shown. Statistical significance is indicated on the graphs as follows: (*) $p < 0.05$. The number of mice in each group is 5.

7.4. Discussion

Various hematological diseases can potentially be treated using gene therapy and several clinical trials have investigated strategies to accomplish this (Aiuti et al., 2013; Cavazzana-Calvo et al., 2010; DiGiusto et al., 2010). The nature of each disease influences the strategy used to obtain a desired therapeutic effect. A gene therapy that transduces HSCs with a therapeutic gene which provides the HSCs with a survival advantage does not need very high engraftment rates (Aiuti and Roncarolo 2009; Fischer et al. 2010). The transduced cells will out-compete non-transduced cells and repopulate the bone marrow. A gene therapy which transduces HSCs with a therapeutic gene, that does not provide the HSCs with a survival advantage, needs high long-term engraftment rates to ensure that therapeutic levels of the expressed protein can be obtained (Liu et al. 1999; Sidransky et al. 2007). A gene therapy with a well-designed vector construct will not be able to provide a therapeutic effect if the modified cells cannot engraft. It is thus important to investigate techniques that can improve the ability of transduced HSCs to engraft. Many techniques have been investigated in previous studies to improve the transduction rate of HSCs with variable degrees of success (Liu et al. 2009; Uchida et al. 2011). However, only primitive HSCs are capable of providing a long-term therapeutic effect and therefore need to be focused on. The transduction of primitive HSCs is influenced by many factors including the cycling state of cells. Thus, lentivirus vectors are capable of transducing quiescent cells; however, this occurs at lower rates than cycling cells (Davy and Doorbar 2007; Zhao and Elder 2005). The primitive HSCs are quiescent and need at least 24h to start cycling (Moore and MacKenzie 1999). During the transduction procedures that can take up to 7 days, these primitive HSCs start differentiating and lose their engraftment capacity.

Of the various techniques that have been investigated for the expansion of HSCs, the use of SR1 is one of the most promising (Boitano et al., 2010). The engraftment of NSG mice (Ishikawa et al., 2014) was used to determine if the addition of SR1 to the transduction medium during the transduction period could increase the engraftment capacity of the transduced cells. The percentage total human CD45+ cells in relation to mouse CD45+ cells in the bone marrow of transplanted mice was determined. This indicated that the percentage of transduced cells in the bone marrow of mice transplanted with cells transduced in the presence of SR1 is on average 2.12 fold higher compared to mice transplanted with cells transduced without SR1. The transplantation success rates indicate that the use of SR1 in the transduction protocol increased the ST-SRC rate 4.2 fold and the LT-SRC rate 2.2 fold. EGFP cells of the 20% O2/SR1 sample were also observed in the differentiated cells demonstrating their ability to reconstitute the immune system, and higher levels of genetically modified immune cells were observed. The engraftment of NSG mice demonstrated that the use of SR1 in the transduction medium does improve the engraftment capacity of transduced cells. This finding will benefit the field of gene therapy as it will help to overcome the limitation experienced in terms of the low engraftment success of transduced HSCs.

7.5. Conclusion

The use of SR1 in the transduction medium during the transduction period increased the engraftment capacity of transduced cells. The higher percentage of genetically modified immune cells produced by the transplanted cells has the potential to provide a better therapeutic effect.

Chapter 8

Opportunities and barriers to establishing a cell therapy programme in South Africa

8.1. Introduction

South Africa, based on the World Economic Forum (WEF) 2011-2012 Global Competitiveness Index (GCI) evaluation (Schwab, 2011), is ranked 50th out of 142 countries and 66nd with respect to GDP per capita (USD 7 158). However, when one breaks this down into individual components, South Africa's ranking drops to 131 in the health sector, and to 111 regarding the availability of scientists and engineers (Table 11) (Schwab, 2011). It is apparent that South Africa as a country faces significant challenges in the areas of health care and scientific capacity. This chapter focuses on how the health care needs of the country could be addressed from a biotechnology perspective, with particular emphasis on cell-based therapy which includes, but is not limited to, stem cells.

Cell-based therapy has been used successfully for several decades for the treatment of an ever-expanding number of hematological and other disorders (Medical Research Council of the United Kingdom, 2012). This includes the use of human cells, tissues, or cell and tissue-based products in clinical research/practice (U.S. Government printing office, 2012). Patients in developing countries have limited access to cell therapy as a therapeutic option. The first priority in developing countries is to ensure that basic medical services are established and maintained (Department of Health, 1997); the need for specialized forms of treatment such as cell-based therapies is therefore questionable (Department of Health, 2004). This is exemplified in South Africa by the resources required to manage infectious diseases including human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and tuberculosis (Bell et al. 2003), which draws resources away from specialized treatments (Bollinger & Stover, 1999) as it requires prolonged and costly

patient care. South Africa also has a high prevalence of non-communicable diseases including cardiovascular diseases and diabetes (World Health Organization 2011). By providing cost-effective life-long cures for chronic diseases (communicable and non-communicable), stem cell therapy could reduce the dual disease burden and free up resources that are currently used for chronic patient care (Chhabra et al. 2009). Economic growth could also be stimulated by a cell therapy programme, as has been the case in industrially advanced countries where cell therapy is an important income generating component of the health biotechnology sector (UK Stem cell Initiative, 2005). Cell therapy is a high-risk venture and is still in its infancy in South Africa (Price Waterhouse Cooper, 2010). It is therefore critical to identify whether the current situation in South Africa is suitable for the development of a cell therapy programme.

Table 11 World Economic Forum Global Competitiveness Index estimates for 2011-2012

Sub index	Factor	Rank (out of 142)
	Overall ranking	**50**
A	Basic Requirements	85
	Health and primary education	**131**
	Business impact of HIV/AIDS	132
	Tuberculosis incidence	141
	HIV prevalence	139
	Business impact of tuberculosis	135
	Life expectancy	130
	Infant mortality	111
	Business impact of malaria	103
	Malaria incidence	90
C	Innovation and sophistication factors	39
	Innovation	41
	Availability of scientists and engineers	**111**

Data extracted from the WEF Global Competitiveness Report 2011-12.

8.2. Opportunities

8.2.1. A strong market pull

Although South Africa is advanced in stem cell technologies compared to other African countries (Kassah, 2011), the availability of stem cell therapies is still limited. No public cord blood bank is active in South Africa although there are two private cord blood banks; also the bone marrow registry is demographically misrepresented (Heyns, 2007), which provides minimal access and benefit to the majority of the country's population. Hematologists and medical centres that provide stem cell therapies are mainly situated in Gauteng and the Western Cape and are only available from private institutions (Netcells, 2011). There is a strong appeal to make the available stem cell therapies more readily accessible for the currently unmet clinical needs and to develop novel therapies for diseases which burden South Africa.

Despite the fact that a significant proportion of the national budget is allocated to the management of HIV/AIDS and tuberculosis, their impact on mortality and morbidity in South Africa remains high. The group most affected by these diseases is the economically-active sector of the population (Erasmus & Nkau, 2003). Although infectious diseases are currently the most important contributor to South Africa's heavy burden of disease (World Health Organization, 2004), the World Health Organization Global Burden of Diseases Report of 2004 predicts that the relative contribution of infectious diseases in low and middle income countries will drop as we approach 2030 and that the contribution of cardiovascular and other non-communicable diseases will increase (World Health Organization, 2004). On the

basis of the current market needs in South Africa and these predicted changes, cell-based therapy will need to address infectious diseases as a short term priority, and lifestyle diseases as a long term priority. The specific fields of future interest for South Africa include: hematopoietic stem cell transplantation as a second line treatment for leukemia and other hematological malignancies (Gunnellini, Emili, Coaccioli, & Liberati, 2012), hematopoietic transplantation of engineered autologous cells to provide resistance to HIV/AIDS (Kiem et al. 2012), mesenchymal stem cell transplantation for cardiovascular regeneration (Elnakish et al., 2012) and the treatment of diabetes (Pileggi, 2012). The scope of the disease burden is large and a well-developed strategic plan is therefore needed.

8.2.2. Innovative capacity

For a cell therapy programme to address these needs, a significant amount of innovation is required. Innovation is not just the discovery of a novel solution to a problem through the successful development of a product or service, but if an innovation is to be dynamic it needs to be driven from several perspectives. These include institutions that facilitate the creation, dissemination and use of novel and economically useful knowledge (Nelson and Nelson 2002; Thorsteinsdóttir et al. 2004) (Figure 26). Research and educational institutions need to provide optimal support for health-related research, and long term investment from stakeholders is essential. This needs to be combined with innovative firms that develop new health products or innovative processes which integrate various types of knowledge. Governments are responsible for supporting health biotechnology by setting up institutions, developing policies and providing legal-regulatory frameworks such as

those dealing with intellectual property (IP) (Nelson and Nelson 2002). South Africa has a GCI ranking of 46 out of 142 for innovation with “government’s procurement of advanced technological products” being a major disadvantage (Schwab, 2011). Inter-institutional/departmental collaboration and the understanding and fulfilment of each institution’s task in serving a common purpose, is a challenge that needs to be met (Thorsteinsdóttir et al., 2004). The recognition of this shortcoming provides an opportunity for the procurement of future advanced technological products, including cell-based therapies.

8.2.3. Recognition of cell therapy’s potential

Cell therapy is on the brink of expanding rapidly as potential therapies, once validated through clinical trials, become available in the clinic. This is evident from the large number of investigations that use stem cells of various types in clinical trials involving a wide range of disorders (Figure 27) (www.clinicaltrials.gov). For example, certain autologous cell products are in late clinical trials or are already in clinical use internationally (Medical Research Council of the United Kingdom, 2012) including adipose derived stem cells used for reconstructive breast surgery (BlueCross BlueShield of North Carolina, 2013), chondrocytes and mesenchymal stem cells for cartilage repair (Melero & Al-Rubeai, 2007; Stuart, 2009), hematopoietic stem cell transplants as the standard second line treatment for lymphoma (Gunnellini et al., 2012), keratinocytes and mesenchymal stem cells used for burn wounds (Arno et al., 2011), satellite cells for skeletal muscle regeneration (Tedesco, Dellavalle, Diaz-manera, Messina, & Cossu, 2010), and bone marrow stem cells applied to denuded donated trachea for airway replacement (Elliott et al., 2012). It is important for South

Africa to strategically position itself to benefit from future therapies as they become available. An accredited clean room facility is an important component of a cell therapy programme, and is used for the isolation, expansion and manipulation of stem cells. A cell therapy programme should also include entities involved in the donation, procurement, testing, preservation, storage and distribution of human tissues and cells (European Commission, 2004). The program will be able to co-ordinate the translation of stem cell research into cell based medicinal products for patient-enrolled clinical trials and therapies.

The need for a public cord blood bank, which will provide cord blood units to individuals of all ethnic groups in South Africa, is well recognized and is being investigated (Crookes et al. 2007). Private umbilical cord blood stem cell bank services exit in South Africa, but are controversial due to the negligible recall rate of stored samples that occurs only in very rare cases when the donor or more often direct family needs the cells (Chima & Mamdoo, 2011). On the contrary, a public bank is universally accepted and would assure availability of stem cells to any histocompatible individual in the population or abroad (Pepper 2010).

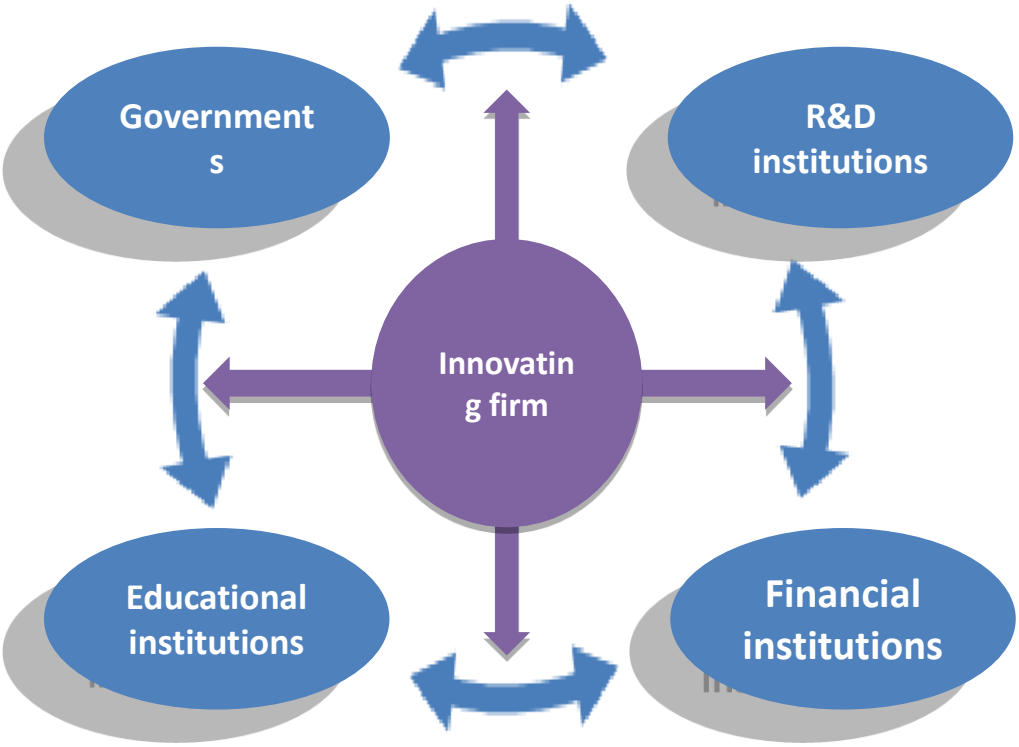

Figure 26. Main institutions in the innovation process.

With permission from Thorsteinsdóttir et al., 2004 (Pileggi, 2012)

8.3. Weaknesses

8.3.1. Lack of human resources

Human capital is an important catalytic ingredient for innovation (Kahn et al. 2004) and for the provision of health care services (Department of Labour South Africa, 2008). Specialists are necessary to drive the knowledge economy, and tend to move easily between countries due to the great global demand (Kahn et al. 2004). The highest rates of economic growth and the fastest developments in science and technology occur in countries with the greatest intellectual resources (Iredale, 2011). South Africa loses 40 000, mostly skilled people per annum to international countries, and gains 11 000, mostly unskilled people per year from poorer African countries (Rogerson, 2007). South Africa also faces major health care problems including an inequitable distribution and a significant attrition of trained health personnel. It is a common phenomenon that skilled health personnel migrate to richer countries and South Africa has not been excluded from this “brain drain” in the past (Rogerson, 2007), losing an estimated 3600 health personnel per annum. As a result, the country is forced to recruit expensive foreign expertise or to accept poorly trained workers from poorer countries (Bohlman, 2010; Iredale, 2011; IRIN, 2002; Padarath et al., 2004). This could affect the successful establishment and maintenance of a cell therapy programme, due to the lack off, for example of clinicians (hematologists and oncologists) and skilled scientists and laboratory personnel. There are currently around 25 clinical hematologists in South Africa, most of whom are in the private sector. This is equivalent to a ratio of 1 hematologist per two million people. In a country such as the United Kingdom, the ratio is 13.2 per million (Pool, communication with authors, 2011). Even though South Africa has high

quality scientific research institutions (Schwab, 2011), the inability to retain expertise for the development of novel technologies including cell therapy, is the first and perhaps the most important barrier to establishing cell therapy as a discipline in South Africa. It is therefore necessary to provide conditions and incentives for skilled personnel that will encourage them to remain and work in the country (The Presidency of South Africa, 2007). South Africa has implemented a policy which aims to recruit highly qualified professionals in the health care sector to overcome these shortcomings (Department of Health, 2010).

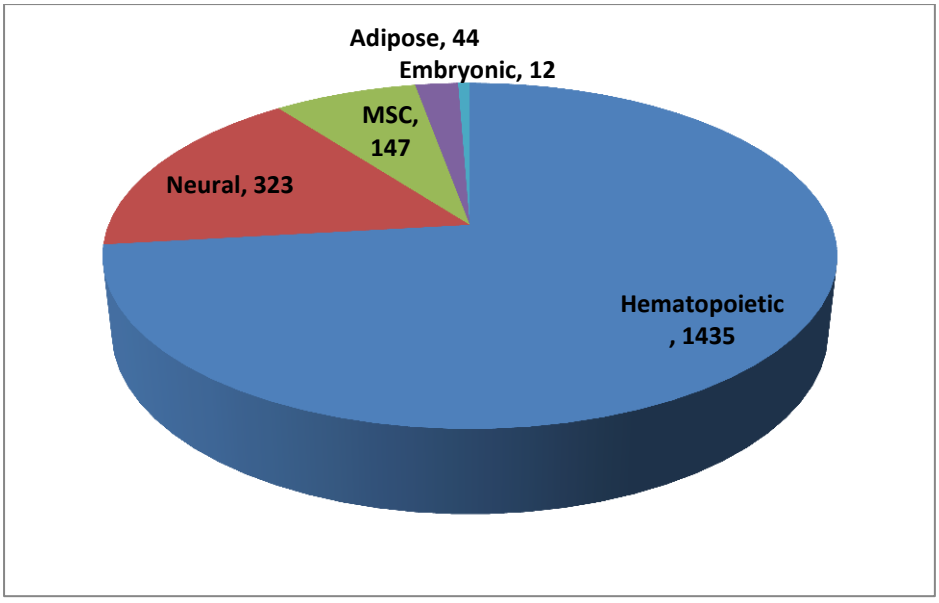

Figure 27. The numbers of open clinical trials currently underway in which hematopoietic, neural, mesenchymal, adipose, and embryonic stem cells are being used. Data from www.clinicaltrials.gov, last accessed 28-11-2015.

8.3.2. Barriers affecting successful innovation

An often spoken about weakness in the innovation / commercialization chain is the so-called “innovation chasm” or the “valley of death”. This refers to the inability of academic research to reach the market as products and services. The National Biotechnology Strategy (NBS), which was adopted in 2001, has the aim of inducing an entrepreneurial culture and innovation. This will soon be replaced by a new Bio-economy Strategy, which has built on the experience of the past 10 years (Gastrow, 2008). In this time, the National Department of Science and Technology has put into place several instruments to bridge the ”innovation chasm”, the most recent of which are the Technology Innovation Agency (TIA) and the National Intellectual Property Management Office (NIPMO) (Moore 2009). However, in terms of tangible outputs such as biotechnology firms, employment, patents and commercialization revenues, a limited amount of progress has been made over the past decade (OECD, 2007). One of the reasons for this lack of growth may be the lack of increase in the number of projects entering the innovation pipeline, which for all intents and purposes is “empty”. This might in part be attributed to higher education institutions and funding agencies that do not implement the guidelines of the NBS (OECD, 2007). The government implemented the new Intellectual Property Regulations from Publicly Funded Research and Development (IPR-PFRD) Act in 2010 to promote a commercial outflow from research projects (Presidency, 2008) and to drive a better return on publically-funded research investment (Moore 2009). Although there has been criticism that the Act could negatively affect academia, intra-firm research alliances and international research collaboration (Staphorst, 2010), it is too early to determine whether this is the case.

8.4. Threats

8.4.1. Limited funding

South African companies have to compete in the international arena and need to develop new products with substantially less capital (Sherwin, 2011). Government expenditure on research and development (R&D) was R21 billion according to the 2008/2009 R&D survey (Labadarios et al., 2007) and although few in number, funding bodies that fund biotechnology companies do exist, including the Industrial Development Corporation and the TIA (Technology Innovation Agency, 2012). Despite their best intentions, they still leave a major funding gap (Al-bader et al., 2009) which in some cases can be bridged by venture capital. The capital available from venture capital firms for biotechnology is, however, limited but interest in the field is emerging. Bioventures (Northlands, South Africa) has successfully supported several biotechnology projects in South Africa such as Rysen Biosciences (Sherwin, 2011). At this stage local funding is insufficient for the process of product development and foreign funds are needed. The latter will in part depend on the global perception of South Africa's biotechnology capacity.

8.4.2. Ethics and Education

Aside from technical, political and financial challenges, cell therapy does provoke strong ethical and cultural concerns, due in part to ignorance and a lack of education (Pepper, 2010a). The South African bone marrow registry contains more than 65 000 entries (Heyns, 2007), which is largely represented by Caucasians (Heyns, 2007). This is of concern as the non-Caucasian population is largely underrepresented and is therefore unable to benefit fully from the therapeutic benefit that bone marrow

transplantation can provide (Anglo American, 2012). This imbalance might in part be historical but is also the result of a lack of education regarding donation and the advantages thereof. Also, many people in South Africa have attached cultural and religious beliefs and norms to various body parts, and are reluctant to donate or receive stem cells (Davis and Randhawa 2006). This should be taken into account when setting up a cell therapy programme, since it might influence public support and therefore the market. Obtaining and maintaining an adequate number of cord blood samples for a public bank that is representative of the total population, could be challenging. The Public Understanding of Biotechnology programme was created to inform the public of biotechnology opportunities in South Africa, and also to inform people about biotechnology products that might affect them (Department of Science and Technology, 2007). News and scientific articles have given people false hope and understanding of current stem cell therapies and as a result desperate patients are enticed to spend large sums of money on unproven stem cell treatments (Pepper 2010a). South Africa has not been spared in this respect, and needs to prevent these exploitative practices before they hamper local public perception and funding opportunities (Mahomed & Slabbert, 2012). There is thus an urgent need for a national information centre that can provide information concerning public understanding of the present and future applications of stem cells.

8.4.3. Inadequate regulatory environment

A major concern is that the cell therapy environment is in part regulated by legislation which is incomplete, redundant and in some cases scientifically flawed. The scientific landscape at the time of the drafting the legislation did not require

carefully balanced rules and guidelines for complex and controversial issues regarding human stem cells (Pepper 2009). Thus although some aspects of cell therapy appear to be adequately regulated (Jordaan 2012), the absence of stem cell regulations in other areas and the inability to enforce existing legislation means that patients do not enjoy protection and are exposed to untested and unsound treatments (Pepper 2012). Although recently enacted, an urgent revision of the National Health Act (NHA) is required, particularly as far as cell based therapies are concerned (Pepper 2012). Several physicians are already practicing cell therapy in South Africa, and there is a need for these practices to be regulated. The immediate establishment of a national regulatory body is essential for the protection of patients and guidance of the development of cell therapy in the country. One of the body's main roles would be to provide best practice guidelines and training for physicians. These guidelines could be drafted on the basis of international best practice standards. The body could thus promote access to cell therapy, while at the same time protecting patients from "bogus therapies" (Ledford, 2010).

8.4.4. Market barrier

Will private individuals or the government be able to pay for the cost of cell therapy? The uptake of cell therapy will depend on several factors which include cost, public perception and the regulatory environment. It is possible that treatments will initially not be paid for by the state or covered by medical aids, and that patients will have to pay out-of-pocket. It is likely therefore that cell therapy will initially not be accessible to everyone, but that this will become more affordable over time. The decision of medical aids to cover a new treatment is based on the clinical effectiveness and

safety of the treatment. In the case of higher cost treatments, financial cost-sharing mechanisms would have to be formulated between the insurance company and the company providing the service (Fulton, Felton, Pareja, Potishcman, & Scheffler, 2009). It is expected that the main portion of the cell therapy market could therefore initially be the private sector (Lehohla, 2009). The National Department of Health is struggling to provide basic care with its allocated budget, and at least initially, would simply not authorize the payment of specialized treatments of this nature (Lehohla, 2009). However, a convincing motive to fund cell therapy is the possibility of obtaining a once-off treatment, with a significantly lower overall cost when compared to conventional long term patient care (Bollinger & Stover, 1999). As an example, HIV/AIDS is the main contributor to the disease burden and therefore constitutes the main domestic market, and cell therapy treatments for this disease could establish a good market-related foothold in South Africa (Kiem et al. 2012).

8.5. Strengths

8.5.1. Public sector initiatives

Although the local biotechnology industry is small by international standards, it has significant potential. A number of structures and interventions aimed at strengthening the biotechnology sector have been implemented, including as mentioned previously, the TIA and the NIPMO. Incubators have also been created to facilitate the innovation process by supporting the development of entrepreneurial companies with both technology and business incubation. The Innovation Hub, Africa's first internationally accredited Science Park, was created with the goal of assisting at least 20 biotechnology companies with the commercialization of life sciences research, products and services (Khumalo, 2007). The International Centre for Genetic Engineering and Biotechnology (ICGEB) which is dedicated to biotechnology, was opened in Cape Town in 2007 (South African Government, 2007). It is an inter-governmental centre of excellence for research and training in biotechnology and genetic engineering, which focus on the needs of the developing world (Ripandelli, 2008). Hosting the facility has established the country as a preferred destination for science and technology initiatives, and has helped to attract highly skilled scientists to Cape Town ("Global Biotech Lab Opens in SA – SouthAfrica.info," 2007). Health-specific biotechnology innovations are also harnessed to address public health problems, such as the development of vaccines through the South African AIDS Vaccine Initiative (Cape Town, South Africa) (Motari et al., 2004). In the health biotechnology sector, stem cell research, banking, and therapy are already taking place in South Africa. Two private cord blood stem cell banks exist, namely Cryo-Clinic and Netcells Biosciences. The acute need for a

public stem cell bank has been recognized and a feasibility study is underway even though it demands significant capital investment and it is not a health priority given the heavy burden of communicable and non-communicable diseases (Jordaan, Woodrow, and Pepper 2009).

Although the South African government has identified the biotechnology sector as an important contributor to economic growth and the well-being of all South Africans, no national initiative has yet been undertaken to develop cell therapy in the country. A carefully structured strategy must be put in place to overcome the identified barriers. According to the United Kingdom stem cell initiative report (UK Stem cell Initiative, 2005), this type of venture needs to be a government initiative in order to be successful. It is recommended that a cell therapy initiative should be comprised of higher education institutions, research councils, private funding bodies, and a specialized research ethics committee. Further responsibilities include the establishment of public-private partnerships and co-ordinating bodies to support research, and the development of a program of public dialogue (UK Stem cell Initiative, 2005). If such a strategy can be implemented it will support the potential ability of South Africa to establish a successful cell therapy programme and to attract foreign investment.

8.6. Conclusion

Cell therapy could provide South African patients with a suitable alternative to conventional treatments and is likely to have a positive impact on the country's economy. This is a high profile venture that requires innovation and careful planning and implementation. It is not practical to consider all possible cell based therapies in the light of the regulatory, staffing and resource limitations. South Africa, as a developing country, has many strengths but also has barriers which need to be overcome if a thriving cell therapy industry is to be developed. The government has launched several major strategic initiatives to remedy the shortcomings in the biotechnology sector, but lacks a focus on cell therapy. We recommend the establishment of a national cell therapy programme which would be dedicated to the coordination of development of stem cell research and therapy. This group should be responsible for making the available treatments more accessible in the short term, mainly by establishing clinical hematological facilities in collaboration with universities and thereby training clinical hematologists. It could assist the bone marrow registry to improve the demographic representation of the registry. It should regulate cell therapy treatments and clinical trials in collaboration with partners in the international stem cell arena and also serve as a connection point to provide education and information to the public regarding stem cell treatments. The national cell therapy program's long term strategy should be to facilitate the development of locally-inspired stem cell solutions. Setting up a cell therapy programme in South Africa at this time is important, but will require strong leadership and the co-ordinated efforts of role-players in several sectors to initiate clinical translation of potential to actual treatments.

Chapter 9

Conclusion

Since the first clinical trial that made use of genetically manipulated cells in 1990, gene therapy has not advanced to provide a safe and efficient treatment for any disease on a routine basis. Clinical trials have demonstrated the potential of gene therapy technology with symptoms being alleviated in a number of patients. However, the technology still faces various challenges regarding safety and efficiency. The majority of stem cell gene therapy clinical trials make use HSCs. This is due to the extensive history of HSC transplantation and the numerous hematological diseases that can potentially be treated in this way. Factors that limit the success of gene therapies include inadequate techniques to safely and efficiently suppress or express target genes, the limited number of CD34+ cells obtained from patients and the inadequate engraftment of transduced CD34+ cells.

Many clinical trials that have made use of lentivirus transduced HSCs have observed little or no therapeutic effect due to low engraftment rates. Initial batches of CD34+ cells obtained from patients have been a limiting factor and various conditions have been investigated in an attempt to expand HSCs in culture without differentiation. A low concentration of DMOG (0.1mM) was found to be sufficient to mimic hypoxic conditions and maintain HSCs in culture providing similar but not identical cellular subpopulations. Stemregenin 1 not only maintained HSCs in culture but also expanded the primitive HSC population. This study demonstrated that the expansion of HSCs in culture medium containing SR1 has the potential to alleviate limitations experienced with low initial CD34+ cell numbers and low engraftment rates. This could benefit gene therapy as a whole as well as HSC transplantations. The use of DMOG in combination with SR1 increased the proportion of primitive HSCs but did not provide additional benefit to increasing the total number of primitive HSCs.

However, the use of 1% O2 with SR1 provided similar engraftment levels compared to the use of SR1 at 20% O2 with 3.56 fold less transplanted cells. Thus fewer cells are required to observe the same effect.

The success of obtaining a sufficient number of transduced HSCs does not only depend on the initial number of HSCs, but also on the efficiency of transducing the primitive population. This study showed that techniques previously used to improve the transduction of CD34+ cells such as genistein and spinoculation, do not promote the transduction and preservation of the primitive HSC population. However, the use of SR1 resulted in a 6-fold increase in the number of transduced primitive HSCs.

The use of SR1 for the expansion of HSCs before and during transduction could overcome the lack of reported engraftment rates experienced in clinical trials. Therefore, gene therapy techniques could possibly, with the use of SR1, provide a “cure” for many diseases including HIV. The improved transduction of primitive HSCs should be confirmed with engraftment in immunocompromised mice as well as the safety of the cells using transcriptome analysis. If the engraftment of the genetically manipulated cells can be improved and a therapeutic effect obtained, HIV gene therapy can potentially provide significant relief to the HIV burden. To implement such an intricate procedure, proper coordination and infrastructure is needed. We evaluated the opportunities and barriers in South Africa regarding setting up a cell therapy platform, and we recommend the establishment of a national cell therapy programme. This program should be dedicated to the coordination of the development of stem cell research and therapy in the country.

Gene therapy has come a long way and could, with final optimization of processes and systems involved, provide a solution to various diseases, including the HIV pandemic.

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- Oncotarget Journal. Progresses towards safe and efficient gene therapy vector. S. Chira, C.S. Jackson, L. Oprea, F. Ozturk, M. Pepper, L. Diaconu, C. Braicu, L. Raduly, G. Calin, I. Berincan-Naegoe. 2015
- Cytotechnology. In vitro immunophenotypic and differentiation capacity assessment of human adipose derived mesenchymal stromal cells transduced with GFP lentiviral vectors. F.A van Vollenstee. C.S. Jackson, D. Hoffmann, M. Potgieter, C. Durandt. M.S. Pepper 2016
- Stem cell Research and Therapy. Opportunities and barriers to establishing a cell therapy programme in South Africa. C.S. Jackson and M.S. Pepper. 2013
- Springer. Clinical safety and applications of stem cell gene therapy. C.S. Jackson, M. Alessandrini and M.S. Pepper. 2016

Manuscripts in preparation

- Clinical applications of genetically modified hematopoietic stem cells. C.S. Jackson, R. Myburgh, P. Salmon, K. Krause, R.F. Speck and M.S. Pepper

- Targeting the ARNT complex with DMOG and Stemregenin 1 improves primitive hematopoietic cell expansion. C.S. Jackson, C. Durandt, P. Brunet de la Grange, I. Janse van Rensburg, V. Praloran and M.S. Pepper

- Analyzing techniques to improve the transduction of hematopoietic stem cells. C.S. Jackson and M.S. Pepper.

Conference presentations

- **ICTERM (International Conference on Tissue Engineering and Regenerative Medicine) 2014:** Targeting the ARNT complex with DMOG and Stemregenin 1 improves the expansion of primitive hematopoietic cells
- **SAGS (South African genetics society) 2014:** Improving the transduction of hematopoietic stem cells. C.S. Jackson and M.S. Pepper.
- **GRI (Genomic research institute) 2014:** Improving techniques used to expand and transduce hematopoietic stem cells. C.S. Jackson, C. Durandt, P. Brunet de la Grange, I. Janse van Rensburg, V. Praloran and M.S. Pepper
- **PathRed (Pathology Research and Development Congress) 2015:** Improving lentivirus transduction of primitive hematopoietic stem cells. C.S. Jackson and M.S. Pepper.
- SSAJRP (Swiss South African Joint Research Program): Annual Sympsium: **Improving lentivirus transduction of primitive hematopoietic stem cells.** C.S. Jackson and M.S. Pepper.
- SSAJRP (Swiss South African Joint Research Program): Annual Sympsium: **Expansion of hematopoietic stem cells.** C.S. Jackson, C. Durandt, P. Brunet de la Grange, I. Janse van Rensburg, V. Praloran and M.S. Pepper
- SSAJRP (Swiss South African Joint Research Program): Annual Sympsium: **Techniques used to provide HIV resistance to hematopoietic stem cells.** C.S. Jackson and M.S. Pepper.

Posters

- **WBMT 2014:** Targeting the HIF complex with DMOG and Stemregenin 1 mediates the improved expansion of primitive hematopoietic cells. C.S. Jackson, C. Durandt, P. Brunet de la Grange, I. Janse van Rensburg, V. Praloran and M.S. Pepper
- **WBMT 2014:** Improving lentivirus transduction of primitive hematopoietic stem cells. C.S. Jackson and M.S. Pepper.

Annexures

A 1. Introduction

Permission for the use of Figure 1 in this thesis was obtained from Terese Winslow and shown in appendix A 2. A. The information leaflet and informed consent forms used to obtain consent from pregnant mothers was approved by the human ethics committee of the University of Pretoria and shown in appendix A. 2. B.

A. 2. Contents of appendices

A. 2. A.

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17-03-2016

Author Signature (sign above line) Date

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A 2 B.

PATIENT INFORMATION LEAFLET AND INFORMED CONSENT

(Each patient must receive, read and understand this document before the start of the study)

STUDY TITLE
Isolation of haematopoietic stem cells from umbilical cord blood (UCB).

INTRODUCTION
This information leaflet is to help you to decide whether you would like to participate in a research project. Before agreeing to partake in this study, please familiarise yourself with all procedures involved and do not hesitate to ask the investigator about anything that you might be uncertain about. You should not agree to take part unless you are completely satisfied about all procedures involved.

WHAT IS THE PURPOSE OF THIS STUDY?
Researchers at the University of Pretoria would like to investigate the healing properties of cells found in the afterbirth (placenta). These cells could potentially be used to treat patients with various kinds of diseases such as HIV, cancer and blood diseases. In order to use these cells to treat humans in the future, researchers must first study these cells in a laboratory. The collection of these cells and the research will not use any unethical procedures.

WHEN AND HOW IS THE BLOOD FROM THE PLACENTA COLLECTED?
Under normal conditions, the umbilical cord and placenta is discarded after a baby is born, since they serve no further purpose for either the mother or the baby.

Researchers could however use the normally discarded blood for research purposes. After the birth of the baby, the umbilical cord is cut and cord blood will then be collected from the cord. Collecting this blood does not harm the mother or the baby in any way. The collection can only take place at the time of delivery and is performed by your doctor.

WHAT WILL BE EXPECTED OF ME?
Apart from some personal information about yourself and the baby, you will only need to give the researchers permission to collect the blood upon delivery.

All your personal information will be regarded as confidential during this study. Each participant will be assigned a specific code and this code will be the only information that the researchers will have.

You hereby give the researchers permission to request your HIV status from your doctor since it is important for our work that we only work with HIV-negative blood. If you do not wish us to know the result of your HIV test, we will not be able to include you in the study. In the case of an HIV positive result, you will be counselled by qualified medical personnel.
It might however be important for the doctors or researchers involved in this study to convey medical information to medical personnel or appropriate Research Ethics committees. In such a case, you hereby authorise your investigator to release your medical records to regulatory health authorities or an appropriate Research Ethics committee. These records will only be utilised by them in connection to them carrying out their obligations toward this study, while always acting in your best interest.

HAS THE STUDY RECEIVED ETHICAL APPROVAL?
A study protocol for this study was submitted to the Research Ethics Committee and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki, which deals with the recommendations guiding doctors in biomedical research involving humans. A copy of this may be obtained from the investigator should you wish to review it.

WHAT ARE MY RIGHTS AS A PARTICIPANT IN THIS STUDY?
Your participation in this study is entirely voluntary and you can refuse to participate or withdraw consent at any time without stating any reason. Your withdrawal will not affect your access to medical care or the quality of medical care that you or your baby will receive.

IS THERE ANY RISK OR DISCOMFORT TO MY CHILD OR MYSELF DURING COLLECTION?
No. The cord blood is collected after your baby has been born. The collection is painless, easy, and safe for you and your baby and takes about 5 minutes.

IS THERE FINANCIAL GAIN / LOSS FOR MY ACCOUNT IN THIS STUDY?
There will be no gain or loss for yourself or the baby should you participate in the study. This research could potentially lead to future profitable treatments. However you will not have access to these profits. There will be no additional financial costs for you to participate in the study.

SOURCE OF ADDITIONAL INFORMATION
If at any time you have any questions about the study, please do not hesitate to contact Mr. Carlo Jackson (082-259-4553) or Prof. Michael Pepper (012-319-2190).

For information regarding the collection procedure, you can contact your doctor.

CONFIDENTIALITY

All information obtained during the course of this study is strictly confidential. Data that may be reported in scientific journals will not include any information which identifies you or your baby in this study. Only anonymous details which include date of birth, ethnicity and gender will be used in this study.

INFORMED CONSENT

I hereby confirm that I have been informed by the investigators, Dr. Chrisna Durandt, Mr. Carlo Jackson or Prof. Michael Pepper, about the nature, conduct, benefits and risks of this study. I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the study.

I am aware that the results of the study, including anonymous personal details regarding my ethnicity, gender, age, and date of birth will be processed into a study report.

I hereby give the researchers permission to request your HIV status from your doctor since it is important for our work that we only work with HIV-negative blood.

I may, at any stage, without prejudice, withdraw my consent and participation in the study. I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Patient's name _____ (Please print)

Patient's signature _____ Date _____

I herewith confirm that the above patient has been informed fully about the nature, conduct and risks of the above study.

Investigator's name _____ (Please print)

Investigator's signature _____ Date _____

Doctor's name _____ Please print)

Doctor's signature _____ Date _____

A 2 C.

ETHICS APPROVAL

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

* FWA 00002567, Approved dd 22 May 2002 and Expires 13 Jan 2012.
* IRB 0000 2235 IORG0001762 Approved dd Jan 2006 and Expires 13 Aug 2011.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 11/10/2010

PROTOCOL NO.	**89/2010**
PROTOCOL TITLE	Rendering the Immune System Resistant to HIV
INVESTIGATOR	**Principal Investigator:** Prof. Michael S. Pepper
SUBINVESTIGATOR	None
SUPERVISOR	None
DEPARTMENT	**Dept:** Immunology **Phone:** 012-420-5317 **Mobile:** 072-209-6324 **E-Mail:** michael.pepper@up.ac.za
STUDY DEGREE	Grant
SPONSOR	None
MEETING DATE	26/05/2010

The Protocol and Informed Consent Document were approved on 29/09/2010 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid until the end of December 2012, and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female)BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female)BA et Scien, B Curationis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Prof VOL Karusseit	MBChB; MFGP(SA); MMed(Chir); FCS(SA) - Surgeon
Prof JA Ker	MBChB; MMed(Int); MD – Vice-Dean (ex officio)
Dr NK Likibi	MBBCh – Representing Gauteng Department of Health)
Prof TS Marcus	(female) BSc(LSE), PhD (University of Lodz, Poland) – Social scientist
Dr MP Mathebula	(female)Deputy CEO: Steve Biko Academic Hospital
Prof A Nienaber	(female) BA(Hons)(Wits); LLB; LLM(UP); PhD; DipLDatametrics(UNISA) – Legal advisor
Mrs MC Nzeku	(female) BSc(NUL); MSc(Biochem)(UCL, UK) – Community representative
Prof L M Ntlhe	MBChB(Natal); FCS(SA)
Snr Sr J Phatoli	(female) BCur(Eet.A); BTec(Oncology Nursing Science) – Nursing representative
Dr R Reynders	MBChB (Prét), FCPaed (CMSA) MRCPCH (Lon) Cert Med. One (CMSA)
Dr T Rossouw	(female) M.B, Ch.B. (cum laude); M.Phil (Applied Ethics) (cum laude), MPH (Biostatistics and Epidemiology (cum laude), D.Phil

DR R SOMMERS; MBChB, MMed(Int), MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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Animal Ethics Committee

PROJECT TITLE	Engraftment of treated hematopoietic stem cells into NOG mice
PROJECT NUMBER	H008-13
RESEARCHER/PRINCIPAL INVESTIGATOR	Mr. C Jackson

STUDENT NUMBER (where applicable)	27458599
DISSERTATION/THESIS SUBMITTED FOR	PhD

ANIMAL SPECIES	Mice	
NUMBER OF ANIMALS	30	
Approval period to use animals for research/testing purposes		July 2013-December 2013
SUPERVISOR	Prof. M Pepper	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 24 June 2013
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Engraftment of lentivirus vector transduced hematopoietic stem cells into NOG mice**
PROJECT NUMBER	**H011-15**
RESEARCHER/PRINCIPAL INVESTIGATOR	**C Jackson**

STUDENT NUMBER (where applicable)	**274 585 99**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**Mice**	
NUMBER OF ANIMALS	**30**	
Approval period to use animals for research/testing purposes		**July 2013-December 2013**
SUPERVISOR	**Prof. M Pepper**	

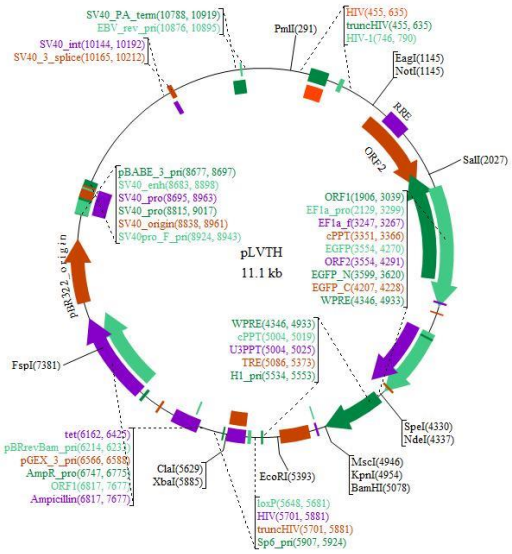
KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

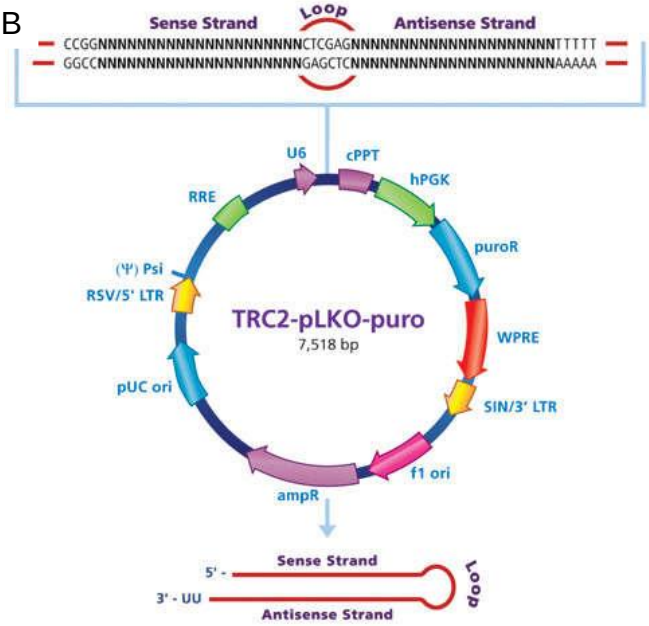
APPROVED	Date **24 June 2013**
CHAIRMAN: UP Animal Ethics Committee	Signature

A 2. D.

A



B



Vector maps of pLVTH and pTRC2-pLKO-puro. www.adgene.com, http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/library-information/vector-map.html.