CONTINUOUS PROLIFERATION AND SIMULTANEOUS MATURATION OF HAEMATOPOIETIC STEM CELLS INTO BLOOD CELL LINEAGES

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Continuous Proliferation and Simultaneous Maturation of Haematopoietic Stem Cells into Blood Cell Lineages

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By

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This thesis is the result of my own work. The material contained within the thesis has not been presented, either wholly or in part, for any other degree or qualification.

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Abstract

For decades, research has been focussed on finding a way to produce artificial blood as a resolution for the insufficient amount of blood components provided by donation and to provide a more transportable alternative with a longer shelf life. Red blood cells (RBCs) are the most common cell type in blood and are responsible for oxygen transport throughout the human body. It is therefore extremely important to find an alternative oxygen carrier whether these are tissue engineered RBCs or a chemically defined oxygen delivery system

The study conducted for this thesis was part of a larger project called Redontap, and was aimed to develop a bioreactor for the manufacture of RBCs. During this research to produce RBCs from adult stem cells *in vitro*, the main goal was to upscale haematopoietic and erythroid cultures. Understanding the biological signals and their temporal magnitude involved in the division, maturation and migration of the $CD34^+$ haematopoietic stem cells (HSCs) and their differentiated progeny would allow for a controlled continuous production of mature blood cells. The differentiation of HSCs into different blood cell types occurs within different bone marrow niches and so mimicry of the erythrocyte niche is likely to result in maximisation of the rate of red blood cell development.

Published research provides evidence that peripheral blood mononuclear cells (PBMCs), including $CD34^-$ cells, will be advantageous for erythroid maturation. For this thesis, $CD34^+$ cells were expanded within a population of PBMCs on a stromal layer to recreate a niche-like environment. This approach was also utilised with umbilical cord blood isolated MNCs (UBMCs) to compare HSC expansion potential and subsequently efficiency in erythroid maturation was analysed. Whereas the cell output was limited, differentiated cells proved positive for a range of RBC surface markers and haemoglobin content.

As part of the aim for upscaling cell culture by translating static cultures to bioreactor processes, bioreactors with volumes varying between 250mL-3L were analysed for cell retention and viability to achieve high cell densities whilst refreshing culture medium, monitoring culture parameters (*e.g.* pH, dissolved oxygen), and introducing an hypoxia environment for mimicking the *in vivo* stem cell niche. In general, this research was focussed on improving dynamic culture conditions for generating higher numbers of cultured erythrocytes than so far has been achieved.

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Contents

Abbreviations

AMSC	adipose mesenchymal stem cell
BCB	brilliant cresyl blue
BM	bone marrow
BMSC	bone marrow mesenchymal stem cell
BSA	bovine serum albumin
cDNA	complimentary DNA
CAFC	cobblestone area-forming cell
CFU	colony forming unit
CFU-E	colony forming unit erythrocyte
cRBC	cultured red blood cell
CT	Cycle Threshold
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DPSC	dental pulp stem cell
EDTA	ethylenediaminetetraacetic acid
EPO	erythropoietin
EM	erythroid maturation
ESC	embryonic stem cell
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FDA	Food and Drugs Administration
Flt3-L	flt3-ligand
G-CSF	granulocyte colony-stimulating factor
gDNA	genomic DNA
GMP	good manufacturing practice
GvHD	graft-vs-host disease
Hb	haemoglobin
HBOC	haemoglobin-based oxygen carrier
hESC	human embryonic stem cell
HPC	haematopoietic progenitor cell
HSC	haematopoietic stem cell
IGF-1	insulin-like growth factor-1
IL-3	interleukin-3

IL-6	interleukin-6
IMDM	Iscove's Modified Dulbecco's Medium
iPSC	induced pluripotent stem cell
MACS®	magnetic-activated cell sorting
MCH	mean cell haemoglobin
MCV	mean cell volume
MNC	mononuclear cell
MSC	mesenchymal stem cell
MSCGM™	mesenchymal stem cell growth medium
MWCO	molecular weight-cut-off
NHS	National Health Service
PB	peripheral blood
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PEG	polyethylene glycol
Pen/Strep	penicillin/streptomycin
PFC	perfluorocarbon
PLGA	poly(D,L-lactide-co-glycolide
PSC	pluripotent stem cells
RBC	red blood cell
RNA	ribonucleic acid
rpm	rotations per minute
RT-PCR	reverse transcription-polymerase chain reaction
SCF	stem cell factor
SEM	scanning electron microscopy
StDev	standard deviation
Temp	temperature
TPO	thrombopoietin
TRALI	transfusion-related acute lung injury
UBMC	umbilical cord blood mononuclear cell
UCB	umbilical cord blood
UI	user-interface
vCJD	variant Creutzfeld-Jacob disease
αMEM	Alpha Modification Minimal Essential Medium

Chapter 1

Introduction

1.1 Overview

The need to provide safe and functional auxiliary blood is a constant pressure for healthcare providers. It is essential for people undergoing particular medical treatments (*i.e.* heart surgery, organ transplant, and blood disorders) in order to maintain life, it is also essential in critical care and trauma to save lives. Currently, blood is provided by means of donation to blood banks. Often blood is not required in its whole form and therefore it has added value for use in multiple applications and is separated into different components (*i.e.* red blood cells, platelets, and plasma) to make more per unit of donated blood. Even this separated products approach is still not providing sufficient to keep up with the needs in medical treatments.

For decades, research has been focussed on attempting to produce artificial blood to find a resolution for the insufficient amount of blood components provided by donation and to provide a more transportable alternative with a longer shelf life. Red blood cells (RBCs) are the most common cell type in blood and are responsible for oxygen transport throughout the human body. It is therefore an extremely important quest to find an alternative oxygen carrier whether these are tissue engineered RBCs or a chemically defined oxygen delivery system. In the first scenario, these cells need to be a copy of healthy RBCs and in the development of the latter product it is essential to create a high quality product with a good shelf life under convenient storage conditions, as well as an excellent oxygen binding and release affinity. This chapter will cover a wide range of techniques to supply RBCs into the human body as an alternative for traditional blood transfusion.

1.1.1 The oxygen carrier protein: haemoglobin

Many research groups focus on novel methods to administer haemoglobin (Hb) into humans as this abundant RBC protein is of paramount importance for the supply of oxygen to tissues of the body [1]. The foremost important property of erythrocytes is critical in situations of

anaemia due to *e.g.* significant blood loss [2-4]. One major aspect of this globally widespread research is the *ex vivo* creation of artificial RBCs; but we will also address oxygen carrier systems designed for *in vivo* studies [5].

1.2 The need for tissue engineering RBCs

There is an increased need for donated RBCs, and although 92% of all RBC units that meet release criteria find a recipient, blood-banking is not tailored for massive supply and for rare blood-group units under extraordinary circumstances [6, 7]. Also, in transfusion medicine there are physical risks for the patient, arising from allogeneic transfusion and from issues regarding the storage of tissues. Currently, the major therapeutic option for oxygenating tissue is transfusion [8]. These issues may be overcome by engineering artificial red cells as an alternative solution for supplying oxygen capacity to the patient.

One of the greatest potentials for transfusion medicine can be found in the developing world where blood is scarce and hazardous. The blood products, if available, are often untested and inadequately processed, stored and transported [9]. A safe and affordable RBC substitute could have a major impact on the lives of transfusion patients.

1.3 Current issues in transfusion medicine

A number of issues arise when it comes to transfusion of blood products into patients. The main risks for the patient are allergic reaction, anaphylaxis, fluid overload, transfusion-related acute lung injury (TRALI), haemolytic transfusion reaction, bacterially contaminated blood, viral contaminated blood, variant Creutzfeld-Jacob disease (vCJD), and transfusion-associated graft-vs-host disease (GvHD) [10, 11]. A number of these risks are usually mild (allergic reaction) and very rare (bacterial infection, GvHD), the number of incidents for other risks has greatly reduced since the mid-1980s (viral infection). Introduction of pre-transfusion blood testing and risk analysis of donor behaviour contributed to this reduction of viral infection, but disease transmission may still occur during the time after infection when the donor is infectious but screening tests are negative.

Other risks on this list, such as TRALI, anaphylaxis, and haemolytic transfusion reaction compose greater, possible life threatening, danger to the patient. The haemolytic transfusion reaction, where immune antibodies in the recipient plasma interact with the surface antigens on donor RBCs, is the one of most serious of complications and may either be immediate or delayed after transfusion. These antibodies develop after a patient's exposure to RBCs expressing antigens they lack, ultimately destroying the transfused RBCs. Some haemolytic transfusion reaction result from plasma containing blood components and generally these transfusion reactions happen within two weeks [8, 11]. In general, the risks of non-infectious complications are generally well-recognised and amendable to technologic solutions. A more critical concern is whether the ageing of RBCs stored in plastic containers, as part of allogeneic transfusion, affects the morbidity and mortality of the patient [7, 12]. Koch *et al* found that for patients undergoing cardiac surgery, a significant increased risk of postoperative complications was associated when patients received RBCs that had been stored for more than 2 weeks, including the reduction of short-term and long-term survival [13].

The storage, processing and screening, of blood is the main task of blood banks and with current guidelines RBCs may be stored up to 42 days at refrigerated temperatures [7, 12, 14]. During this period, the cells undergo 'storage lesion'; a process where the morphology, biochemistry and structure of the RBCs deteriorate [9], ultimately affecting the cell function after administering to the recipient. Haemolysis is the most severe occurrence of storage lesion; the rupture of the RBCs which results in free suspended Hb and the loss of membrane-bound Hb in microvesicles could impose great risk to the recipient [15]. There is a need for prospective studies, with a large cohort of subjects, to analyse the effects of longer term RBC storage for transfused patients [14]. As part of quality control, both the Food and Drug Administration (FDA) and the Council of Europe have implemented a haemolysis standard for the licensing of RBC storage. A mean haemolysis of less than 1% and 0.8%, respectively, is the standard with the proviso of the '95/95 rule' where 95% of the units meet the standard and this is evidenced with a 95% statistical certainty [7, 15].

For each transfusion, the recipient's blood needs to be matched with the donated blood to prevent haemolytic transfusion reactions. The pre-transfusion compatibility testing is crucial and ABO and Rh D are the two antigens to be tested first to find a match. Other antigens are not routinely checked but in special circumstances these may be added to the analysis to improve the match; for example when patients have had a transfusion before. The routine for analysis is electronic as well as immediate spin/antiglobin crossmatching of recipient and donor blood and takes about 30 minutes. It is common practice that when an emergency arises, with insufficient time to perform a cross match, group O cells may be given to the patient [8]. The highest risk of complications is the mismatching of donated blood with the recipient blood and is in most cases avoidable [8, 11]. In general, immunologic incompatibility [8] and storage lesion [14] are the two major challenges in transfusion medicine.

1.4. Acellular oxygen carriers

1.4.1 Haemoglobin-based oxygen carriers (HBOCs)

Cell-free Hb causes problems (*e.g.* toxicity, vasoconstriction [16, 17]) and therefore it needs a certain degree of modification before it can be used in humans [17, 18]. A decade ago, Winslow reviewed the history of blood substitute research and overlooked the current progress on Hb solutions (chemically and genetically modified Hb), increasing oxygen affinity of Hb and the design of safe and effective solutions by increased viscosity and oncotic pressure [18]. At that time it became clear that there was no consensus on clinical applications but it was believed that the first successful products could be used in elective surgery and trauma to maintain tissue oxygenation [5, 18]. Since then a few products based on Hb-conjugated molecules have been tested during clinical trials. The benefit of using this type of oxygen-carriers is the universal compatibility with all blood groups, it is disease free (highly preferable in areas with high risk of HIV/AIDS transmission), and it has a long shelf life compared to blood products [5, 19]. One of these synthetic blood substitutes was PolyHeme, a glutaraldehyde polymerised stroma-free human Hb product, produced by Northfield Laboratories [20]. It reached Phase III clinical trials but the FDA decided that the risks outweighed the benefits and the product was rejected [21].

Another product which had slightly more success is a derivative of HemoPure (HBOC-201) and was produced by Biopure. This HBOC-201 acellular Hb product is a glutaraldehyde-crosslinked bovine Hb for intentional use in humans but after extensive testing in pigs and humans, Biopure decided that due to the adverse events no further clinical trials were warranted [20]. The Biopure-developed product Oxyglobin (HBOC-301) was approved by the FDA and the EC for veterinary use in the USA and EU [22-25]. This evidences that finding an acellular oxygen carrier system is extremely challenging but by continuing development the results so far could be a step forward in creating an all important HBOC therapy for humans.

It is important to remember that a potential clinical product of this kind will not have the same function as donated blood upon administering to patients as continued doses might induce toxic effects.

1.4.2 Perfluorocarbon oxygen carriers

Another category of chemically defined products, aiming for oxygen delivery in humans, is perfluorocarbon (PFC) based [26]. Typical for this product is the absence of Hb [27] and the relatively small size of emulsified particles (~0.2 μm) when compared with erythrocytes (~7-9 μm) [26, 28]. PFCs are synthetic fluorinated hydrocarbons and in emulsion able to dissolve (no binding) large quantities of oxygen proportional to the inspired oxygen and once entered into the blood stream capable of oxygenating local tissues. As PFCs are chemically and biologically inert, they are not metabolised but are initially taken up by the reticuloendothelial system, secreted back into the blood, and transported to the lung where they are excreted via exhalation [5, 26, 29].

To date there have been First and Second Generation PFC emulsions developed and tested. The first product that has been fully approved by the FDA for phase III clinical trial was called Fluosol-DA, developed in Japan and contributed to oxygen delivery under higher oxygen tensions. This product belonged to the First Generation PFC emulsions and 5 years after it was approved in 1989 it was withdrawn due to its limited success, complexity of use, and side effects (*e.g.* anaphylaxis, pulmonary hyperinflation) [5, 26, 30]. In 2010, Castro and Briceno reviewed a range of PFC-based oxygen carrier products and their trials. They included Second Generation PFC emulsions and defined the improvements compared to First Generations PFC emulsions: Second Generation emulsions have a significantly higher PFC content compared with First Generation emulsions, Second Generation products use natural phospholipids instead of the water-soluble emulsifier F-68, and Second Generation products can be stored without freezing [30, 31]. This generation of products have been tested in

various applications and some still ongoing (pre)clinical studies relating to brain injury, haemorrhage shock, ischemia, and cardiac surgery. Despite improvements in properties over first generation products, some second generation products still suffer from unwanted side effects. Therefore these clinical trials have been suspended and abandoned altogether or require more extensive studying before trails could be resumed [30]. There are overall more positive outcomes to discuss which demonstrates that this field is rapidly developing to facilitate the demand for oxygen delivering systems. Oxycyte (Oxygen Biotherapeurtics) is available on the market and there are still ongoing clinical trials (Phase II) for patients with traumatic brain injury [26, 32].

Positive improvements in the field of acellular blood substitutes are of the utmost importance to maintain the momentum of finding an alternative for blood transfusion. An essential part of this process is furthering the understanding of mechanisms upon administering these products to animal and human models and how to interpret the result to modify the acellular blood substitutes to develop a suitable option when current practice of blood transfusions is not possible.

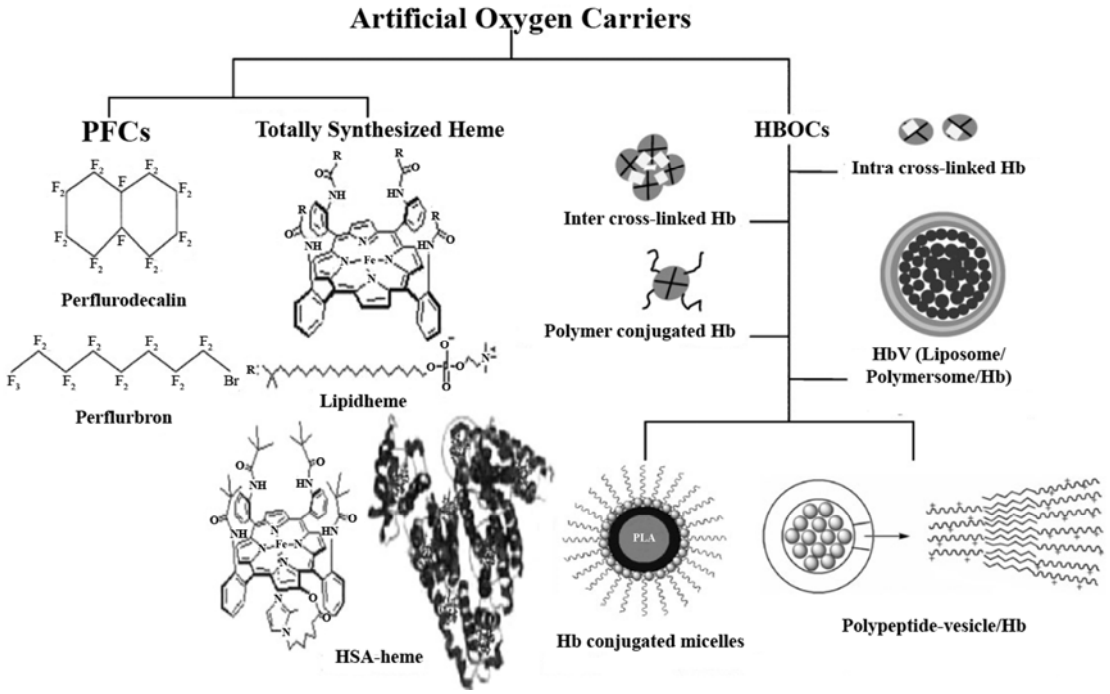

Fig. 1.1. *Overview of artificial oxygen carriers (Li et al 2011)*

1.4.3 Dendrimers and micelles oxygen carriers

Other acellular blood substitutes that are under investigation are dendrimers, biodegradable micelles and lipid vesicles. Twyman *et al* reported in 2006 and in 2012 the progression of the porphyrin-pyridine complex encapsulated by hyperbranched polymers (3,5-diacetoxybenzoic acid) as heme protein models. The Fe(II) phorphyrin is the active unit on Hb, responsible for reversible binding of oxygen. By developing this dendrimer they have shown potential for biomimetic models for use in oxygen storage and delivery [33, 34].

One type of HBOC is Hb conjugated with micelles as is described by Li *et al*. They reported the use of a triblock amphiphilic copolymer PML (PEG-b-PMPC-b-PLA). After conjugating with Hb the micelles could bind and release oxygen reversibly with the outer Polyethylene glycol (PEG) segments protecting the Hb from being attacked by the immunological system. PEG can also stabilise the particles in the aqueous medium of blood and prolong the systemic circulation of micelles [19]. The use of PEG in this type of micelle is described in

the literature [35] as is its use in HBOC lipid vesicles to improve the circulation time [36-38].

From the current literature we can conclude that major progress has been achieved but also that a stable product, highly suitable for the blood transfusion market, has not been established yet.

1.5 Cellular oxygen carriers

1.5.1 Principles of *in vitro* generation of RBCs

A completely different concept, but with the shared goal of finding an oxygen delivery system, is research into a cellular blood substitute. For more than half a century this research attempted to understand the stem cell responsible for the blood cell lineages. The haematopoietic stem cell (HSC) is studied extensively as is the role of erythropoietin (EPO) in the differentiation of HSCs into erythrocytes. The current status is that RBCs have been cultured *in vitro* for many years and these RBCs have been successfully tested in animal models. However, limitations lie within the number of RBCs that can be cultured from one unit of blood and the associated costs of these expensive cultures.

Migliaccio *et al* reviewed the history of *ex-vivo* expansion of RBCs and the principles of massive red cell amplification *in vitro*. By culturing stem cells in the presence of the essential cytokines stem cell factor (SCF) and EPO [39], unilineage production of erythroblasts can be achieved. This breakthrough, in combination with initial culture of cells in expansion media and subsequently in maturation media, achieves optimal *in vitro* erythroid expansion [40]. Many researchers have published their data on various approaches to tissue engineering RBCs [41-50]. The general consensus for producing RBCs *in vitro* is a pre-culture of HSC for erythroid progenitor cells with a subsequent generation of high numbers of erythroblasts followed by a erythroid maturation phase in the presence of a feeder layer to facilitate enucleation [51]. Importantly, maturation without a feeder layer has also been reported now [52] and this development is essential if tissue engineered RBCs is to become a standard clinical therapy.

1.5.2 A clinical trial

To date, only one study reported on the transfusion of cultured red blood cells (cRBCs) into humans: Giarratana *et al*. achieved this major step as reported in 2011. They successfully injected $1x10^{10}$ cultured enucleated cells (68% enucleation rate during culture), generated from autologous peripheral blood HSCs, into a healthy volunteer. The lifespan of these Cr^{51}-labeled cells was 94-100% over the first 5 days and ~50% after 26 days. Together with the positive results from extensive testing cRBC characteristics, this data shows the feasibility of human cRBCs for clinical applications [53].

The fact that after decades of research just one clinical trial has been reported does not mean we are not getting closer to more trials in humans. However we note that the culture of high numbers of erythrocytes is still an enormous challenge. In order to create several blood units for transfusion thousands of litres of media are needed, let alone the vast space required to establish and maintain these cultures [54]. In the human clinical trial mentioned above they isolated stem cells from peripheral blood (PB) which have inferior biological properties for erythroid maturation compared to HSCs isolated from cord blood [55, 56]. Other origins of cells which have been used for erythroid maturation are adult bone marrow (BM) [56], embryonic stem cells (ESCs) [47] and induced pluripotent stem cells (iPSCs) [57].

1.5.3 Tissue engineering RBCs

HSCs from the various sources have been analysed for their biological properties and potential for various applications. The high yield and high expansion potential of cells isolated from cord blood and BM make these excellent sources for erythroid maturation [45, 58]. Most HSCs and progenitor cells in humans express the CD34 antigen while being $CD38^-$ [58]. However, some reports state that CD38 is indefinite for the erythroid potential and that HSCs can be referred to as CD34+; a heterogeneous cell population of which only a small fraction are actually HSCs [54]. This heterogeneity is even further demonstrated by

data that suggest that the erythroid expansion potential of $CD34^-$ outweighs the contribution of $CD34^+$ cells [41] and that $CD3^+/CD14^+$ depletion from mononuclear cells (MNCs) before expansion of this mixed cell population improves the $CD34^+$ cell yields [59]. Most research still uses highly purified $CD34^+$ populations, isolated from donated human tissue, for erythroid maturation. Cord blood isolated CD34+ cells generate on average 10x times more erythroblasts (Figure 1.2) than CD34+ cells isolated from adult blood [60]. These cells are considered adult stem cells. For all cell populations containing HSCs it is well documented what conditions are required for tissue engineering RBCs. The various stages of erythropoiesis are characterised by distinctive cell types, from the self renewal of the stem cell via the colony forming units (CFUs) to the erythroblasts and RBCs [39].

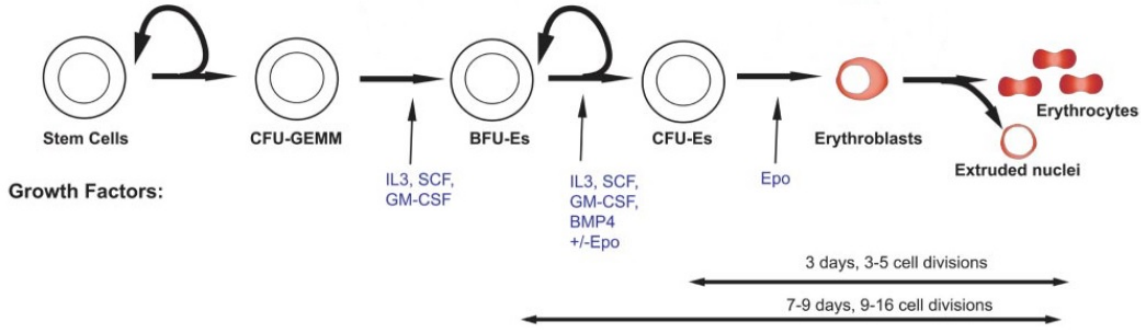

Fig. 1.2. *A schematic overview of erythropoiesis (Hattangadi et al 2011) – Distinctive cell types in each step of erythropoiesis with corresponding growth factors and cell division rate in one differentiation method*

1.5.4 The use of pluripotent stem cells

For the tissue engineering of RBCs, research has mainly focussed on the use of adult human stem cells (*e.g.* HSCs isolated from PB, BM, and umbilical cord blood (UCB)). A possible other cell source for this research could be induced pluripotent stem cells (iPSCs). Although there can be subtle differences, ESCs and iPSCs are both pluripotent stem cells. These cells have been extensively studied for erythropoiesis in mice but essential is the ongoing research of basic science studies and translational studies with human cells. These studies are

essential before haematopoietic derivatives of PSCs can be used for clinical trials. A difficult challenge is possibly the differentiation of PSCs into cells with an adult phenotype without any adverse effects [61].

Compared to erythroid maturation from adult stem cells, erythropoiesis from iPSCs brings extra possibilities but also extra concerns. Possible pre-existing genetic mutations in somatic cells are still a major concern for the production and further use of iPSCs but access to somatic cells are near unlimited which, theoretically, could give access to the manufacture of unrestricted numbers of RBCs. There is limited availability of adult HSCs which currently restricts cell therapy; iPSCs could overcome this hurdle. A recent review regarding the use of iPSCs for safe cell therapy showed the work that has been focussed on creating and selecting the appropriate iPSC cell lines that are not tumorigenic or show teratoma formation *in vivo*. This provides evidence that progress has been made but safety concerns must be assuaged by thorough long-term validation of iPSC(-derived) cells: tumorigenicity, genetic and epigenetic status, and *in vitro* and *in vivo* differentiation capability for various clinical applications [62].

Up to now, published data have shown the generation of erythroid progenitors by using hESC on a murine fetal liver-derived stromal feeder layer where the Hb content gradually changed over time from mostly Hb-ε to almost 100% adult type Hb-β [63]. Most research show similar data but when iPSC-derived HSCs are used in a xeno-free erythroid environment the challenge remains one of quantity and quality (*i.e.* adult phenotype) of these differentiated erythrocytes [64, 65]. The full potential of these promising iPSC derived erythroid maturation conditions *in vitro* has yet to be achieved.

1.6 The HSC environment

The micro-environment of the stem cell is crucial for its fate. This environment includes medium composition, paracrine signalling, mechanical stress, and metabolic activity. Ideally the parameters of this environment would recreate the widely discussed stem cell niche to develop derived daughter HSCs into RBCs, comparable with the *in vivo* unrivalled expansion rates to produce cells of exceptional quality.

When targeting for clinical use, media for tissue engineering RBCs needs to be in compliance with good manufacturing practice (GMP) and it is essential that no xenogeneic components should be used (*e.g.* serum, feeder layers). Several companies have defined specialised serum-free medium for HSC expansion and basic medium for erythroid maturation. Examples are StemSpan (Stem Cell Technologies), Stemline (Sigma-Aldrich), Haematopoietic Progentior Cell Expansion Medium DFX (PromoCell), and StemPro®-34 (Invitrogen) with or without an accompanying cytokine mix. Some research groups have defined their own medium based on a formula with either Iscove's Modified Dulbecco's Medium (IMDM), Alpha Modification Minimal Essential Medium (αMEM), or Dulbecco's Modified Eagle Medium (DMEM) [40]. Common ingredients of non commercial basic medium are transferrin, insulin, bovine serum albumin (BSA), ferrous sulphate, ferric nitrate, thioglycerol, glucose, myo-inositol, and glutamine.

Much research has been focussed on the crucial factors for the culture of HSCs to maximise erythroid expansion and terminal differentiation. For expansion SCF, flt3-ligand (Flt3-L), thrombopoietin (TPO) are commonly used but also interleukin-3 (IL-3), interleukin-6 (IL-6) and granulocyte colony-stimulating factor (G-CSF) are mentioned for use in various cocktail compositions [66, 67].

For erythroid differentiation, EPO is the utmost important factor and is used in all various stages of the maturation process. It is widely known that this cytokine is used in synergy

with a cocktail of SCF, IL-3, insulin-like growth factor-1 (IGF-1), dexamethasone, hydrocortisone, and/or cholesterol-rich lipids. During the three phases of this culture (erythroid expansion, maturation, terminal differentiation) the cocktail recipe usually changes [2, 42, 45, 53].

Erythroid cultures benefit from feeder layers as a surrogate for the cellular *in vivo* microenvironment and this was regarded as essential for quite a time. More recently, data has been published on the terminal differentiation of HSCs that proved successful without a supportive cell layer. The expansion fold rate without was higher (~37,000) than with a feeder layer (~29,000) but enucleation rate was ~68% and ~90% respectively [42, 53] whilst another study obtained a majority of erythroblast and observed no reticuloctyes or erythrocytes after 21 days of culture [68]. Of importance is the efficiency of enucleation in each of these cases and the result will have widespread potential of translating this redesigned culture into the clinic. Although there are reports of unsupported terminal differentiation, it has not been widely published yet for all common cell sources (*e.g.* PB, cord blood). If research groups will be able to succeed, this will most likely have a major positive impact on the quantity and quality of xeno-free erythroid cultures with a clinical aim.

1.6.1 3D (dynamic) cultures

A closed system that would generate cultured RBCs (cRBCs) from stem cells throughout all the various stages of erythroid maturation would be ideal. This would imply continuous refreshment of medium, change of medium composition at essential stages of the culture, and high density cell packaging to create artificial blood units with the sole function of oxygen transport. The high demanding culture conditions for the production of *ex vivo* erythrocytes does not make the translation from a static to a dynamic environment straightforward. For over 20 years, small scale 3D cultures have been setup to analyse

haematopoietic and erythroid cell growth [69-71]. Despite various attempts, no all-inclusive, large-scale dynamic systems have yet been successfully created for the manufacture of RBCs [72]. Most current studies involve small-volume closed-systems such as gas-permeable bags, stirred flasks, flatbed perfusion bioreactors and 3D scaffolds [73].

A novel method to expand HSCs and differentiate erythroid committed cells is the automated suspension bioreactor with online monitoring and control. This system can detect different cell responses and act accordingly regarding O_2 tension and seeding density, but it is merely a very valuable small-scale tool for studying HSC bioprocessing rather than a system for gaining high quantity (and quality) cell output [74]. Another tool for studying erythrocyte production is an 800 mL 3D hollow fibre capillary membrane-based bioreactor scaled-down to a capacity of 2-8 mL. For these miniature compartments the same perfusion unit (containing pump units, pressure control, heating chamber with automated temperature control, and gas mixing unit) was used as for the 800 mL bioreactor compartment. The hydrophilic medium perfusion capillary membranes were fabricated from microporous polyethersulphone with a molecular weight cut-off (MWCO) of ~500,000 Dalton. Static preculture of $CD34^+$ cells was still required but this pioneering device is a step forward to a possible large scale bioreactor for erythroid cultures as successful harvesting of cells with mature RBC characteristics was achieved [75]. More studies utilised hollow fibres, with a similar MWCO to retain medium supplements such as cytokines within the capillaries preventing dilution in the bulk medium, although this is not a common type bioreactor [72].

The use of scaffolds has also been among a method to establish 3D cultures for effectively expanding HSCs and haematopoietic progenitor cells (HPCs). The main purpose is a sustainable culture of HSC although up-scaling of 3D scaffold cultures might prove difficult. The rationale for introducing scaffolds is based on evidence that the co-culture of haematopoietic cells with stromal cells is useful for proliferation, as well as a recreation of a stem cell niche. Various scaffolds have been mentioned in the literature, for example highly

porous poly(D,L-lactide-co-glycolide) (PLGA) and polyurethane scaffolds coated with collagen type I cultured with UCB MNCs (UBMCs) to facilitate cytokine-free HSC expansion. Collagen-coated polyurethane scaffolds were advantageous for support and cell expansion (54 fold after 28 days) and this was supported by positive Wright-Giemsa staining. Other research mentioned 3D collagen gels either with or without embedded mesenchymal stem cells (MSCs) as a model for the haematopoietic niche. Analysis showed that the presence of BM MSCs (BMSCs) in the gel significantly enhanced the number of primitive $CD34^{+}/CD38^{-}$ HPCs when compared to more conventional suspension cultures. Recently, data was published on entrapped cryopreserved BM cells in a porous polyvinyl fluoride 3D scaffold to influence HPC expansion [76-78].

All the above-mentioned 3D scaffolds benefit in their specific way the expansion of haematopoietic cells for potential erythroid maturation. While these systems are excellent research tools for improving expansion conditions, as are the stated small volume bioreactors, the establishment of potential clinical applications will need characteristics improved over those currently available. These characteristics are preferable and ultimately Good Clinical Practise focussed for prospective clinical approval with systems that can either be continuous or fed-batch processes with more emphasis on elevated production of erythroid differentiated cells [79].

1.7 Comparison of artificial RBCs

For clinical use, cRBCs need to mimic RBCs in every possible way. After establishing cultures to produce cRBCs more emphasise has shifted to compare size, shape, Hb content, and volume of both RBC sources. Research should aim for uniformity in a range of tests to check the validity of the *ex vivo* manufactured RBCs, comparable to current practise for blood tests. After global implementation the production process can be validated by testing the cRBCs for approval in transfusions.

1.7.1 Native RBCs vs cultured RBCs

Table 1.1 provides a brief summary of cRBC characteristics and the comparison with RBC characteristics from isolated PB. All cRBCs contain a degree of fetal Hb, the cell size is up to ~40% larger and the total Hb content can be found in a similar range as isolated RBCs (27-33 picograms). The information provided by Giarratana *et al* (2011) showed that with nearly identical RBCs it is possible to perform a blood transfusion. The *in vivo* life span of these cells is promising with a survival rate of 76±10% at day 14 and at day 26 this was 51±12%, as measured by counting the ^{51}Cr sodium chromate labelled cRBCs at various time points post transfusion.

	Cell Source	RBCs (%)	Size (µm)	MCV (fL)	MCH (pg)	Hb type (%)	Blood type	Literature
Adult RBCs	PB	100	8	~90-93	27-33	>95 Hb-A	-	[C, D]
Cultured RBCs	PB-CD34$^+$	82±4	9	113±3	33±2	94±2 Hb-A Hb-F	n.d.	[A]
	CB-CD34$^+$	82±4	9	113±3	33±2	63±13 Hb-F	n.d.	[A]
	CB-CD34$^+$	>95	n/a	~99	42.6±1.2	n.d.	Same as donor	[B]
	CB-CD34$^+$	>90	n/a	~133	30.5±0.2	50.8±10 Hb-A 47.3±7 Hb-F	n.d.	[C]
	PB-CD34$^+$	n.d.	11	138±4.2	35±1.6	88±2.7 Hb-A 10.6±2.8 Hb-F	Same as donor	[E]

Table 1.1. *The characteristics of cultured RBCs compared to RBCs isolated from PB. The characteristics of the cultured RBCs can be found in a similar range except for the content of the various Hb's which is highly variable. Not all studies report on the blood type but a few state similar blood types as the donor. Important to note is that the cRBCs used in the human clinical trial are 11 µm in diameter and contain ~88% Hb-β (Giarratana et al., 2011)*

MCV: Mean Cell Volume, MCH: Mean Cell Haemoglobin, µm: micrometer, fL: femtolitre, pg: pictogram, n.d.: no data, CB; cord blood, PB; peripheral blood. Literature: [A]: Giarratana et al., 2005; [B]: Baek et al., 2008; [C]: Timmins et al., 2011; [D]: Hess, 2010; [E]: Giarratana et al., 2011

1.7.2 The comparison between acellular and cellular oxygen carriers

For the development of acellular oxygen carriers, the research is focussed on delivery systems containing Hb (HBOCs). The majority investigates cell-free Hb and Hb encapsulated in lipid vesicles or polymers. These are non-biological systems which use molecules to 'carry' Hb. No cell culture is involved and these chemically produced HBOCs have a relatively long shelf life, are disease free and have a universal compatibility with all blood groups [5, 19]. So far, the side-effects of this product resulted in very restricted use of HBOCs. Improved product design to eliminate these detrimental effects may results in reconsideration of HBOC for healthcare use [80].

On the other side of the line, researchers aim to use biological oxygen carriers which are *in vitro* produced cells containing intracellular synthesised Hb. This system, which does not have the above-mentioned advantages of the acellular platform, aims to manufacture cRBCs which should be identical to native RBCs. This system has potential to become a tailor-made

cell therapy which could be more patient friendly than chemically defined HBOCs and this might benefit the patient's recovery. The risk for the cellular platform is the chance of exposure to diseases during culture and therefore, among other regulations, GMP is paramount. The success of this product depends on the ability to safely manufacture clinically relevant numbers of cRBCs at a sustainable cost either as an alternative for current blood donation or as therapy aimed at patients who require a transfusion in extraordinary situations.

1.8 *In vivo* animal studies

Most *in vivo* testing of cRBCs have been done in animals (mice) after extensively comparing the characteristics of these cells with isolated erythrocytes; so far only one human clinical trial using autologous cells has been performed [53]. Table 1 showed that cRBCs are similar but not exactly the same in size and content to PB isolated RBCs. A mice study was performed with ~$3x10^6$ 10-day old erythroid differentiated cells and post injection cell expansion was observed with cells located in the BM, liver, lung and spleen undergoing terminal maturation. Four days *in vivo*, an overall 96-fold amplification of the human erythroid cells was detected [46]. Other data also suggested that after the transfusion of reticuloctyes/immature erythrocytes into mice these cells fully mature *in vivo* [42]. These results indicate that cRBC will only need *in vitro* stimulation to a certain level before the body will finish this process of terminal differentiation. This minimum level of erythroid stimulation *in vitro* is essential to ensure that cRBCs produced for transfusion are ready for their task *in vivo*.

1.9 Crucial challenges for the engineering of oxygen carriers

Researchers have made tremendous progress in the field of creating artificial RBCs, however there are still challenges to be faced to make this story a success. There are key issues, in terms of technical challenges, which will determine the success of engineering solutions. A requirement for potential products involves abiding by the regulation of governing bodies which allow for safe human therapies. This regulation demands thorough (clinical) testing of engineered oxygen carriers and guards patient safety.

When it comes to regulation of administering oxygen carriers, there will always be safety concerns for products that might have been exposed to xenogeneic substances during cell culture. Xenogeneic-free cell culture is crucial and the development of suitable complete humanised media for the *ex vivo* maturation of RBCs is essential. Other safety concerns involve possible exposure to infectious agents and neoplastic transformation during product manufacture and are prevented by appropriate GMP procedures. Cell products require evaluation by cytogenetic and genotoxicity assays (in compliance with FDA guidelines) and these products need to be treated as any transfusion product relating to risks for infection, incompatibility, and antigenicity [60].

Current regulation provides the guidance for finding a suitable oxygen carrier and is of paramount importance for patient health protection. Researchers need to overcome the technical hurdles in order to provide a solution for artificial oxygen carriers. One of the main challenges for a cellular platform for transfusion remains the requirement of a great number of cells which may be tackled by enhanced culture techniques. However, CD34+ cells from the same donor generate a similar number of erythroblasts in culture repeated over time, whereas there is great variability in culture production from different donors [60].

Erythroblasts from autologous cells could be a potential cell therapy with a relatively long term therapeutic effect but for transfusion of immediate oxygen carrying capacity, in case of

emergency, only enucleated artificial red cells could provide an alternative to transfusion of RBCs [60]. Efficient enucleation and separation of extruded nuclei is a major obstacle in cRBC culture protocols. First reports which showed a high enucleation efficiency rate used co-cultures but researchers have now achieved high enucleation efficiency without the use of feeder layers for low-density cultures. High-density cultures are essential and therefore high enucleation efficiency rates still need improving in order to achieve successful manufacture of cRBCs [61].

Human ESCs and iPSCs may have potential as a source for *ex* vivo expansion of erythroblasts for a cell-therapy product as RBCs do not have a nucleus. However, it is believed that this research might be more beneficial for important developmental biology issues than it will for generating erythroblasts *ex vivo* for transfusion [60].

A critical issue is the acceptance of oxygen carriers by the recipient's body. So far, the majority of developed and tested acellular platforms did not reach the clinic mainly because of their adverse effect profile. An in depth analysis by Silverman and Weiskopf stated that all HBOCs tested to date caused vasoconstriction and elevation in blood pressure [20]. Therefore, targeted chemical modification of HBOC, to eliminate vasoconstriction, might be the key to success for certain second-generation products [80]. For the cellular oxygen variant (e.g. cRBCs) the situation is somewhat different. As a potential cell therapy, the antigen profile of the graft should be identical as possible to that of the host's to minimise the effects of inflammation and any other undesirable physical response. During the reported clinical trial with autologous cells there were no noticeable differences in antigen expression between the native RBCs and cRBCs, including the ABO and Rh antigens [53]. It was not reported what effect the injection of $1x10^{10}$ cRBCs had on the human subject. This is interesting data, however the administration of such a cell number is a factor of 250 smaller than that in a regular blood unit and this might evoke a different immune response. For that

reason, more in-depth analysis is required into the effect of real life quantities of cRBCs on transfusion subjects.

As stated, there are a number of barriers that need to be overcome in order for artificial oxygen carriers to become an acclaimed treatment. The quality and the quantity need to be sufficient for an alternative therapy in transfusion. When the quality of a potential product is perfect, sufficient product needs to be available in order to become reliable for treatment. With products available in the veterinary sector for acellular products it is shown that this can be done cost effectively. For cellular products, which require high numbers of cells, manufacturing requires large volumes of expensive media and this includes the costs of GMP [60]. This creates a tremendous financial challenge to make it sustainable when compared with current transfusion practice.

1.9.1 Potential clinical applications in the short term

It is evident that despite the reality of manufacturing cRBCs, this practise has not reached the clinic due to many hurdles. The use of artificial cellular oxygen carriers as a sustainable transfusion therapy might happen in the future, but tissue engineered RBCs might have other benefits at the present time. With relatively easy access to HSCs and available protocols for erythroid maturation, the *in vitro* production of RBCs allows for precise control of the various stages of the erythroid progenitor cells to select cells for other applications. Two areas which could benefit from this research are drug discovery and drug delivery [40]. Over the past few years, researchers have started to use erythroid progenitor cells for drug discovery including identification of the toxicity mechanism of the B19 parvovirus [81], screening for compounds that induce γ-globin production [82], and susceptibility of erythroblasts to malaria infection [83]. This stage-specific control of erythroid progenitor cells grants better opportunities to analyse the susceptibility of cells, including rare blood types, for various diseases than is the case with isolated cells.

RBCs have been reviewed as a vehicle for intravascular drug delivery with strategies involving encapsulation into cells and coupling drug molecules to the cell surface [84]. Human isolated RBCs for a drug delivery system is a novel and promising technique but will also evoke the same issues as occurs in current transfusion practise. *Ex vivo* manufactured RBCs for drug delivery systems might overcome these issues as these cells have a potential to be tailored to the patient's, possibly rare, antigen profile, minimising an immune response. All developmental progress made in the field to develop therapies involving cRBCs will likely contribute to establish, at some point in the future, tissue engineered RBC transfusion products.

1.10 Outlook

Over the past decades tremendous research has been performed to bring us to the stage where we are now. The first data of a human clinical trial with *in vitro* produced erythrocytes is hugely important. On the route to full clinical use of cultured RBCs, more *in vivo* experiments with humans are paramount to establish a robust argument for this method as an alternative to current blood transfusion. In the mean time, cRBCs could have their use in the development of drug discovery and drug delivery, indicating applications beyond transfusion.

Controlling the antigenic profile of cRBCs and the use of non-autlogous cells are essential pieces of this complex jigsaw. Critically, upon administration of these cells the recipient's immune system should not reject the oxygen carriers including minimal inflammatory response. Ideally, the developed product should reduce the risks for the patient when compared to current transfusion products. It is also vital to develop a device that is ultimately capable of producing at least multiple units of RBCs from a select number of HSCs in a clinically safe manner. Despite major progress, there are still major challenges to overcome in manufacturing cRBCs including the increase of enucleation efficiency and complete allogeneic culture conditions.

As with any medical treatment regulation from governing bodies is required to safeguard quality control for patient's safety. A degree of uniformity in this market will be required if tissue engineered RBCs for transfusion becomes common practise in order to avoid discrepancy between the use and quality of cellular products. A potentially successful outcome will produce a rationale for widespread clinical use if production costs can compete with the price of current transfusion practice.

1.11 The research project

In the previous paragraphs the wider scope of producing artificial RBCs has been described. The title of this particular research study was officially formulated as "Continuous proliferation and simultaneous maturation of HSCs into RBCs". This is a tissue engineering approach to produce RBCs from adult stem cells *in vitro*.

The research was part of a larger project involving European research groups and a company (Applikon Biotechnology, Delft (NL)) which produces bioreactors for cell culture. The overall aim of this project was the bioreactor development for the manufacture of RBCs. During this project, intensive contact was maintained with all research partners and in particular with Applikon Biotechnology. The work done in chapter 5 was performed in partnership with Applikon. Experiments were performed in the laboratory located in Liverpool, using equipment provided by Applikon, and data was fed back to the company located in Delft (NL) in order to improve and optimise the bioreactors for cell culture.

During this project, the research was divided in three research topics:

i. Haematopoietic Cell Culture (Chapter 3)
ii. Manufacture of Red Blood Cells (Chapter 4)
iii. Development of the Bioreactor (Chapter 5)

The first topic, described in chapter 3, involved the long term culture of HSCs isolated from PB and UCB. This chapter reports on the efficiency of HSC isolation and subsequent proliferation of these stem cells including the progress made by including MNCs for HSC expansion. Chapter 4 shows how this research is continued by differentiating the expanded cells, including MNCs, into RBCs. The research described in chapter 5 was aimed at the upscaling of successful erythroid maturation cultures. This chapter contains results, obtained after analysis of bioreactors, including the culture parameters and cell viability. Together

these chapters provide a process for upscaling cell culture with the overall aim of producing clinically relevant numbers of RBCs.

Chapter 2

Materials and Methods

2.1 Culture media

2.1.1 Medium for haematopoietic stem cell expansion

A selection of media was used for the expansion of HSCs:

I-Medium:

ISCOVE incomplete medium (BioChrom AG, T0046) supplemented with 5mM glucose (Sigma-Aldrich, G7021), 0.4mM myo-inositol (Sigma-Aldrich, I7508), 2mM glutamine (Invitrogen, 25030-024), 10% (v/v) fetal calf serum (FCS) (BioWhittaker, DE14-701FH), 1% (v/v) penicillin/streptomycin (Pen/Strep) (Sigma-Aldrich, P4458), and 50 ng/mL SCF, TPO, and Flt3-L.

I-Medium *[Serum Free]*:

ISCOVE incomplete medium (BioChrom AG, T0046) supplemented with 5mM glucose (Sigma-Aldrich, G7021), 0.4mM myo-inositol (Sigma-Aldrich, I7508), 2mM glutamine (Invitrogen, 25030-024), 2.5 mg/mL BSA (Sigma-Aldrich, A3912), 50 µg/mL holo-transferrin (Sigma-Aldrich, I6634), 25 µM β-mercaptoethanol, 10 µM $Fe(II)SO_4$, 1% (v/v) Pen/Strep (Sigma-Aldrich, P4458), and 50 ng/mL SCF, TPO, and Flt3-L.

P-Medium *[Serum Free]*:

HPC Expansion Medium DFX (PromoCell, C-28021) supplemented with cytokine mix E for HPC-expansion Medium DFX (PromoCell, C39891).

D-Medium *[Serum Free]*:

IMDM with GlutaMAX™ (Gibco, 31980), $1.6x10^{-4}$ 1-thioglycerol (Sigma-Aldrich, M6145), 120 µg/mL holo-transferrin (Sigma-Aldrich, I6634), 10 µg/mL insulin Sigma-Aldrich, T0665), 1% (v/v) Pen/Strep (Sigma-Aldrich, P0906), and 10 mg/mL BSA (Sigma-Aldrich, A3912) as described as a basic medium by Douay et al (2009) [86], supplemented with 50 ng/mL SCF, TPO, and Flt3-L.

S-Medium *[Serum Free]*:

StemLine® II Hematopoietic Stem Cell Expansion Medium (Sigma-Aldrich, S0192)

2.1.2 Basic medium for erythroid maturation (Douay and Giarratana, 2009) [86]

The basic medium that was used for erythroid maturation was IMDM with GlutaMAX™ (Gibco, 31980) containing $1.6x10^{-4}$ M 1-thioglycerol (Sigma-Aldrich, M6145), 120 µg/mL holo-transferrin (Sigma-Aldrich, I6634), 10 µg/mL insulin (Sigma-Aldrich, T0665), 1% (v/v) Pen/Strep (Sigma-Aldrich, P0906), and 10 mg/mL BSA (Sigma-Aldrich, A3912).

2.1.3 Cytokines for HSC expansion and erythroid maturation

Human-SCF (Stem Cell Factor):

-(R&D Systems, 255-SC-050) and (Miltenyi Biotec, 130-093-992)

Human-EPO (Erythropoietin):

-(R&D Systems, 287-TC)

Human-IL-3 (Interleukin-3):

-(R&D Systems, 203-IL/CF)

Human-TPO (Thrombopoietin):

-(Miltenyi Biotec, 130-094-013) and (Gibco, PHC9514)

Human-Flt3-L (Flt3-Ligand):

-(Miltenyi Biotec, 130-093-855)

Human-G-CSF (Granulocyte Colony-Stimulating Factor):

-(Miltenyi, 130-096-346)

2.1.4 Medium for the human lymphoblast cell line U937

U937 cells were either cultured in low glucose DMEM (Gibco, 21885) supplemented with 5% FCS (Gibco, 10099-141) and 1% Pen/Strep (Sigma, P4458)

and

RPMI 1640 medium (Gibco, 21875) supplemented with 5% FCS (Gibco, 10099-141) and 1% Pen/Strep (Sigma, P4458).

2.1.5 Medium for primary cultures and dental pulp stem cells (DPSCs)

DMEM, GlutaMAX™ (Gibco, 31966) containing 10% (v/v) FCS, and 1% (v/v) Pen/Strep (Sigma-Aldrich, P4458).

2.1.6 Medium for mesenchymal stem cell expansion

Mesenchymal Stem Cell Growth Medium (MSCGM™) (Lonza, PT-3001) contains Mesenchymal Stem Cell Basal Medium and a MSCGM™ SingleQuots® Kit.

2.2 Cell isolation

2.2.1 Purification of MNCs from peripheral blood

Samples of 30mL PB were isolated from healthy donors and 0.6 mL of heparin (Multiparin, 74301/5) were added per sample to prevent coagulation. Each blood sample was mixed with 20 mL 1mM EDTA (Gibco, 15575) in Phosphate Buffered Saline (PBS) (Sigma-Aldrich, D1408) (4°C), up to a total volume of 50 mL. Leucosep™ 50 mL tubes (Greiner Bio-One, 227290), preloaded with 15 mL histopaque (Sigma-Aldrich, 1077), were filled with 25 mL of mixed blood and centrifuged at 2000 rotations per minute (rpm) for 20 min at 20°C, no brakes. Buffy coats from the same donor were pooled in a 50mL tube, washed with up to 50mL of 1mM EDTA in PBS (4°C) and centrifuged at 1700rpm for 10 min at 4°C. Obtained cell pellets were washed with 50 mL of 1mM EDTA in PBS (4°C) and centrifuged at 1500rpm for 10 min at 4°C. Supernatant was discarded and MNCs were either resuspended in medium, 300 µL buffer or freezing buffer, depending on application.

2.2.2 Enrichment of $CD34^+$ cells using MACS® technology

Purified MNCs were resuspended in 300 µL magnetic-activated cell sorting (MACS®) buffer (kept cold on ice), containing 2mM EDTA and 1% BSA in PBS, in a 15mL falcon tube and 100 µL FcR blocking reagent (Miltenyi Biotec, 130-046-702) was added well mixed. Next, 100 µL CD34 MicroBeads (Miltenyi Biotec, 130-046-702) were added to the cell suspension and mixed well, followed by a 30 minute incubation time at 4°C while slowly rotating on a Spiramix 5 Roller Mixer (Denley). After incubation the cells were washed with MACS® buffer and centrifuged at 1500rpm for 5 min. The cell pellet was resuspended in 500 µL MACS® buffer, ready for magnetic separation.

For magnetic separation, a MS column (Miltenyi Biotec, 130-042-201) was placed in the magnetic field of a MiniMACS® Separator (Miltenyi Biotec, 130-042-102). The column was

rinsed with 500 µL MACS® buffer before the cell suspension was added, followed by 1 mL MACS® buffer. The column was rinsed four times with 500 µL MACS® buffer. Next, a new MS column was placed in the MACS® separator and prepared as stated above. Cells from the first column were eluted into a second MS column using 1mL MACS® buffer and plunger. A second column with cell suspension was washed three times with 500 µL MACS® buffer. Afterwards the cells were eluted, as described above, into a collection tube and used for further processing.

2.2.3 Isolation of MSCs from human bone marrow

Samples of BM (5-20mL) were collected from human donors by standard surgical procedures. Each sample was diluted 1:1 (v/v) with 1mM EDTA in PBS (4°C), put through a 100 µm cell strainer to remove any bone debris, and transferred to a 50mL Leucosep™ tube, preloaded with 15mL Histopaque and subsequently centrifuged at 2000rpm for 20 min at 20°C, no brakes. Buffy coats were transferred to a 50mL tube, washed with up to 50mL of 1mM EDTA in PBS (4°C) and centrifuged at 1700rpm for 10 min at 4°C. Obtained cell pellets were washed with 50 mL of 1mM EDTA in PBS (4°C) and centrifuged at 1500rpm for 10 min at 4°C. The supernatant was discarded and MNCs were either prepared for culture or resuspended in 300 µL MACS® buffer or freezing buffer, depending on application.

For culture, each cell pellet was resuspended in 5 mL primary culture medium and transferred to a 25 cm^2 tissue-culture treated flaks (BD Falcon, 353136) to allow cell adherence. Medium was refreshed weekly until the flasks was 70-90% confluent. Cells were harvested for subculture in MSC medium and analysed for MSC phenotype using Fluorescent Activated Cell Sorting (FACS) using appropriate antibodies (CD90, CD105, CD29, CD73, CD34, and CD45).

2.2.4 Isolation of MSCs from human adipose tissue

Adipose tissues (5-20mL) were collected from human donors by standard surgical procedures. On arrival, tissue was washed in a 100mL container with lid, filled with 30-50 mL PBS containing 2% Pen/Strep (Sigma-Aldrich, P4458) by shaking vigorously for 10 seconds. PBS was removed and the washing procedure was repeated until the culture medium was clear.

To digest adipose tissue and harvest the MSCs, tissue was cut in 2 mm^2 pieces using 22mm blades (Swann-Morton, 22-0208), placed in a 50ml tube and subsequently digested by 10% collagenease (Sigma-Aldrich, C2674) in DMEM (Gibco, 31966) containing 2% Pen/Strep, incubated overnight at 37°C whilst slowly rotating on a Spiramix 5 Roller Mixer (Denley). Collagenase activity was stopped by adding 10% FCS (BioWhittaker, DE14-701FH) and collagenase-digested tissue was transferred to a 50 mL tube through a 100 μm cell strainer (BD Falcon, 352360) to obtain a cell suspension free of undigested tissue.

Cell suspension was centrifuged at 1500rpm for 5 min and supernatant was removed. To remove erythrocytes, the cell pellet was resuspended in Red Blood Cell Lysing Buffer (Sigma-Aldrich, R7757) for 5 minutes at RT followed by a washing step with 20 mL of PBS. After centrifugation, cells were resuspended in 10 mL of primary culture medium, transferred to a 50 mL tube through a 40 μm cell strainer (BD Falcon, 352340) and subsequently the cell suspension was transferred to a 75 cm^2 tissue-culture treated flaks (BD Falcon, 353136) to allow cell adherence. Medium was refreshed weekly until the flask was 70-90% confluent. Cells were harvested for subculture in MSC medium and analysed for MSC phenotype with FACS using appropriate antibodies (CD90, CD105, CD29, CD73, CD34, CD45).

2.3 Cell culture

2.3.1 Culture of HSCs and MNCs

$CD34^+$ cells were isolated from MNCs from PB or UCB. The $CD34^+$ cells were cultured as a liquid cell culture in 24 and 48 well plates using media for haematopoietic cell expansion either without or with a stromal layer. The stromal cells were either harvested from BM or adipose tissue and cultured on a tissue culture (TC) treated surface prior to culture. For subculture, attached cells were detached using a cell scraper (Greiner Bio-one, 541070), the cell suspension transferred to a falcon tube, and the culture surface was washed thoroughly to harvest all the cells. The obtained cell suspension was transferred to a falcon tube, and cells were centrifuged at 1500rpm for 5 minutes, resuspended in appropriate medium, subcultured in wells or a flask depending on the application, and stored in a 37°C incubator with 5% CO_2 infusion.

2.3.2 Erythroid maturation (Douay and Giarratana, 2009) [86]

Either MNCs or $CD34^+$ cells, isolated from either PB or UCB were used for differentiation towards the erythroid lineage. In general, erythroid culture is divided into three different phases:

Phase I (Day 0-8): During this phase of erythroid culture, complete IMDM (Douay and Giarratana, 2009) [86] was supplemented with the following additions with the following final concentrations: 100 ng/mL SCF, 5 ng/mL IL-3, 3 IU/mL EPO, 10^{-6} M Hydrocortisone (Sigma-Aldrich, H2270).

Phase II (Day 8-11): For phase two, complete IMDM (Douay and Giarratana, 2009) [86] was supplemented with 3 IU/mL EPO only. Maturing cells were cultured on a stroma of MSCs and resuspended at ~$5x10^6$ cells/mL.

Phase III (From Day 11): For phase three only complete IMDM (Douay and Giarratana, 2009) [86] was used. Maturing cells were cultured on a stroma of MSCs to allow elimination of nuclei, which are engulfed by the stromal layer.

For better preservation of maturating erythroid cells during phase II and III, human AB serum (Sigma, H4522) could be added to the medium up to a final concentration of 5%. During this phase of culture, non-adherent cells were removed gently to obtain a greater percentage of enucleated cells.

2.3.3 Culture of U937 cells

Cells were seeded in suspension either in a T75 flask (BD Falcon, 353136), a T225 flask (BD Falcon, 353139), a 250 mL bioreactor (Applikon), or in a 3 L bioreactor (Applikon) at a cell density of 2-5 x 10^5 cells/mL. When cultured in flasks, cells were reseeded once a cell density of 1-2 x 10^6 cells/mL was reached.

2.3.4 Subculture of adherent cells

When cells were 70-90% confluent culture medium was removed from the TC treated flask (BD Falcon), cells were washed with PBS and Trypsin-EDTA solution (Sigma-Aldrich, T4174) was introduced to the flask and incubated for 5-10 min at 37°C to allow cells to detach. Following detachment, trypsin was neutralised by adding medium containing 10% FCS, the cell suspension transferred to a 10 mL falcon tube (BD Falcon, 352096) and centrifuged at 1500rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in appropriate medium. Aliquots of cell suspension were transferred to flasks containing medium and stored in a 37°C incubator with 5% CO_2 infusion. Medium was refreshed every 7 days. When required a haemocytometer in conjunction with an inverted

light microscopy was used for cell counting. Images of cells were obtained using an inverted light microscope in conjunction with the software platform Axiovision (Zeiss, UK).

2.3.5 Inhibition of cell expansion for stromal cells

To inhibit stromal cell expansion, after reaching confluence, mitomycin C (Sigma, M4287) was added to the culture medium to avoid over confluence cultures. First, the powdered stock of 2 mg was dissolved in 4mL of dH_2O to obtain a stock solution of 1 mg/mL. Next, either 4 μL of mitomycin C solution was added per 1 mL of cell culture medium, filter sterilised, and incubated at 37°C for 3-4 hours, or 1 μL of mitomycin C solution was added per 1 mL of cell culture medium, filter sterilised, and incubated at 37°C for 24 hours. After inhibition, medium was discarded and cells were washed with PBS before appropriate medium with either MNCs or $CD34^+$ cells was added to the culture.

2.4 Freezing and thawing of cells

Freezing of cells:

Freezing media were prepared with Medium A (100% FCS) at room temperature and Medium B (FCS with 40% DMSO (Sigma, D2650)) at 4°C. Cryovials (Simport, T311-2) were put on ice. Cells were resuspended in 1mL of Medium A, then 1 mL of Medium B was added slowly whilst swirling gently until the two media were at a 50:50 ratio. Promptly the cell suspension was aliquoted in the cryovials; 1 mL suspension per vial. Cryovials were placed in a Mr Frosty at -80°C for 24 hours before storage in liquid LN2.

Thawing of cells:

The vial with frozen cells was thawed in a water bath at 37 °C by gently moving until a small ice crystal is left. The cell suspension was then transferred to a 50 mL conical tube and 1 mL of cold appropriate medium containing 2% FCS was added drop-wise to the conical tube whilst swirling gently. After three minutes, 2 mL of the thawing medium containing 2% FCS was added slowly to the cell suspension whilst swirling gently and after another three minutes this process was repeated with 4 mL of thawing medium; this is the same volume as the cell suspension in the conical tube. The procedure was repeated every three minutes, whilst swirling gently, until the final volume reached 32 mL. Next, the tube was centrifuged at 250g for 10 minutes at room temperature, the supernatant removed and the cell pellet was resuspended in 20mL of thawing medium and centrifuged at 200g for 8 minutes at room temperature. The supernatant was removed and the cell pellet resuspended in appropriate medium at 0.5 x 10^6 cells/mL. A cell count was performed using the viability assay trypan blue (Sigma, T8154) and a haemocytometer.

2.5 Bioreactor culture methods

2.5.1 Cell culture using bioreactors

Various bioreactors were used for culturing U937 cells and UBMCs. The bioreactors have working volumes ranging from 50mL-3L. The bioreactors were provided by Applikon Biotechnology (Delft, The Netherlands). The bioreactors were connected to My-Control Units (Applikon) to control and monitor parameters using the My-Control user-interface (UI) and BioXpert Lite software (Version 1 and 2) for Windows.

2.5.2 Bioreactor equipment

The bioreactor equipment consisted of: 3L bioreactor vessel, 2L bioreactor vessel, 2x 250 mL bioreactor vessel, 2x My-Control Unit, 2x dissolved oxygen (DO) sensor (6mm), 2x pH sensor (6mm), DO sensor (10mm), pH sensor (10mm), 6x cable for connecting sensors with My-Control Unit, 2x heating blanket for 250 mL bioreactor, 1x heating blanket for 2L/3L vessel, 2x thermometer for My-Control Units, 2x stirrer for 250 mL vessel, 1x stirrer for 2L/3L vessel + motor, 2x (porous) sparger for 250 mL vessel, 2x (porous) sparger for 2L/3L vessel, acoustic cell separation device for 2L and 3L vessel; BioSep, tubing (Cole-Parmer), peristaltic pumps (Masterflex), air filters (Sartorius), medium bottles (Duran), gas tubing for CO_2, air, and N_2 supply, gas pressure regulators, rotameter, medium sample systems, BioXpert Lite software for recording parameters (*i.e.* stirrer speed, temperature, DO, pH). My-Control Unit: UI for controlling (and calibrating) the pH, DO, temperature, rotation speed of stirrer, and the addition speed of medium.

Fig. 2.1. UI of the My-Control unit (Screen print of the control system)

2.5.3 Cleaning the bioreactor

Before use, all parts to be exposed to the cell suspension were rinsed with dH_2O and 70% ethanol before allowing to air dry. After use all exposed surfaces, including tubing, were cleaned using Virkon (SLS, CLE1556) and 0.1 M NaOH (Sigma, S8045) solution, rinsed with dH_2O and 70% ethanol before the equipment was air dried. pH sensors were stored in appropriate storage solution before reuse.

2.5.4 Preparing the bioreactor for culture

The pH sensor was calibrated using the UI of the My-Control Unit using a 2-step calibration method that was temperature dependent. Next, the pH sensor was cleaned with dH_2O and fixed in the head plate of the bioreactor as was the DO sensor. To facilitate heat exchange during autoclaving, 10mL dH_2O were added to the vessel. All other relevant tubing (*e.g.* air supply, medium addition, air exchange filter) were connected to the various head plate inputs and all remaining connections were sealed off from atmospheric conditions to maintain sterile conditions after autoclaving. The bioreactor, with all connected tubing and vessels, was autoclaved and was set aside to cool down overnight. The DO sensor, in either PBS or

appropriate medium, was calibrated under sterile conditions using the UI of the My-Control Unit in the presence of air.

The fully calibrated and sterilised bioreactor was placed in a laminar flow cabinet to allow addition of appropriate medium and cells under sterile conditions, via a head plate port, and afterwards the bioreactor was again sealed. Next, the bioreactor sensors were connected to the My-Control Unit, the stirrer was connected to the stirred motor, the heat blanket was placed around the bioreactor vessel, the thermometer was placed in the thermometer pocket, the oxygen/nitrogen/CO_2 supply tubing including filter was connected to the My-Control Unit, the tubing for the alkali addition (pH control) was connected to a My-Control pump, and other tubing (*e.g.* medium addition) was connected to an external pump. Data was recorded using BioXpert Lite software for Windows (32bit).

2.5.5 Controlling the dynamic culture

Controlling of the bioreactor culture was performed by the My-Control Unit with pH and temperature respectively set at 7.4 and 37°C under standard conditions. DO was normally set at 20% and stirrer speed at 75, 100 or 150rpm. All changes in parameter readings were recorded with BioXpert Lite.

Fig. 2.2. *BioXpert Lite interface for monitoring the pH, temperature (Temp), DO, and the stirrer speed of the bioreactor (Screen print of the interface)*

2.6 Analysis

2.6.1 Fluorescent Activated Cell Sorting (FACS)

Phenotypic analysis of cells by means of cell antibody markers was performed using FACS. Cells were harvested from flasks and/or wells by trypsinisation of adherent cells or using a cell scraper for liquid cell suspensions. Cell suspensions were washed once with PBS, transferred to tubes and centrifuged at 1500rpm for 5 min. The resulting cell pellet was then resuspended in PBS and aliquots of cell suspensions (10-50 µL) were transferred into FACS tubes (BD Falcon, 352008). A FACS antibody (3 µL) was added to each cell suspension, incubated at 4°C for 30 minutes and 200-300 µL of sheath fluid added to each tube. Cells analysed for Hb-ß were first fixed using paraformaldehyde/sucrose and then permeabilised using the BD Cytofix/Cytoperm™ Kit (BD Biosciences, 554714) prior to incubation with the antibody. Cells were analysed using a FACsort flow cytometer (BD-Biosciences, UK) and CellQuest software (BD Biosciences, UK). Non-specific fluorescence was determined using negative control samples. All FACS antibodies and isotype controls can be found in Table 2.1. Analysis of data was performed using WinMDI v2.9 for Windows (32bit). Typical graphs for analysis can be found in Figure 2.3.

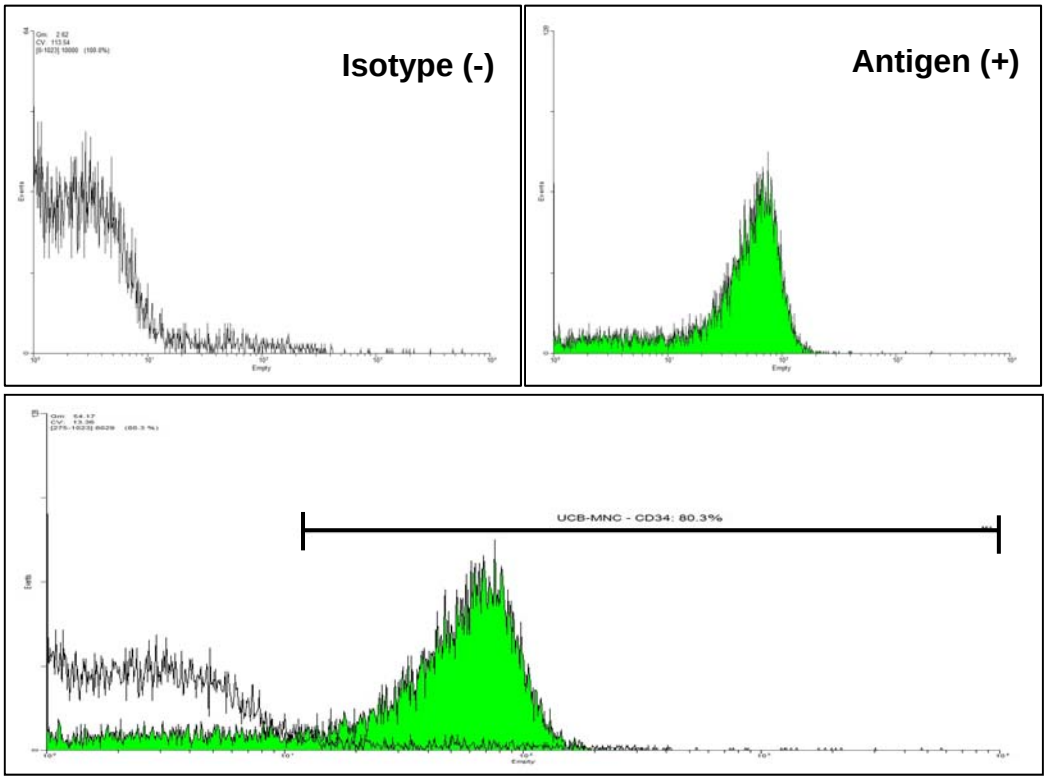

Fig 2.3. *Flow-Cytographs with the negative control (Isotype) and the antibody specific marker (Antigen) shown above and the subtraction (below)*

Antigen	Specification	Reactivity	Conjugate	Isotype	Supplier
CD34	Mouse	Human	FITC	IgG1	BD Biosciences
CD29	Mouse	Human	PE-Cy5	IgG1	BD Biosciences
CD71	Mouse	Human	PE	IgG1	R&D Systems
CD44	Mouse	Human	PE	IgG1	BD Biosciences
CD90	Mouse	Human	FITC	IgG1	BD Biosciences
CD105	Mouse	Human	FITC	IgG1	BD Biosciences
CD45	Mouse	Human	FITC	IgG1	BD Biosciences
CD235a	Mouse	Human	PE	IgG1	R&D Systems
CD73	Mouse	Human	PE	IgG1	BD Biosciences
Hb-β	Mouse	Human	FITC	IgG1	Santa Cruz Biotechnology
CD36	Rat	Human	FITC	IgG2b	R&D Systems
CD45	Mouse	Human	PE-Cy5	IgG1	BD Biosciences
CD133	Mouse	Human	PE	IgG1	MACS® Miltenyi
CD38	Mouse	Human	FITC	IgG1	BD Biosciences
CD117	Mouse	Human	PE	IgG1	BD Biosciences
Isotype	Mouse	Human	FITC	IgG1	R&D Systems
Isotype	Rat	Human	FITC	IgG2b	R&D Systems
Isotype	Mouse	Human	PE	IgG1	BD Biosciences
Isotype	Mouse	Human	PE-Cy5	IgG1	BD Biosciences

Table 2.1. *FACS antibodies used in flow cytometric analysis*

2.6.2 Real-Time Polymerase Chain Reaction (qRT-PCR)

2.6.2.1 Primer design

As previously stated we selected genes based on the literature (section 2). When an appropriate range of genes was selected, highly specific and efficient primers were designed in house using Beacon Designer 7.0 (Biosoft). The primers are listed in Table 2.2.

Target Gene	Accession Number	Sense Primer	Anti-Sense Primer	Tm (°C)
GAPDH	NM_002049	TTG GTA TCG TGG AAG GAC	GTA GAG GCA GGG ATG ATG	55.4
CD34	M81104	TTC CAC TCG GTG CGT CTC	CCC AGA AGG CAG CAA ACT C	59.9
GATA2	M68891	TCA GAC GAC AAC CAC CAC	TCT TGC TCT TCT TGG ACT TG	55.5
HoxB4	NM_024015	AAA GCA AGA AGA AGG AAG AAA G	CAG AGG AAA CAA GAC AGA TGG	52.7
Hb-ß	NM_000518	TTC ACT AG CAA CCT CAA ACA G	CAC CAC CAA CTT CAT CCA C	53.7
Tal1	NM_003189	GCC TGA GTT ATT GAG ATT GTC	CTG AGG TGA GAG GAT TGC	53.4
RunX1	BC136381	CCT CGT GCC TCC CTG AAC	GTA TTG GTA GGA CTG ATC GTA GG	55.5
EPO	NM_000799	CTG ACA CTT TCC GCA AAC	CAA GCA ATG TTG GTG AGG	55.1

Table 2.2. *Primer details qRT-PCR*

2.6.2.2 RNA Isolation

Prior to cellular RNA isolation the following solutions were prepared: 44mL of 100% ethanol were added to 6mL of buffer RPE and 10µL of 1M β-mercaptoethanol (Sigma-Aldrich UK) were added to 1mL of buffer RLT.

Isolated $CD34^+$ UCB cells were transferred to an eppendorf tube and 350 µL buffer RLT were added and incubated for 5 minutes at RT to obtain cell lysate. The lysate was then transferred to a QIA shredder column and centrifuged at 16,000g for 2 minutes. 350 µL of 70% ethanol were added to the tube with eluted liquid and a total of 700 µL were then transferred to an RNeasy Mini column placed in a 2 mL collection tube and centrifuged at 12,000g for 15 seconds. Next, 700 µL of buffer RW1 were added to the column, centrifuged at 8,000g for 15 seconds and the flow through discarded. Then 500 µL of buffer RPE were added to the column and centrifuged at 8,000g for 15 seconds and the flow through was

discarded. This step was repeated again but instead centrifuged for 2 minutes and afterwards the RNeasy Mini column was placed in a new 2mL collection tube and centrifuged at 16,000g for 1 minute. Finally to collect the isolated RNA the column was placed in a 1.5 mL RNase-free tube and 30 µL of UltraPure™ DNase/RNase-Free dH_2O (Gibco, 10977) added to the column and centrifuged at 8,000g for 1 minute. RNA samples were stored at -80°C until required for further processing. All products were purchased from Qiagen (RNeasy Mini Kit) unless stated otherwise.

2.6.2.3 Reverse Transcription

A RT^2 First Strand Kit (QiaGen, 330401) containing 5X gDNA Elimination Buffer (GE), 5X Reverse Transcription Buffer (BC3), RT Enzyme Mix 3 (RE3), Primer and External Control Mix (P2), and RNase-free H_2O was used to synthesise cDNA from isolated RNA. A Genomic DNA Elimination Mixture was prepared by adding 8 µL RNA sample to 2 µL of GE buffer in a sterile PCR tube. Contents were mixed gently with a pipette followed by brief centrifugation and incubated at 42°C for 5 minutes. Next, tube was chilled on ice immediately after for one minute. Parallel, the RT cocktail was prepared by adding 4 µL BC3 Buffer, 1 µL P2 Mix, 2 µL RE3 Mix, and 3 µL H_2O to a sterile PCR tube. First strand cDNA synthesis was executed by mixing 10 µL of RT cocktail gently with the 10 µL Genomic DNA Eliminiation Mixture, incubated at 42°C for exactly 15 minutes and immediately followed by incubation at 95°C for 5 minutes to terminate the reaction. The resulting cDNA was stored at -20°C until required for further processing.

2.6.2.4 RT-PCR

The synthesised cDNA was either diluted 10 or 100 fold with UltraPure™ DNase/RNase-Free dH_2O for subsequent RT-PCR reactions. Prior to RT-PCR reactions the following solutions were prepared. Stock Primer Mixes were prepared from 100 µL Sense Primer and 100 µL of associated Anti-Sense Primer (Invitrogen) according to Table 2.3. Subsequently, a PCR Reagent Stock solution was created from 7.5 µL iQ SYBR Green Supermix (Bio-Rad,

170-8882), 4.5 µL UltraPure™ DNase/RNase-Free dH_2O, and 1 µL Stock Primer Mix per well. All PCR reactions were carried out in iQ 96-Well PCR plates (Bio-Rad, 223-9441).

Diluted cDNA was added to each well in triplicates of 2 µL and 13 µL of PCR Reagent Stock solution was added per well with cDNA to obtain a total working volume of 15 µL. Well content was gently mixed using a pipette whilst avoiding air bubbles. Negative controls were included for each Primer Stock Solution by replacing the cDNA with UltraPure™ DNase/RNase-Free dH_2O, in triplicate.

RT-PCR reactions were carried out using a Bio-Rad iCycler (Version 2.039) and the standard iCycler operating platform (Bio-Rad). The PCR profile included a 3 minute 95°C initialisation step followed by 40 cycles of a 2 two-step amplification programme: 30 seconds at 95°C (denaturation) and 30 seconds at the optimum annealing and extension temperature for each specific primer (Table 2.2, Tm). Threshold cycle (C_t) values were calculated when the fluorescence from SYBR Green binding to double stranded DNA entered its exponential phase during amplification. Subsequently, PCR products were subjected to melting curve analysis.

Target Gene	Sense Primer-nanomoles	Sense Primer-Volume (µL)	Antisense Primer-nanomoles	Antisense Primer-Volume (µL)
GAPDH	18.0	180	24.3	243
CD34	17.6	176	13.9	139
GATA2	23.5	235	18.6	186
HoxB4	11.8	118	13.0	130
Hb-ß	17.8	178	21.6	216
Tal1	21.4	214	21.2	212
RunX1	20.6	206	21.7	217
EPO	25.3	253	23.7	237

Table 2.3. *Lyophilised, in-house designed, primers dissolved in DNase/RNase free dH_2O*

2.6.3 Histology

2.6.3.1 Preparation of a blood/cell smear

Cell suspensions were diluted to appropriate cell densities for analysis (*i.e.* whole blood was diluted 1:2 in PBS). A drop of cell suspension (5-20µL) was placed near the edge of the glass slide. Another glass slide, the spreader, was used to create a smear by placing it at a 45° angle and backing into the drop of cell suspension so that the drops spread evenly along the edge before pushing the spreader across the slide to create the smear. The slide with smear was air dried before the staining procedure was started.

2.6.3.2 Brilliant cresyl blue stain for reticuloctyes

Brilliant cresyl blue (Fluka, Sigma-Aldrich) stock solution was diluted 1:80 to 1:100 with PBS. For a single test 20 µL blood or 20 µL cell suspension was added to 20 µL brilliant cresyl blue (BCB) solution in a tube. This solution was mixed thoroughly and a thin smear was prepared after 30 minutes of incubation at room temperature. For test in series, thin smears of BCB solution were prepared on microscope slides. Air-dried slides were stored up to 2-3 weeks. When used, a small drop of blood was quickly smeared over the stain layer and the still wet preparation was placed in a moist chamber for 5-10 minutes before air drying. Samples were observed using light microscopy (Zeiss).

2.6.3.3 Methylene blue stain for observation of cell nucleus

Glass slides with air dried cell smear were subjected to staining using a 0.05% solution of methylene blue in dH_2O (Sigma-Aldrich). Samples were incubated with the stain for 5 minutes at room temperature followed by rinsing in dH_2O. Samples were air dried at room temperature before being observed using light microscopy (Zeiss)

2.6.3.4 Preparation of cells for scanning electron microscopy (SEM)

Prior to cell seeding, cover slips (Sarstedt, 83.1840.002) were coated with Poly-L-Lysine (Sigma, P4832) for 5 minutes. The solution on the coverslip was discarded and cover slips were washed with PBS and air dried for 2 hours. Cells ($1x10^4$-$1x10^6$) were seeded on the coated cover slips. Next, cells were fixed with 1% (w/w) gluteraldehyde (Sigma, G5882) in PBS for 1 hour, cells were washed 3 times for 5 minutes with PBS followed by 1 wash in deionised water. Cells were then dehydrated using a graded ethanol series; 15%, 25%, 50%, 50%, 70%, 2x 100% (v/v) ethanol for 10 minutes each. Cover slips were dried with hexamethyldisilazane (Fluka, 52620) for 3 minutes prior to mounting onto aluminium specimen stubs (Agar Scientific Ltd) using carbon double sided sticky tabs (Agar Scientific Ltc). Afterwards, these specimens were gold sputter coated (Emitech K575X Sputter Coater) and analysed using a scanning electron microscope (LEO 1550).

2.6.4 Statistics

Statistical analyses were carried out using independent sample T-tests using the software package Microsoft Excel. All used p-values were stated with the corresponding results (Chapter 3 and 4). All error bars shown were calculated using the 'STDEV' formula (Standard Deviation) in the software package Microsoft Excel.

Chapter 3

Results - Haematopoietic Cell Culture

3.1 Introduction

The HSC is fundamental for the development of RBCs. HSCs can be found in various tissues throughout the human body. The most used sources are BM, UCB, PB [87]. Another source for HSCs are iPSCs which can be created from a range of adult terminally differentiated cells (e.g. fibroblasts).

The use of pluripotent cells is more complicated for establishment of erythroid cultures and there are also a number of ethical issues to consider. In contrast, the ease of access and relatively low costs make the use of UCB, and particularly PB, more favourable as sources for potential scaled up erythroid differentiation.

As described previously, HSCs are reported to possess CD34 receptors which, in turn, provide a potential method to distinguish these cells from other cells [88]. Therefore, CD34 specific antigen can also be used in separating these cells from the rest of the cell population. The most common technique used for the isolation of HSC from blood is the utilisation of magnetic bead cell separation to target specific stem cells (*i.e.* those expressing $CD34^{+}$) [88]. This method is described in Chapter 2 and Figure 3.1. and shows the principle of this cell separation system. In short, the technique utilises magnetic beads labelled with specific antigens to target the CD34 receptor of the stem cells.

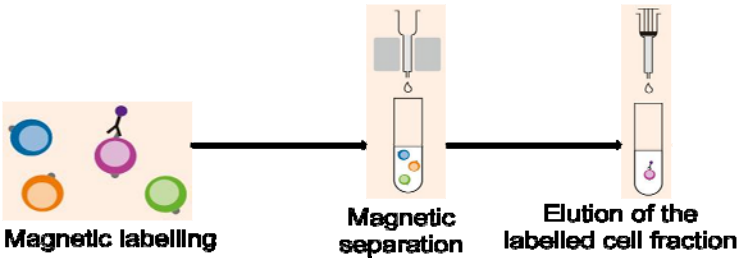

Fig. 3.1. *Magnetic Cell Separating Technique (Miltenyi Biotech)*

After mixing and incubating with a heterogeneous cell population, the mixture of beads and cell suspension is passed through a magnetic column where the magnetic beads and targeted

cells are retained. After all non cell-bead conjugates have been washed through, the targeted cells will be eluted.

This chapter focuses on the establishment of HSC cultures. The objectives were 1) to isolate the cells from PB and 2) to determine how to optimise the conditions in order to maintain these stem cells in culture for more than 7 days.

The hypothesis of this chapter is: Can the long term culture of HSC be improved by introducing an optimised medium composition and a stromal cell support to the cell culture environment?

3.2 Suspension cell culture

All cultures involving HSCs are suspension cell cultures. This means that the cells have no automatic impulse to adhere and spread onto the tissue culture-treated culture plastic. For example, Figure 3.2 shows the differences between cells in suspension and adherent cells. The advantage of suspension cell cultures over adherent cell cultures is based on the ease of scale up due to only volume, as opposed to surface, restrictions for the concentration of cells. In addition, easy cell subculture whereby the culture can be diluted to stimulate growth.

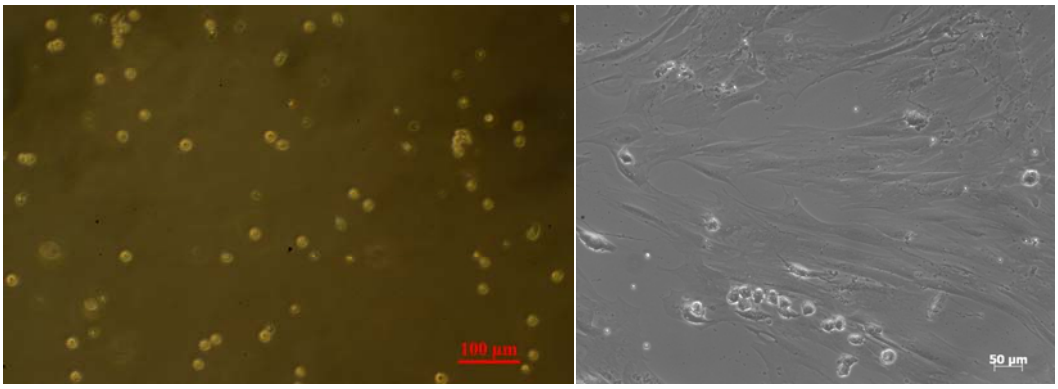

Fig. 3.2. *Suspension Cell Culture vs. Adherent Cell Culture - The suspension culture with UCB isolated cells have a round morphology (left) whereas the MSCs in the adherent culture have a stretched, elongated morphology (right)*

During this study, for example, to setup an expansion culture with a T25 culture flask the working volume of 5 mL of medium requires an adherent cell culture of 2,000 cells/cm^2 whereas for a suspension cell culture at least 30,000 cells/mL are needed. This means that to initiate a culture 50,000 and 150,000 adherent or suspension cells will be required respectively. A number of cultures were performed in well plates (6, 12, 24 or 48 wells) and required medium volume and cell number proportional to the numbers stated above.

3.2.1 Isolation and culture of PB isolated $CD34^+$ cells

An essential step in this project was the acquisition of $CD34^+$ cells (HSCs) isolated from human tissue. As described in Chapter 1, blood is an ideal source for HSCs as it is accessible and taking blood samples is common practice in clinical environments. In the first set of experiments PB was used.

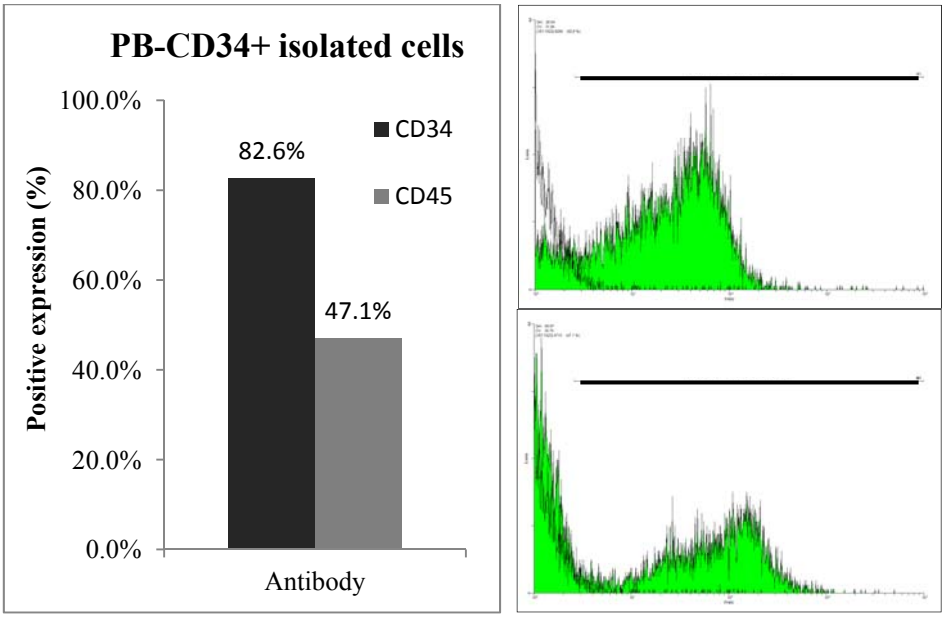

Fig. 3.3. *The $CD34^+$ purity of this cell population is 82.6% which can be derived from the graph on the right (top). The expression of CD45 is 47.1%, indicating a minimum of 35.5% of the cells are HPCs*

Buffy coats were prepared from 30mL of blood and subsequently $4x10^7$ MNCs were isolated. After applying the CD34 MicroBead magnetic separation approximately $3x10^4$ cells were collected. Figure 3.3 illustrates the results of flow cytometry analysis which shows that of the cell population 82.6% expressed CD34 and 47.1% expressed the haematopoietic cell associated marker CD45 [89].

When comparing the number of isolated $CD34^+$ cells between two age groups a difference in isolation efficiency was found. Blood isolated from the age group younger than 30 years contained a higher proportion of CD34 positive cells than blood isolated from donors older than 30 years, normalised for the number of isolated MNCs (Figure 3.4).

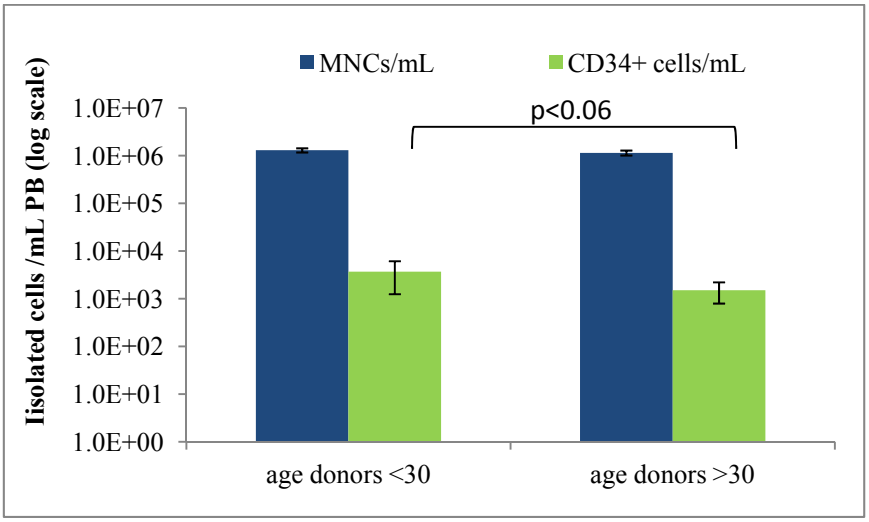

Fig. 3.4. *Isolated cell number per donated mL of blood from donors younger and older than 30 years. Donors aged younger than 30 years have a slightly higher number of CD34$^+$ cells/mL of blood*

Once successful isolation of PB-CD34$^+$ cells was established, cultures with PB CD34$^+$ isolated cells were setup with the aim of expanding HSCs. In each instance, between 30 and 40mL of PB were isolated from healthy voluntary donors. Subsequently, MNCs and CD34$^+$ cells were isolated as described in Chapter 2. Once cultures were either initiated for HSC expansion or erythroid maturation (described in Chapter 4), the isolated cells did not always expand at a rate consistent with that in the literature. Generally, the expansion potential was very low. Cultures with a cell density between $3x10^3$ cells/mL and 10^4 cells/mL, the recommended cell density for UCB-CD34$^+$ cultures, struggled to expand and remain viable for more than 2 days. Overall, the results obtained showed that the CD34$^+$ cell isolation from small volumes of PB for culture, and therefore limited numbers of isolated PB MNCs (PBMCs) was restricted.

To prevent early termination of PB-CD34$^+$ cell cultures as a result of low cell densities in culture, a higher number of PBMCs was required initially. Therefore CD34$^+$ cells were isolated from leukocyte cones which should contain more MNCs compared to 30-50mL PB. These cones, prepared by the National Health Service (NHS) contained 10mL of Buffy Coats and RBCs .These cones (NC24) were prepared daily by the NHS (United Kingdom)

with buffy coats pooled from a number of donors. Figure 3.5 shows a buffy coat cone with a volume of 10mL. In an aseptic environment the buffy coat was transferred to a leukocyte tube containing separation medium (Chapter 2). The procedure was similar to isolating MNCs from PB.

Fig. 3.5. *Leukocyte cone with a capacity for 10mL Buffy Coat*

With the current isolation method, a theoretical maximum of $1.3x10^8$ MNCs could be used for isolation. Prior to CD34 magnetic cell separation approximately $6.5x10^7$ viable PBMCs were isolated per leukocyte cone. With the selected separation technique no more than $6x10^4$ cells were isolated. After a 2 day culture period in commercially available serum-free medium, further described as P-medium (HPC expansion medium (PromoCell)) supplemented with HPC Cytokine Mix E (PromoCell), all viable cells ($1.8x10^4$) were analysed by FACS. This HPC Cytokine Mix E contained the cytokines SCF, TPO, Flt3-L, and IL-3. Of the total isolated cell population, 88.4% of the cells were positive for CD34 (Figure 3.6).

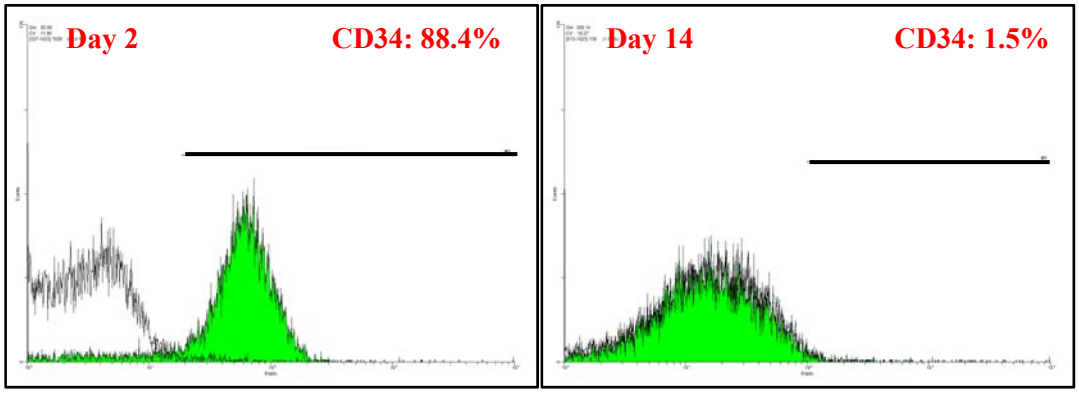

Fig. 3.6. *In two experiments the CD34 expression after 2 days (left) and 14 days (right) of $CD34^+$ isolated PBMCs from Leukocyte Cones. In the first experiment we harvested $1.8x10^4$ cells after two days (88.4% $CD34^+$). In the second culture we harvested $1.9x10^4$ cells after 14 days (1.5% $CD34^+$)*

This experiment was repeated and after a culture period of 14 days it was found that only 1.5% of the cell population was CD34 positive (Figure 3.6). During this culture period cells were counted at days 7 and 14 and $9x10^4$ and $1.9x10^4$ cells were found respectively. These data show that a HSC culture was not able to maintain a CD34 positive phenotype beyond 7-14 days. Results in the literature depict a similar picture and long term culture of HSCs remains a challenge. The current results also showed that isolating $CD34^+$ cells from PBMCs is still a challenge.

3.2.2 Isolation and culture of UCB isolated $CD34^+$ cells

Another source for HSCs is UCB. To study the expansion of HSCs isolated from UCB frozen aliquots of pre-isolated $CD34^+$ cells from fresh blood (kindly provided by Stem Cell Biology Lab, University of Leipzig) were used. For the expansion of UCB-$CD34^+$, $2.95x10^5$ cells were cultured in serum-free P-medium containing HPC Cytokine Mix E at $3x10^4$ cells/mL. After 10 days, $15.6x10^6$ cells were harvested and subsequently analysed for the expression of CD34, CD44, and Hb-β using Flow Cytometry (Figure 3.7). The results show that the UCB-$CD34^+$ cells retained their HSC phenotype after a 10 day culture in P- medium. At day 10, $7.8x10^6$ cells were subcultured and after an additional 3 days of HSC expansion a total of $17.6x10^6$ cells were then collected which were processed for freezing and stored in LN_2 for future use.

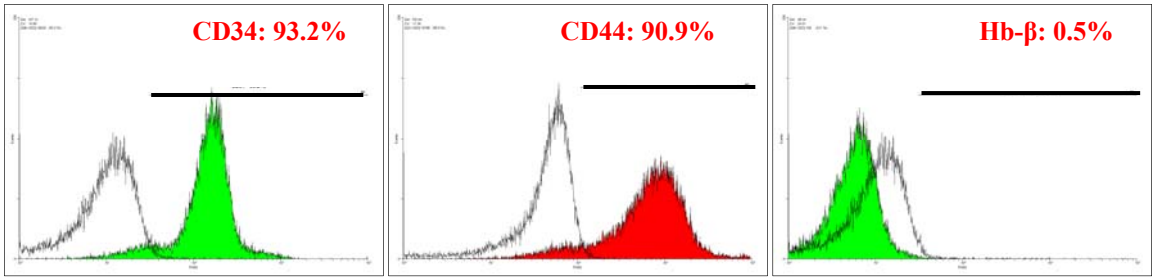

Fig. 3.7. *After 10 days of Haematopoietic Progenitor Expansion of $CD34^+$ cells isolated from UCB the cells maintained their primitive phenotype with a positive expression of CD34 (93.2%) and also highly positive for the leukocyte marker CD44 (90.9%) while negative for the oxygen carrying molecule Hb-β (0.5%)*

Two types of serum free media, other than P-medium, were tested for the expansion of HSCs. One type of medium is a chemically defined basic medium that is also used in the erythroid maturation of stem cells: D-medium. The other medium was the commercially available StemLine II HSC medium and will hereafter be described as S-medium. Both D-medium and S-medium were tested during HSC culture in their basic form and when supplemented with SCF (100ng/mL) and IL-3 (5ng/mL).

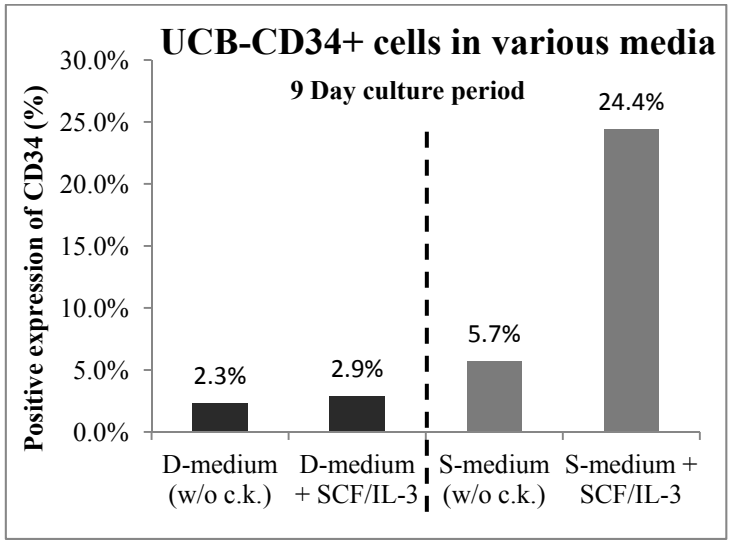

Fig. 3.8. *After 9 days of culture the $CD34^+$ expression dropped from 93.2% to below 6% for all conditions except for cells cultured in HSC expansion medium (S-medium w/ SCF/IL-3): 24.4%*

The cell number and cell viability were recorded for these culture conditions at day 0, day 4, and day 9 (Table 3.1). Both culture conditions containing cytokines SCF and IL-3 showed a high cell viability over 9 days (>95%). The cultures without cytokines did not possess any expansion potential. At day 4 the culture containing D-medium without cytokines showed a decrease in cell number and viability and after 9 days the cell viability dropped to below 25%. The cell culture with S-medium, designed for HSC expansion, contained SCF and IL-3 and showed an expansion of 13.1 fold compared to 2.6 fold for the cell culture with D-medium containing cytokines.

Over 90% of this HSC population used during this experiment was positive for CD34. Cells were cultured at $5x10^4$ cells/mL. These results show that during a 9 day culture, the CD34 expression decreased dramatically for both D-medium and S-medium lacking cytokines (Figure 3.8). The loss of CD34 phenotype for cells cultured in S-medium with SCF/IL-3 was less dramatic, but lower when compared to the expression on day 0.

	Day 0		Day 4		Day 9	
	Cell no.	Viability	Cell no.	Viability	Cell no.	Viability
D-medium (w/o c.k.)	$2.5x10^5$	>95%	$1.5x10^5$	>80%	$6x10^4$	<25%
D-medium + SCF/IL-3	$2.5x10^5$	>95%	$3.2x10^5$	>95%	$6.5x10^5$	>95%
S-medium (w/o c.k.)	$2.5x10^5$	>95%	$2.8x10^5$	>95%	$1.1x10^5$	>70%
S-medium + SCF/IL-3	$2.5x10^5$	>95%	$1.02x10^6$	>95%	$3.28x10^6$	>95%

Table 3.1. *The cell number and cell viability decreased for cultures without cytokines. The cell number increased dramatically for the condition with the better optimised HPC expansion medium (S-medium + SCF/IL-3). Cell viability remained high (>95%) for culture conditioned which contain SCF and IL-3*

After establishment and analyses of cultures with UCB-$CD34^+$ cells, the CD34 Magnetic Cell Separation method was used to isolate cells from frozen aliquots of UBMCs. This method is a standard method to isolate CD34+ cells and is widely used in the literature. HSCs isolated from UBMC have a higher expansion potential and a unit of UCB contains a higher number of $CD34^+$ cells compared to cells isolated from a unit of PB [56].

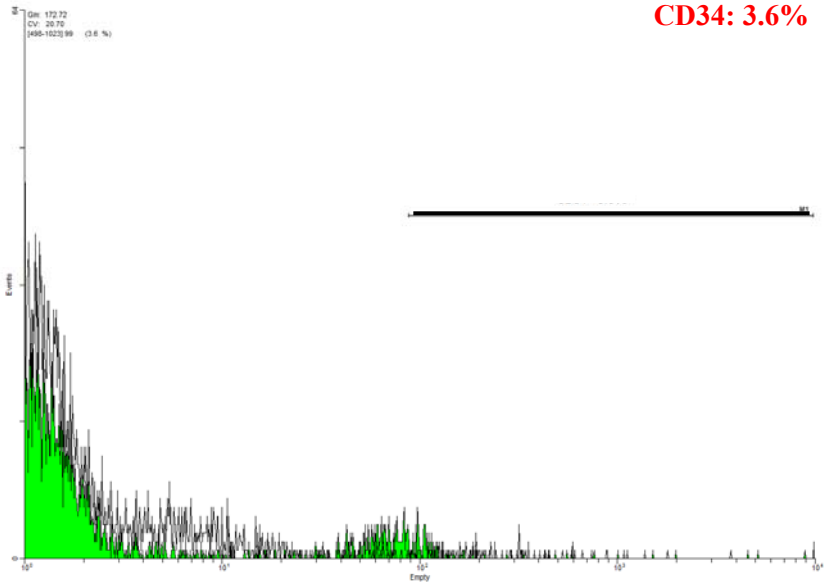

Fig. 3.9. *CD34 expression of cells isolated from UBMCs using MACS®. The number of $CD34^+$ cells was ~1500 out of a total isolated $4.2x10^4$ (~3.6%)*

From $5.9x10^6$ thawed UBMCs approximately $4.2x10^4$ cells were isolated using the CD34 magnetic cell separation method. Of these cells, ~1500 cells were CD34 positive; ~3.6% (Figure 3.9). In a separate cell isolation experiment with a significantly higher number of thawed UBMCs (~$15x10^7$) approximately $1.9x10^5$ cells were isolated. Analysis with flow cytometry showed that ~11.2% of these cells were CD34 positive after a culture period of 7 days in P-medium with cytokines. After 10 days of culture the percentage of $CD34^+$ cells in the population dropped to 3.7% (Figure 3.10). For flow cytometric analysis, $1x10^5$ cells and $5x10^5$ were used for day 7 and day 10 respectively. At day 10, this was the total number of cells harvested.

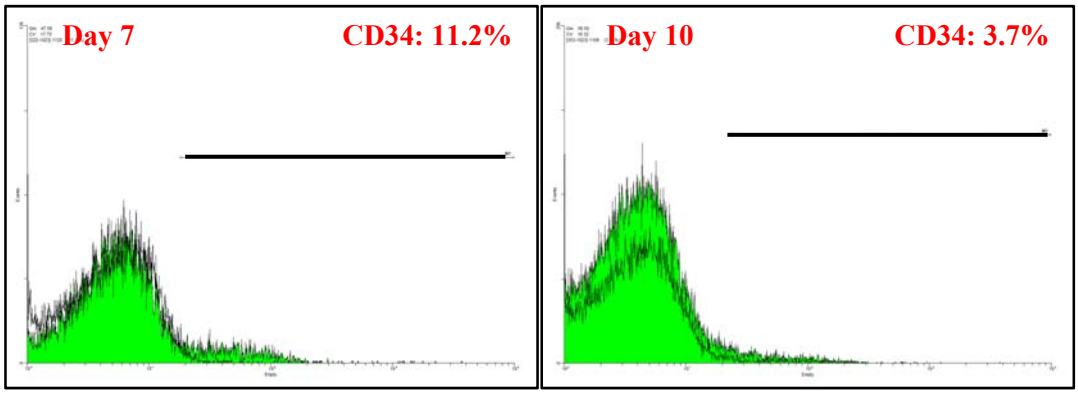

Fig. 3.10. *The CD34 expression of CD34 isolated UBMCs decreases over time.*

It was observed that not all cultures with $CD34^+$ isolated UBMCs maintained their phenotype throughout culture. To stimulate prolongation of CD34 positive phenotype, a supportive stromal cell layer of human MSCs was used. The MSCs were either isolated from BM, BMSCs, or adipose tissue: adipose MSCs (AMSCs). The medium used was defined especially for expansion of HSCs by the Stem Cell Biology Lab (University of Leipzig). This medium was named I-medium and during this set of experiments the medium contained FCS and cytokines (SCF, Flt3-L, and TPO). Due to insufficient cell numbers this setup could not be tested for P-medium.

Morphological images of the co-cultures with UCB-$CD34^+$ cells and either adipose or BM stromal cells are shown in the figures below (Figure 3.11-14). The total number of cells

recorded in the culture without a stroma was lower than the number of cells cultured on a stroma after both 7 and 14 days. The UCB-$CD34^+$ cells on an adipose stromal layer showed a round morphology and were distributed on the elongated stromal cells after 7 days of culture. During an additional 7 days of culture the cell number increased and the morphology remained similar. For the cells cultured on a BM stromal layer a range of cell sizes was observed and in some instances the cells were clustered together on day 7. The cell number was lower over the first 7 days of the culture and over the additional 7 days the cell expansion rate increased, resulting in a higher cell number when compared to the cultures on an adipose stromal layer. The cell cultures without a stromal cell layer were less viable at day 7 and day 14. These cultures contained more cell debris than the cultures containing a stromal layer.

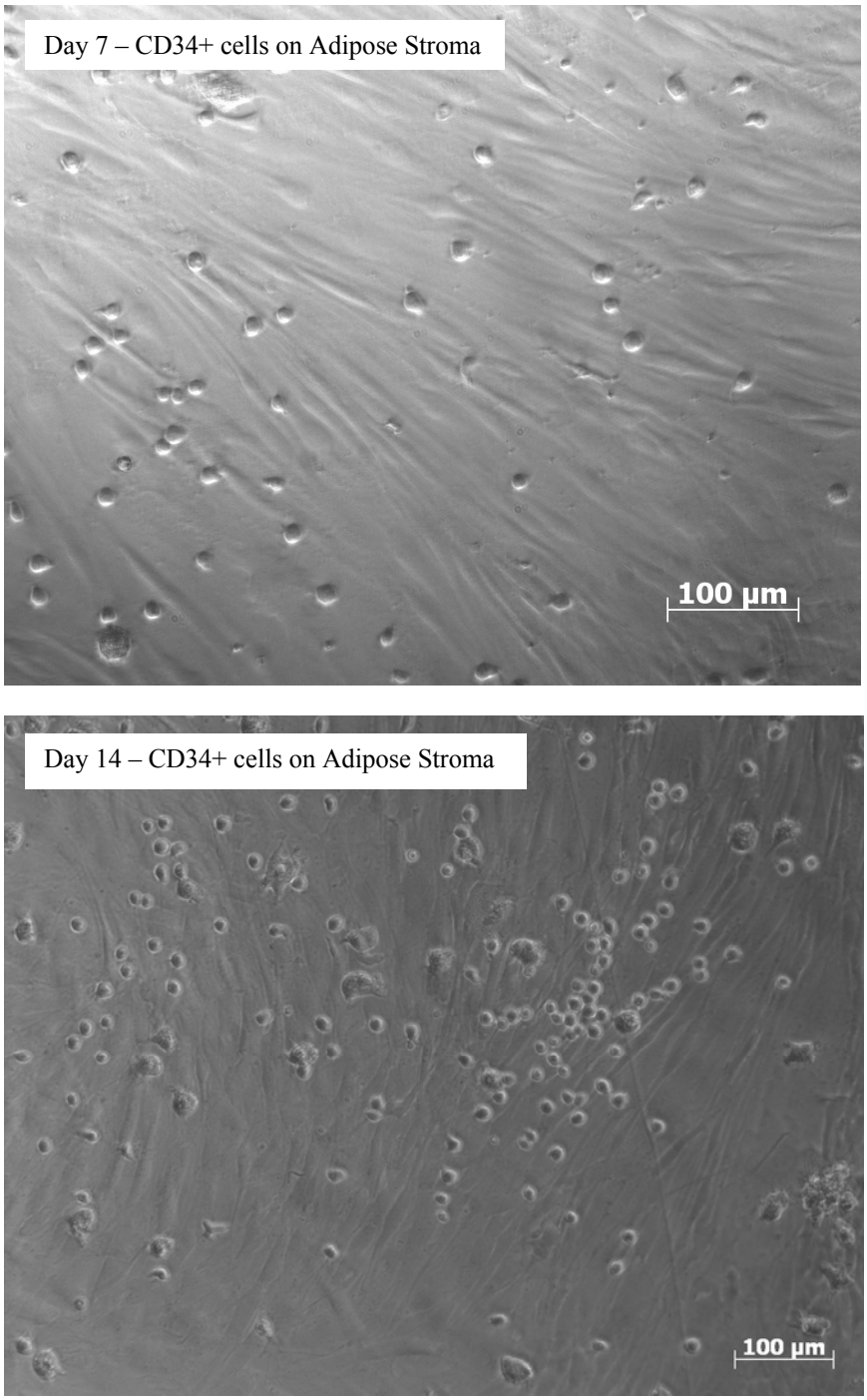

Fig. 3.11. *UCB-CD34^{+} cells cultured in I-medium on adipose isolated MSCs showed a round like morphology on the elongated stromal cells at day 7. Few cells were close to each other whereas other cells were more spread out on the stromal layer. On day 14 the cell density had increased compared to day 7. The cells had a round like morphology with a few cells now slightly bigger than the majority of the cell population. The cells are spread out on the stromal layer*

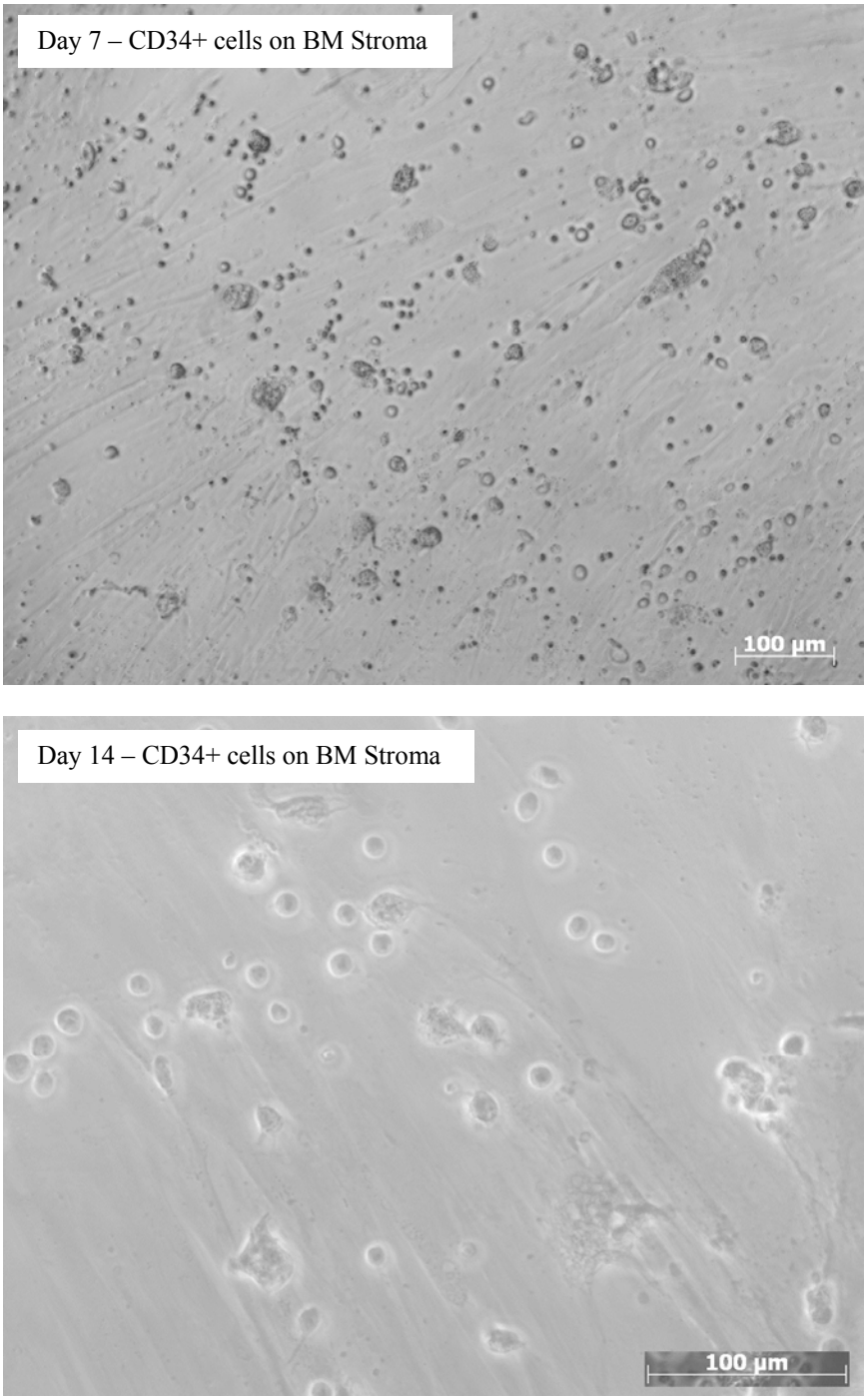

Fig. 3.12. *UCB-CD34$^+$ cells cultured in I-medium on BM isolated MSCs showed a variation in morphologies and different sizes at day 7. Only a minority of the cells had a round-like morphology comparable to the healthy CD34$^+$ cells from Figure 3.2. On day 14 the majority of the cells had a round-like morphology and an increased cell density can be observed*

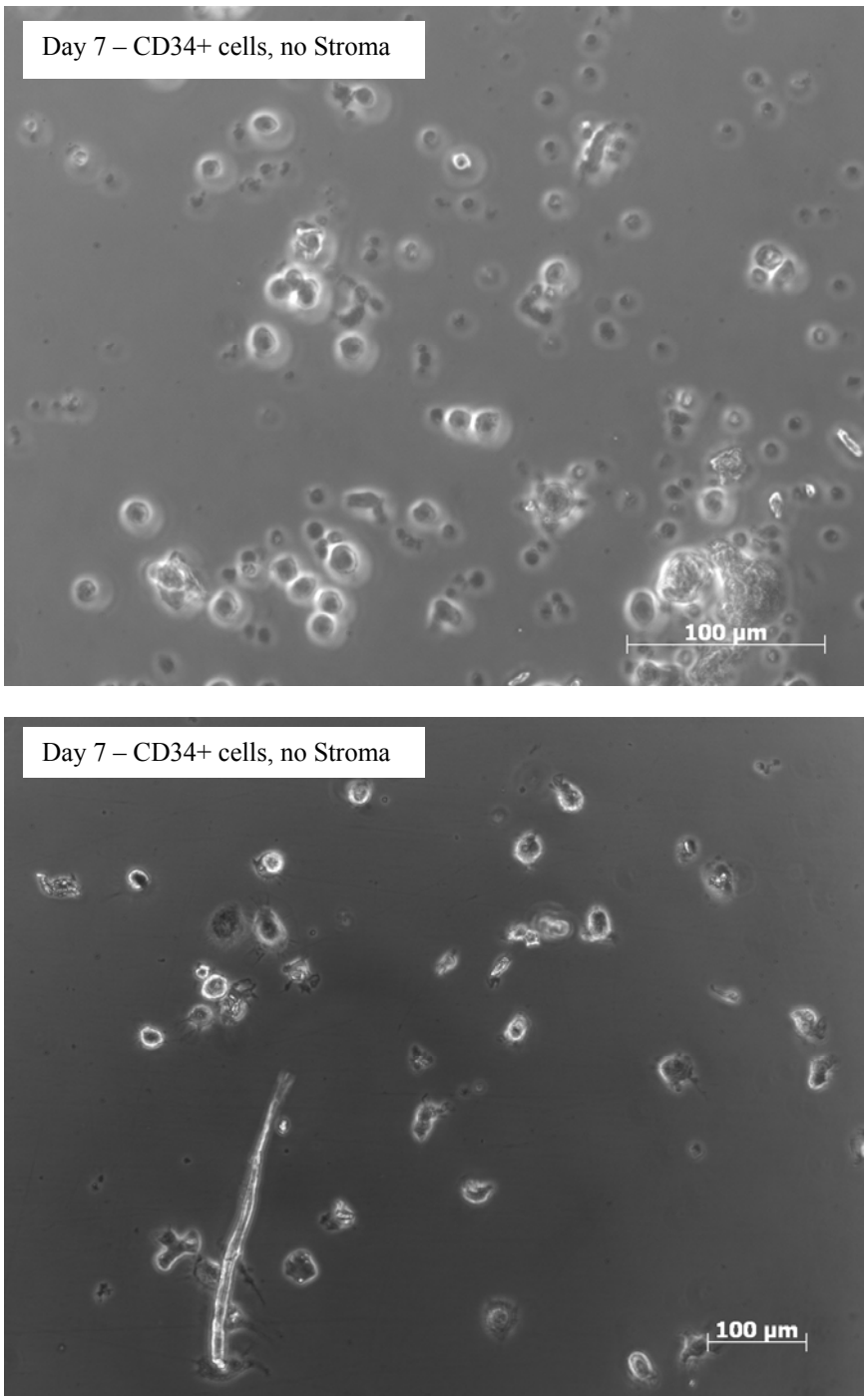

Fig. 3.13. *UCB-CD34^{+} cells cultured in I-medium without a stromal cell layer at day 7 did not have a round like morphology for the majority of the cell population and a lot of cell debris could be found in the culture. At day 14 the number of viable cells has decreased to below 20% and a lot of cell debris could be observed, similar to day 7*

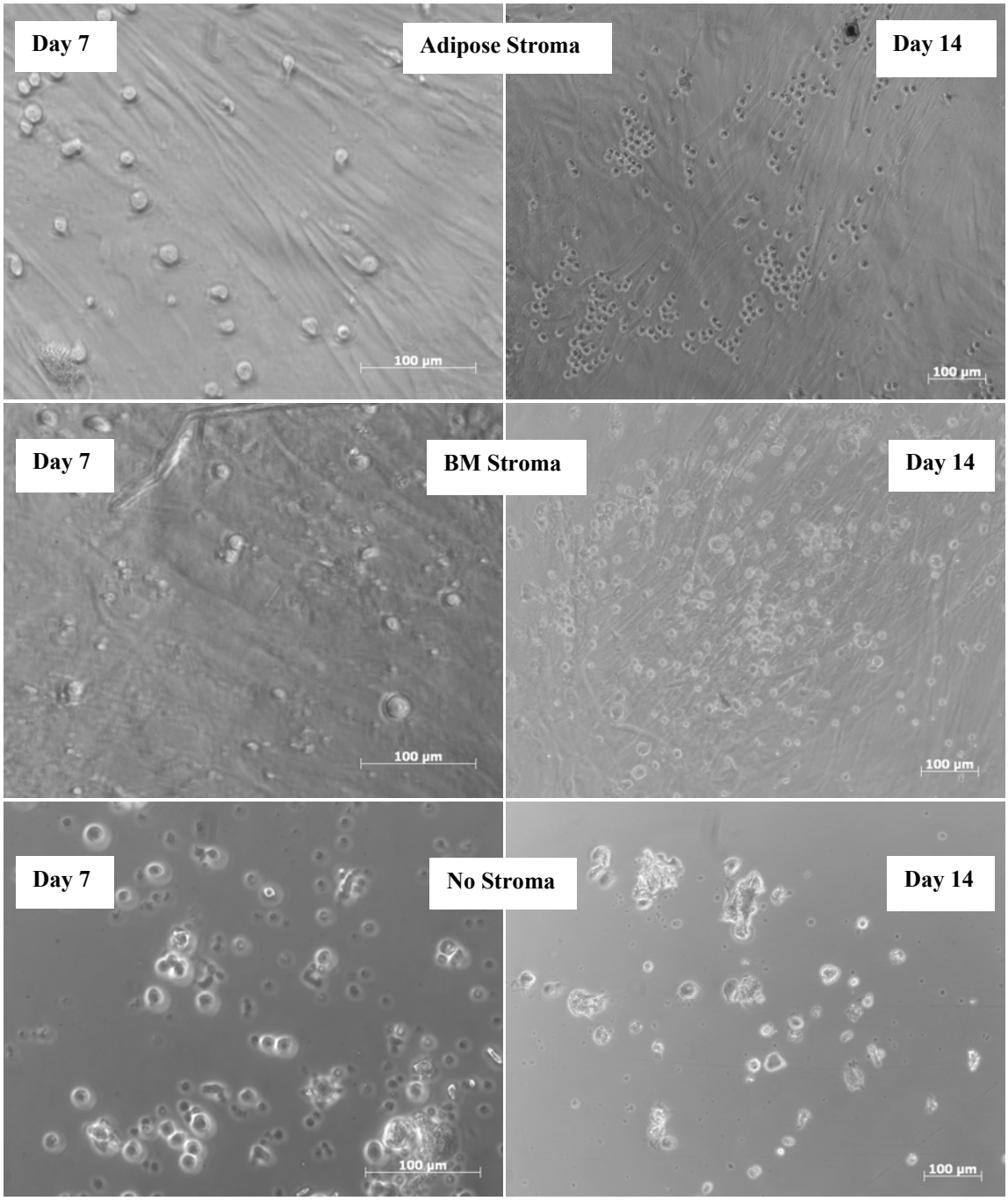

Fig. 3.14. *Morphology of UCB-CD34$^+$ cells in I-medium – This overview shows the various stromal cells and the influence on CD34$^+$ cell morphology. After 14 days, the cells on the supportive cell layer show a morphology similar to the UCB isolated suspension cells in Figure 3.2*

Results obtained with flow cytometric analysis showed for a culture without a stromal layer the expression of CD34$^+$ increased between days 7 and day 14 whereas for the BMSC and AMSC stromal layers the CD34$^+$ expression decreased (Figure 3.15). During harvest of the cells in suspension, a minority of stromal cells became detached from the tissue culture

plastic. When cultured on an AMSC stroma, the relative expression of CD34 increased between day 7 and day 21. Due to infections no data was obtained for the cultures with BMSC stroma and without a stroma on day 21. The population of cells cultured without a supportive stromal layer showed a higher $CD34^+$ expression than the cells on a stromal layer but the cell morphology was different to $CD34^+$ cells freshly isolated from MNCs. When compared to previous expansion cultures with UCB-$CD34^+$ cells, the percentage of $CD34^+$ was lower for cultures without a supportive stromal layer.

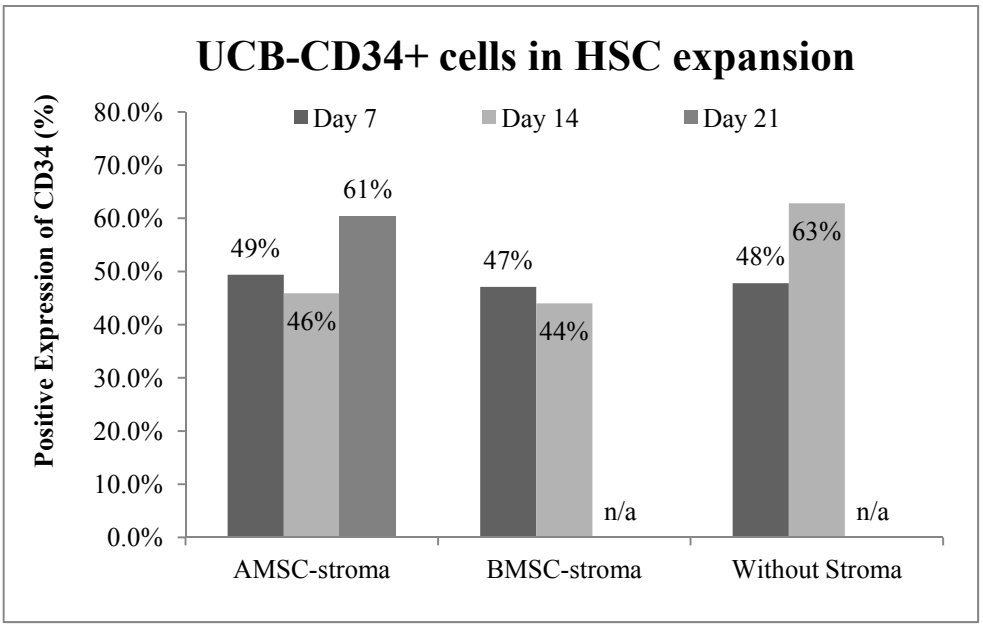

Fig. 3.15. *The expression of the HSC phenotype marker CD34 on cells cultured with and without a supportive MSC stromal cell layer. Due to unexpected premature termination of cultures without a stromal layer and with a BMSC stromal layer no results could be obtained for the last data point*

3.3 Analysis of PB-CD34$^+$ cell cultures

After results were obtained with the various expansion options used for UCB-CD34$^+$ cells, the expansion method for PB-CD34$^+$ cells was optimised. The cells were cultured on a BMSC stroma in P-medium, I-medium, or D-medium, all cultures containing SCF, Flt3-L, and TPO (50 ng/mL). P-medium and D-medium were serum-free, I-medium contained FCS.

The expression of CD34 was analysed with flow cytometry and the results in Figure 3.16 shows that the percentage of cells expressing the HSC associated antibody decreased dramatically over 28 days. PB-CD34$^+$ cells cultured in P-medium showed the lowest expression of CD34 between day 7 and day 28 (<20%). Cells cultured in D-medium showed the highest expression at day 7 and over time this decreased the most quickly to ~10% positive expression. On day 21, PB isolated cells cultured in I-medium showed overall the highest expression of CD34$^+$ (~20%) whereas this was over 50% at day 7 of culture. The initial CD34$^+$ expression at day 0 of the culture could not be measured due to insufficient cell numbers for analysis with flow cytometry.

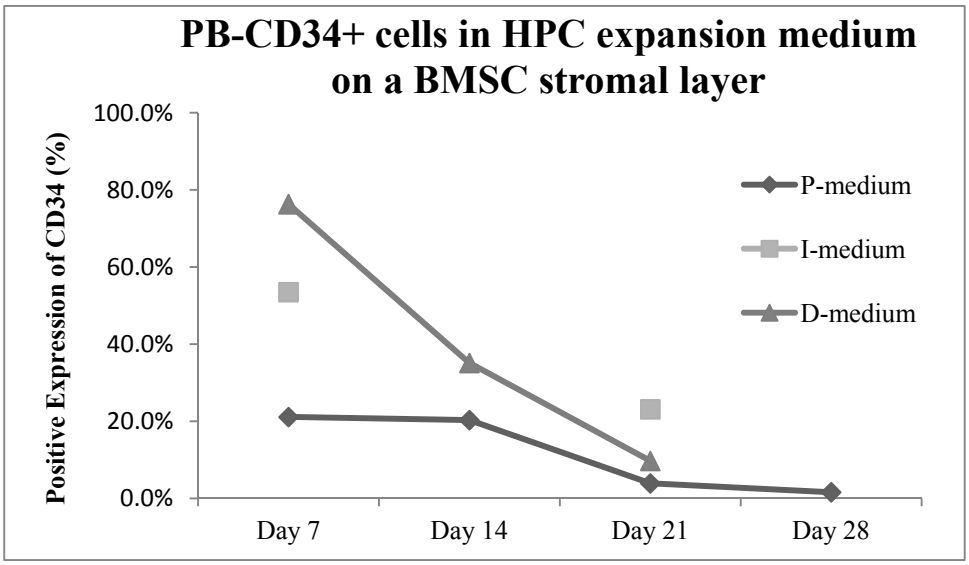

Fig 3.16. *The CD34$^+$ expression of cultured PB-CD34$^+$ cells drops over a 21 day culture period. No data for I-medium on day 14 due to insufficient cell numbers for analysis. No data for I-medium and D-medium on day 28 due to unexpected premature termination of the cultures*

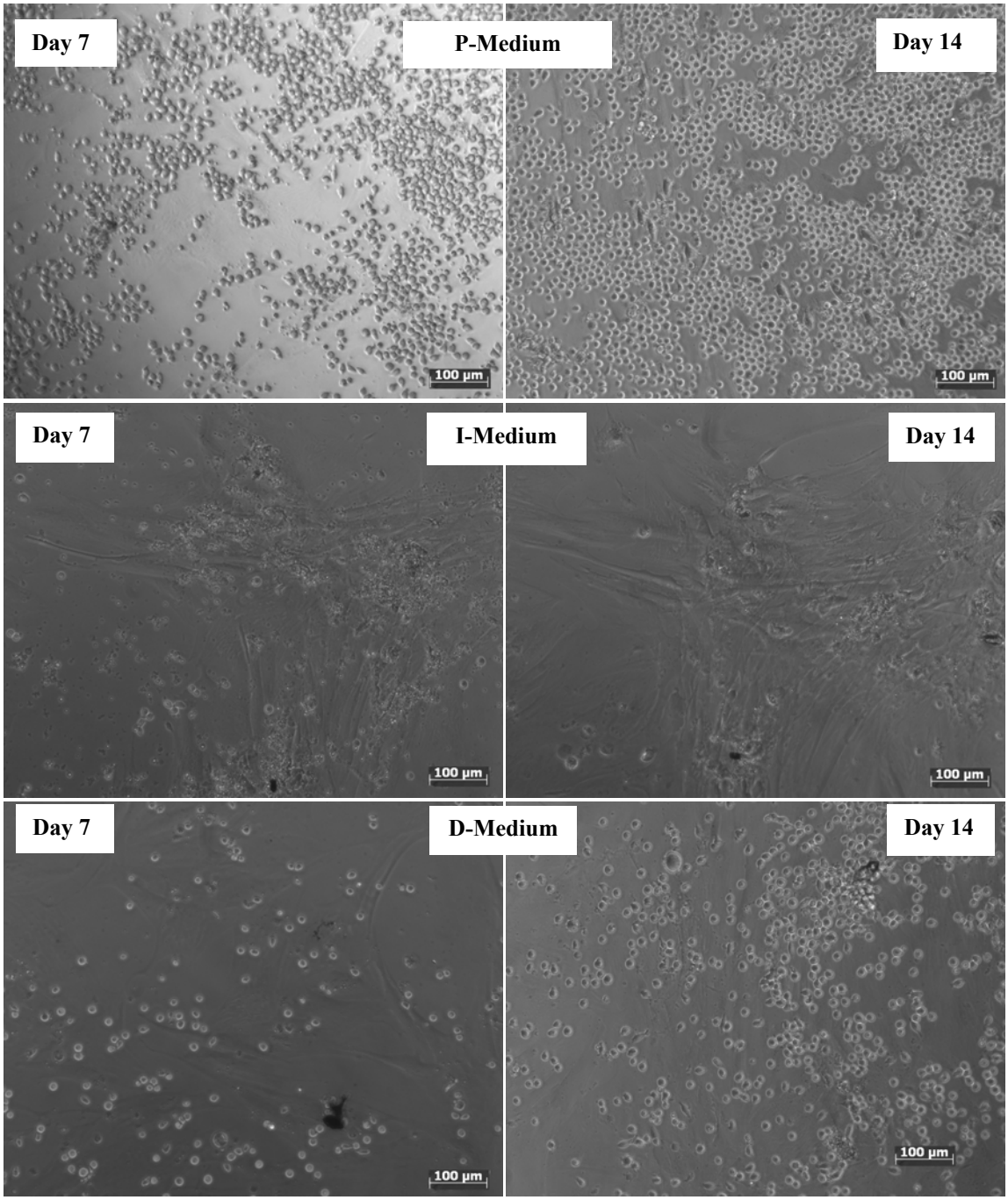

Fig. 3.17. *Morphology of PB-CD34$^+$ cells on a BMSC stromal layer*

The cell number recorded in D-medium was lower than recorded for the P-medium, but the fraction of CD34$^+$ cells was higher for D-medium on day 7 (Figure 3.17). Due to insufficient cells, no flow cytometric analysis could be performed for cells cultured in I-medium on day 14. On day 21 the cultures with I-medium and D-medium were infected.

RT-PCR was used to confirm a list of genomic markers expressed in isolated cells which were able to rapidly proliferate, and then able to differentiate into RBC progenitor cells (Chapter 4).

The focus of the first stage of this research was the isolation of HSCs from UCB. The $CD34^+$ cell population isolated from UCB was analysed for haematopoiesis-related genes, as stated in the literature, in order to create a genomic fingerprint. Genes with a significant role in the regulation of the fate of HSCs are members of the GATA, SCL/Tal-1 and RunX families as well as HOX-B4. The cell population was also tested for the characteristic HSC/erythroid markers CD34, Hb-ß, and EPO [89]. The expression of the preliminary set of genes linked to haematopoiesis is listed in Table 2.1 and the data is shown in Figure 3.18 and Table 3.2. GAPDH is constitutively expressed and served as an internal baseline for expression control (Figure 3.18).

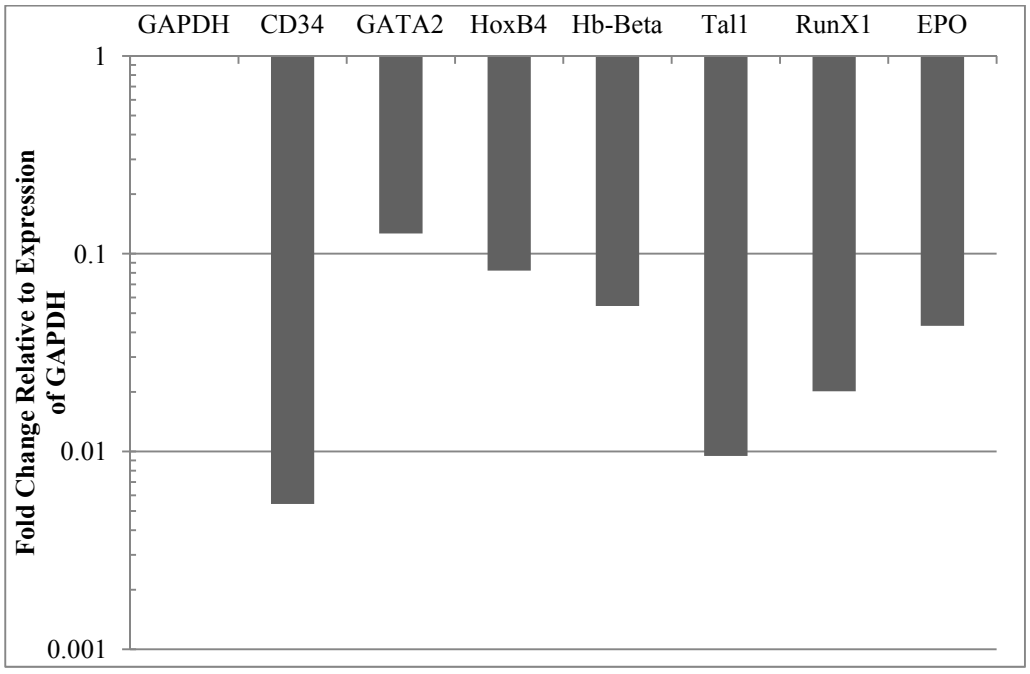

Fig. 3.18. *List of genes in $CD34^+$ UCB cell-derived MNCs, after isolation from UCB. GAPDH is the housekeeping gene.*

The preliminary genes list gives a summarisation of haematopoiesis-associated genes. The results showed positive expression of the genes in a $CD34^+$ cell population isolated from UCB when compared to the positive control (*i.e.* GAPDH). A 1:10 working concentration of cDNA in dH2O had an advantage over a 1:100 concentration due to the lower C_t values which indicated stronger positive reactions.

	Concentration 1:10		**Concentration 1:100**	
	Average C_t	StDev	Average C_t	StDev
GAPDH	21.4	0.2	25.1	0.2
CD34	28.5	0.7	32.6	0.3
GATA2	25.8	0.7	28.1	0.5
HoxB4	24.7	0.3	28.7	1.1
Hb-Beta	26.0	0.3	29.3	0.5
Tal1	27.9	0.2	31.8	0.2
RunX1	27.6	0.3	30.7	0.3
EPO	26.0	0.2	29.6	1.0

Table 3.2. *Preliminary gene expression profile of haematopoiesis genes which are expressed in $CD34^+$ UCB cell-derived MNCs. GAPDH is the housekeeping gene.*

3.4 Expansion of $CD34^+$ cells within a MNC population

The obtained results showed that there were issues with establishing viable HSC expansion cultures with the aim of providing stem cells for the production of RBCs. Further optimisation was needed in order to create enhanced viable HSC expansion cultures. In the literature it can be found that Van Den Akker *et al* (2010) published data which suggested that the majority of *in vitro* erythroid expansion potential resides in CD34 negative cells; these cells significantly increased the erythroblast yield from PB [41]. This principle was applied by incorporating MNC-$CD34^-$ cells for expansion cultures prior to their incorporation for erythroid differentiation studies.

3.4.1 Expansion of $CD34^+$ cells within PBMCs

Expansion cultures with PBMCs were initiated to analyse a possible increase in the output of $CD34^+$ cells and potential benefit for long term viability of HSCs. $CD34^+$ cells within the isolated MNCs population were allowed to proliferate within a heterogeneous cell population which possibly mimics the more natural *in vivo* conditions. This approach also allowed PB-$CD34^-$ cells to show potential for HSC expansion. Data was analysed using flow cytometry and the figures shown in Figure 3.19 and Figure 3.20 depict the typical flow cytometry graphs upon on which the CD34 expression profiles were based.

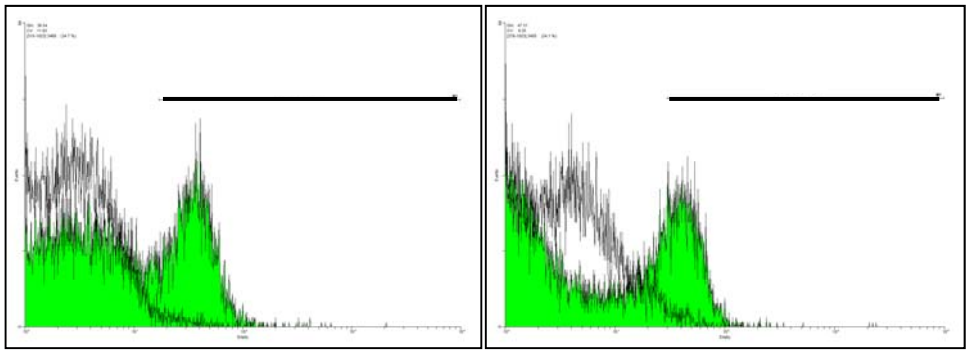

Fig. 3.19. *Two typical flow cytometry graphs of positive CD34 expression in PBMCs cultured on a BMSC stroma for 21 days (left) and without a stroma cell layer for 14 days (right)*

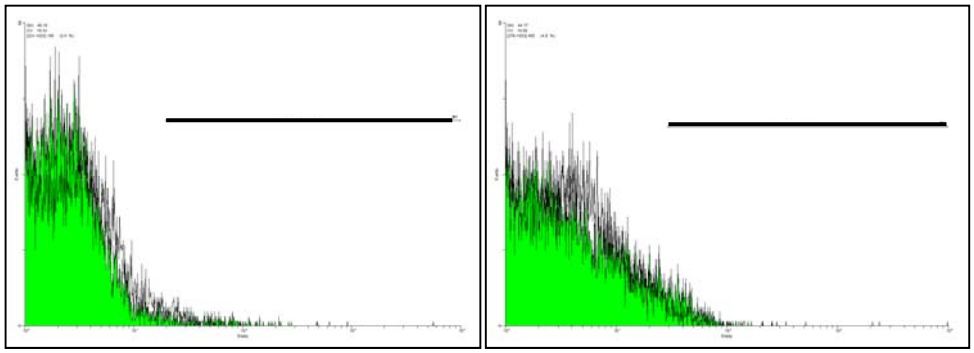

Fig. 3.20. *Two typical flow cytometry graphs of negative CD34 expression in PBMCs cultured on an AMSC stroma for 14 days (left) and without a stroma cell layer for 21 days (right)*

Three types of previously tested media and two types of stromal cells (BMSC and AMSC) were used to analyse the $CD34^+$ profile of PBMCs over 21 days. I-medium contained FCS whereas P-medium and D-medium were serum free. On day 14, when PBMCs were harvested from the co-cultures, a small number of stromal cells were harvested as the stromal layer was disrupted. This could have had an effect on the percentage of CD34 expression within the analysed cell population cultured under the following conditions: AMSC/I-Medium, BMSC/P-Medium, and BMSC/D-Medium.

The results show the effect of culture conditions on the CD34 expression profiles and the variation in culture conditions. An overview with all combined profiles is shown in Figure 3.21 with separate detailed profiles in Figures 3.22, 3.23, and 3.24. During the first few days of culture a high number of cells died in the first few days. Afterwards, the expansion rate increased resulting in a higher number of cells.

The CD34 expression profiles were different for each of the media types used (Figure 3.21). To study the results in more detail, expansion profiles were separated for each medium type (Figures 3.22, 3.23, and 3.24). Data obtained from cultures in I-medium showed that a combination of I-medium and a stromal cell layer elevated the $CD34^+$ expression over 30% after 21 days. This was the highest expression amongst all culture conditions. The culture

without a stromal layer reached its peak at day 14 after which the expression dropped. For all conditions, the sharpest rise in CD34 expression was recorded during the first 7 days of culture.

Data obtained from cultures containing an AMSC stromal layer showed that the $CD34^+$ expression was lower than expected, but could be linked to the harvest of not only the PBMCs but also a number of stromal cells. After 21 days the highest level of CD34 expression was recorded at almost 35% for PBMCs cultured on a BMSC stromal layer.

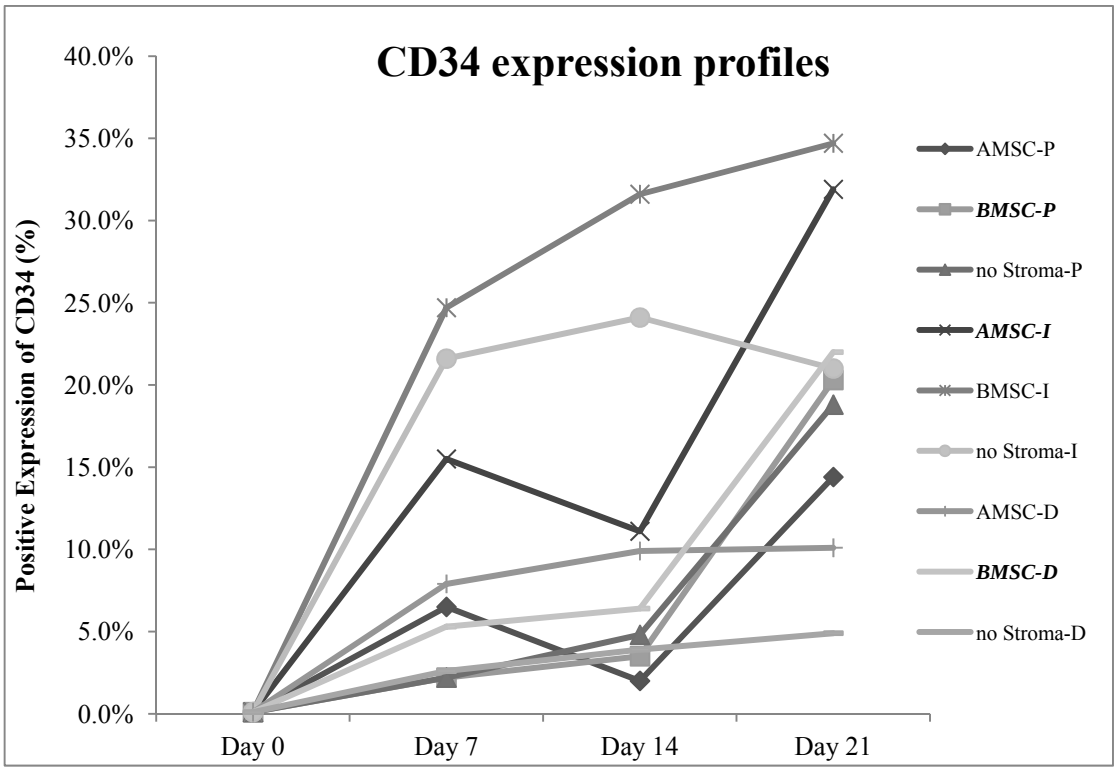

Fig. 3.21. *A complete profile overview. The results showed that PBMCs cultured either in I-medium or on a BMSC stromal layer contained the highest percentage of $CD34^+$ cells. (I=I-Medium, P=P-Medium, D=D-Medium; medium contains 50ng/mL SCF/TPO/Flt3-L)*

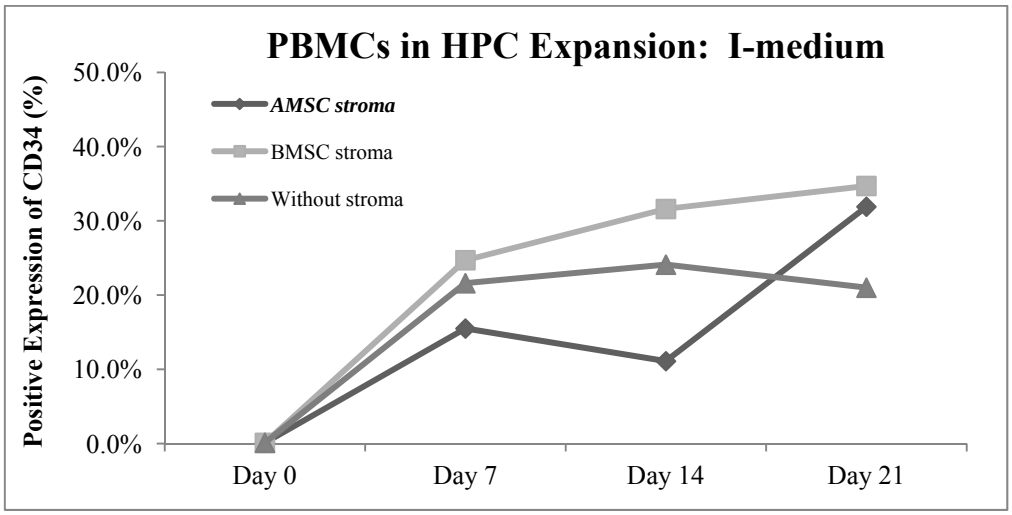

Fig. 3.22. *Percentage of CD34$^+$ cells within their native PBMC population, cultured in I-Medium with SCF/Flt3-L/TPO over 21 days*

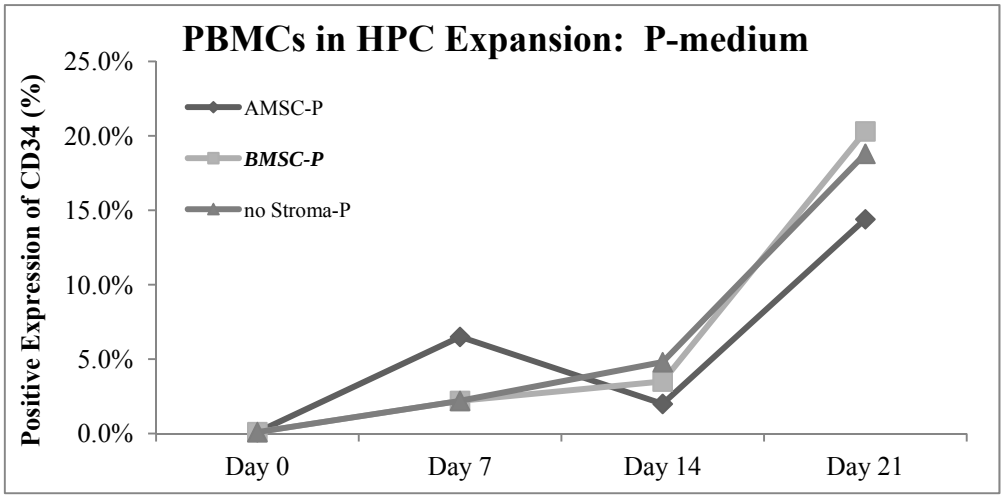

Fig. 3.23. *Percentage of CD34$^+$ cells within their native PBMC population, cultured in P-Medium with SCF/Flt3-L/TPO over 21 days*

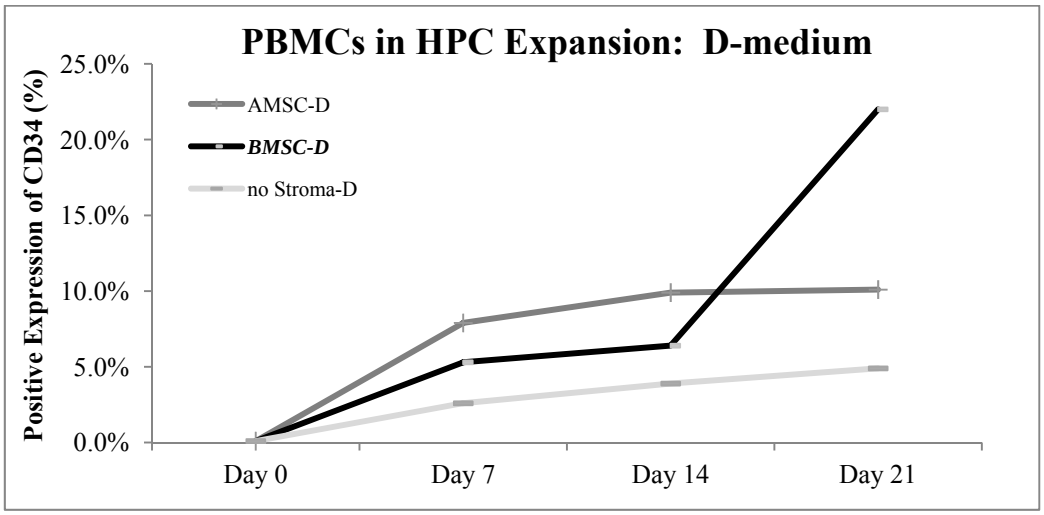

Fig. 3.24. *Percentage of CD34$^+$ cells within their native PBMC population, cultured in D-Medium with SCF/Flt3-L/TPO over 21 days*

The results for PBMCs cultured in P-medium were different from the results obtained for I-medium. The relatively low expression of CD34 for PBMCs isolated from the BMSC stromal layer on day 14 could be linked to the harvest of a number of stromal cells as the stromal layer was severely disrupted after isolation of suspension cells (data not shown).
There are similarities in the expression pattern for all culture conditions with P-Medium in this experiment. For the initial days of culture the expression was ~5% or lower after which the expression rapidly increased to 15-20% during the next 7 days. The maximum positive expression reached was just over 20% (BMSC stromal layer) after 21 days.

The PBMCs cultured in D-Medium showed a CD34 expression profile that was not as elevated when compared to the profile for two other medium types. The culture profile for the BMSC stromal layer only showed a relatively high expression for CD34 after 21 days when compared to the expression profiles for P-medium. During the 21 day period in D-Medium, a positive expression for CD34 was 10% and 5% respectively for PBMCs cultured on an AMSC stromal layer and cultured without a stromal layer. During the harvest of PBMCs on day 14, a number of stromal cells were isolated which could be linked to a lower CD34 expression.

3.4.2 Expansion of $CD34^+$ cells within UBMCs

The experiments with the PBMCs for HSC expansion potential showed that the BMSC stromal layer had the most promising results. Together with the data obtained from cultures using I-medium this resulted in the use of a BMSC stromal layer and I-medium in order to analyse the HSC expansion potential of UBMCs. The conditions under which these cells were cultured were (A) serum free I-Medium without cytokines with a stromal layer, (B) serum free I-Medium containing cytokines SCF/Flt3-L/TPO without a stromal layer, and (C) serum free I-medium without cytokines or stromal layer. To stimulate improved adherence of the BMSC stromal cells, to prevent premature detachment, the tissue cultured treated

plastic was coated with 0.3% gelatine. Subsequently the BMSCs were cultured for 72 hours before becoming confluent. Proliferation of BMSCs was inhibited by incubation with Mitomycin C overnight.

The UBMCs were cultured at $3x10^4$ cells/mL and cells were exposed to serum-free 'I-medium' either with or without cytokines. The cell numbers were monitored to obtain additional quantitative data in order to draw reliable conclusions regarding the increase in the number of $CD34^+$ cells. The previous experiments showed that a period of 21 days and shorter might not have shown the full potential of MNC long term cultures for HSC expansion. Over a period of 28 days, cells were analysed using a haemocytometer and flow cytometry (Figures 3.25 – 3.29). Due to low cell numbers, cells were analysed for two antibody markers only (CD34 and CD45). The latter antibody is associated, in combination with CD34, as a HPC marker [90]. CD45 was only applied to cell samples isolated from cultures containing either cytokines or a supportive stromal layer.

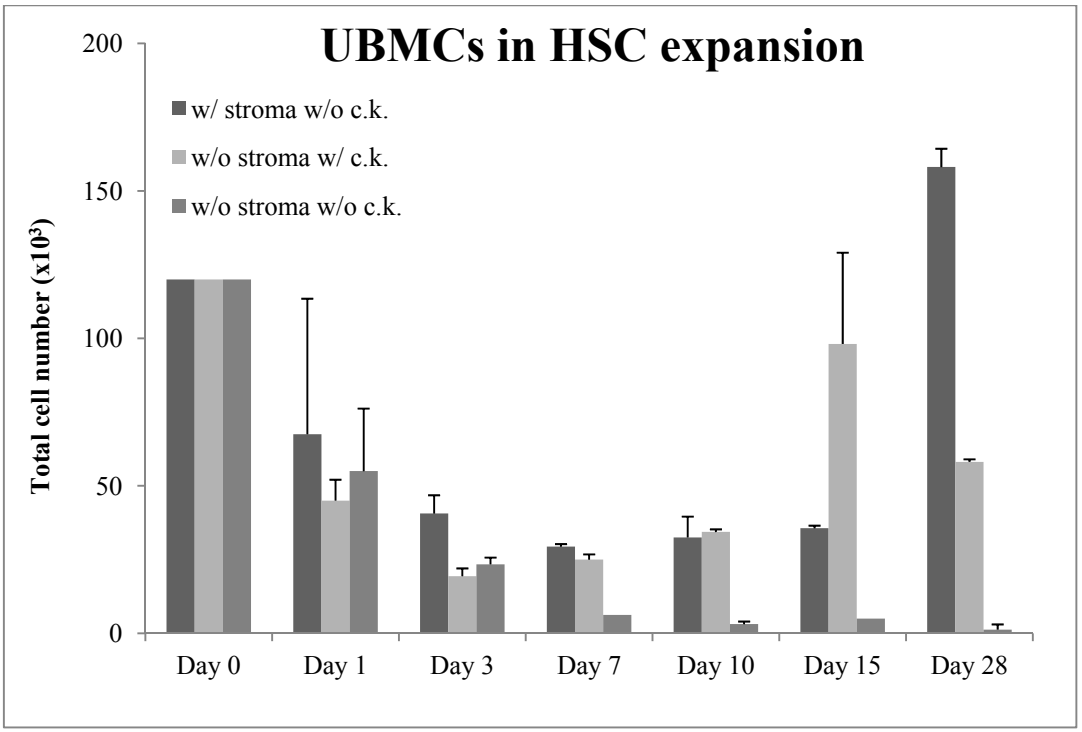

Fig. 3.25. *An overview of the cell numbers for UBMCs in HSC expansion (n=3, error bars represent StDev). [w/ stroma w/o c.k. = with a BMSC stromal layer/without cytokines] - [w/o stroma w/ c.k = without a stromal cell layer/with cytokines (SCF/TPO/Flt3-L)] - [w/o stroma w/o c.k. = without a stromal cell layer/without cytokines]*

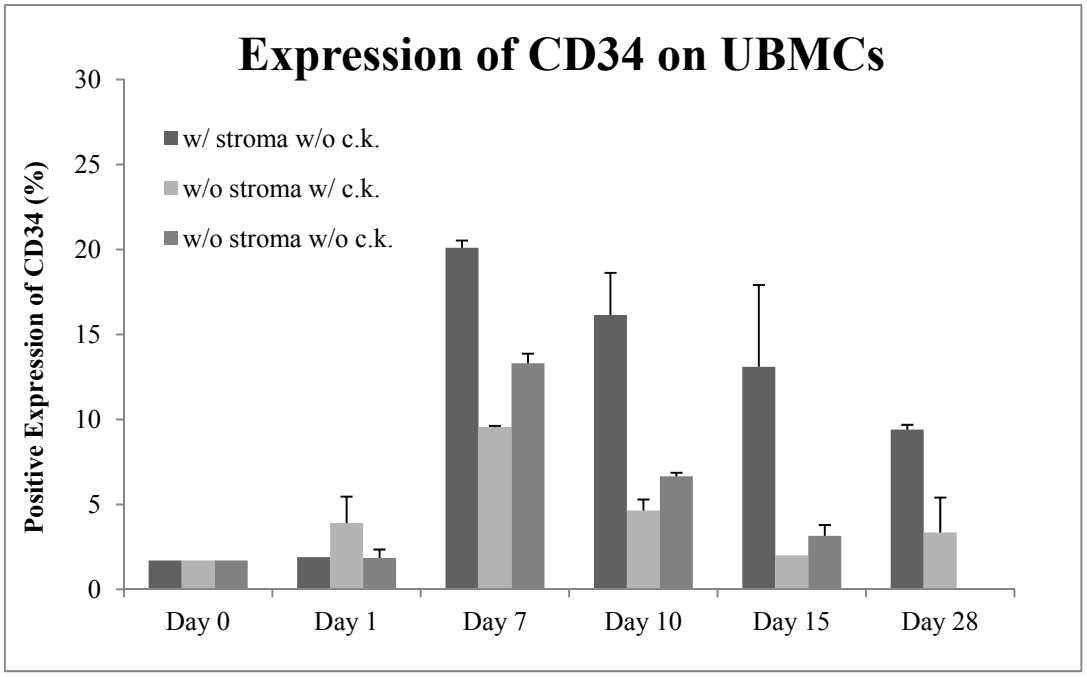

Fig. 3.26. *The relative positive expression of CD34 within each culture condition for up to 28 days (n=3, error bars represent StDev). [w/ stroma w/o c.k. = with a BMSC stromal layer/without cytokines] - [w/o stroma w/ c.k = without a stromal cell layer/with cytokines (SCF/TPO/Flt3-L)] - [w/o stroma w/o c.k. = without a stromal cell layer/without cytokines]*

For all three culture conditions at least 75% of the cells did not survive the first 7 days. With either a stromal cell layer or cytokines available, the number of UBMCs increased over the next 14 to 21 days. The culture without a supportive cell layer and no cytokines was a non-viable cell culture condition. The UBMCs supported by a BMSC cell layer hardly proliferated during the first two weeks of culture (~$35x10^3$ cells on day 15) before the cell number increased dramatically to ~$160x10^3$ cells. In comparison, the cell numbers for a culture with cytokines SCF, Flt3-L, and TPO were ~$100x10^3$ cells (day 15) and ~$60x10^3$ (day 28) cells. The relative expression of CD34 during all culture conditions increased over the first 7 days when most of the cells died. The relative number of cells with a $CD34^+$ phenotype decreased over the subsequent 21 days. The cells cultured on a stromal cell layer showed the overall highest expression throughout the entire culture period; ~20% at day 7 (~10% at day 28). The highest $CD34^+$ expression for cultures with cytokines was ~10% at day 7 (~3% at day 28) whereas this was ~13% for the cultures without cytokines and without a supportive cell layer (0% at day 28).

The result depicted in Figure 3.27 shows information about absolute number of CD34 positive HSCs present in the culture. The number of $CD34^+$ cells remained stable (~$3x10^3$ cells) throughout the culture condition with cytokines for 28 days. For the culture condition without a BMSC stromal layer/without cytokines the number of cells expressing CD34 decreased to ~$0.2x10^3$ (day 10) and ~$0.1x10^3$ (day 15). The condition where the number of $CD34^+$ cells did increase contained a stromal cell layer. During the first 14 days the increase was slow (up to ~$5x10^3$ cells) whereas the increase was more prominent in the last 14 days of the culture; up to ~$15x10^3$ cells.

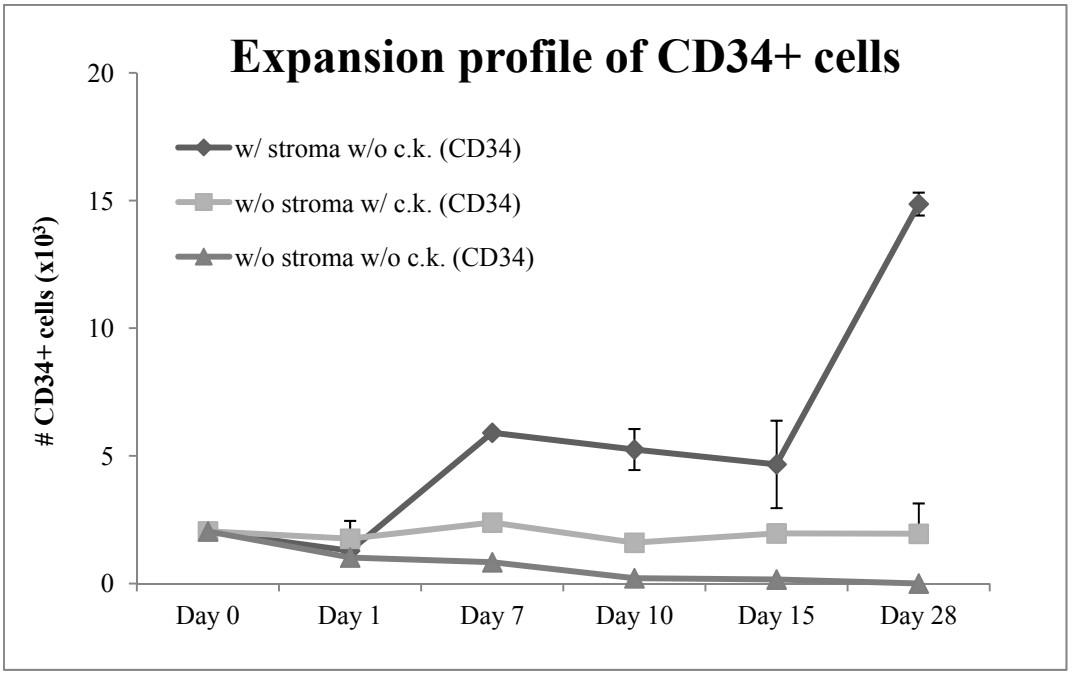

Fig. 3.27. *The absolute number of cells expressing the CD34 marker over a 28 day culture period (n=3, error bars represent StDev). [w/ stroma w/o c.k. = with a BMSC stromal layer/without cytokines]- [w/o stroma w/ c.k = without a stromal cell layer/with cytokines (SCF/TPO/Flt3-L)] - [w/o stroma w/o c.k. = without a stromal cell layer/without cytokines] - [(CD34) = Cells expression the CD34 marker]*

The percentage of cells with a CD45 phenotype cultured on a BMSC stromal layer dropped from 100% to ~30% within 10 days. For UMBCs in medium with cytokines the percentage of $CD45^+$ cells dropped to ~20%. During the next 18 days of culture, both cell populations reached an expression lower than 20% for cells with a CD45 phenotype.

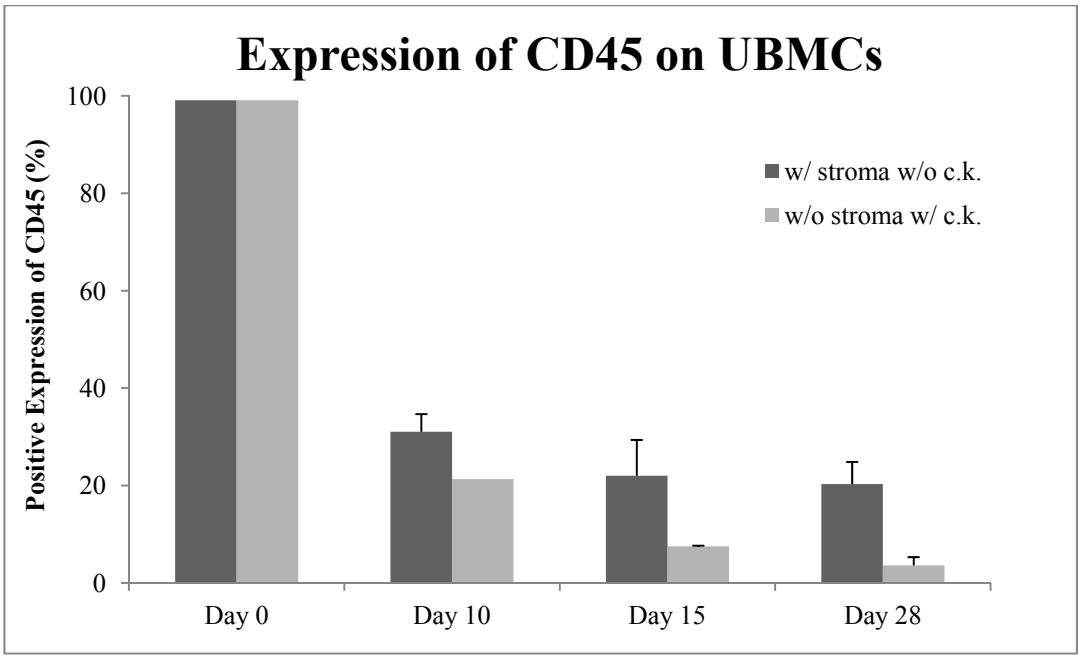

Fig. 3.28. *The relative positive expression of CD45 within each culture condition for up to 28 days (n=3, error bars represent StDev). [w/ stroma w/o c.k. = with a BMSC stromal layer/without cytokines] - [w/o stroma w/ c.k = without a stromal cell layer/with cytokines (SCF/TPO/Flt3-L)]*

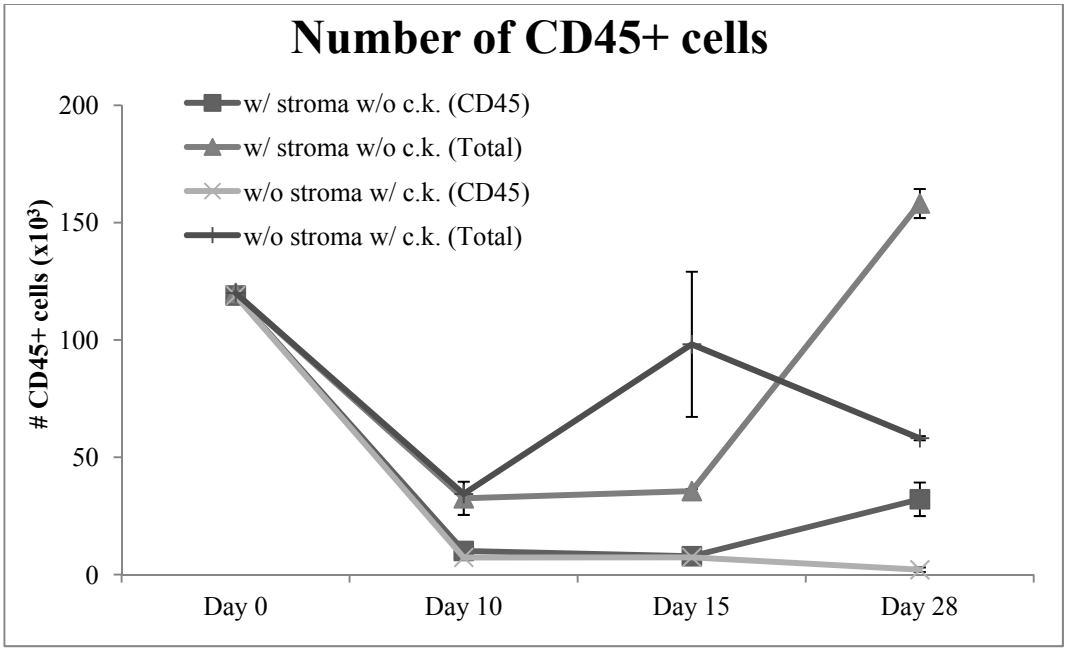

Fig. 3.29. *The absolute number of cells expressing the CD45 marker in direct comparison with the total cell number in the culture (n=3, error bars represent StDev). [w/ stroma w/o c.k. = with a BMSC stromal layer/without cytokines] - [w/o stroma w/ c.k = without a stromal cell layer/with cytokines (SCF/TPO/Flt3-L)] - [(CD45) = Number of CD45 positive cells in the culture] - [(Total) = Total number of cells in the culture]*

The number of cells, overall and CD45 positive cells, decreased during the first 15 days of culture before increasing during the last two weeks of culture. At day 28, a total of ~160x10^3 cells including ~30x10^3 $CD45^+$ cells were counted for the culture containing a stromal cell layer. For the UBMCs cultured with cytokines present in the medium, the overall cell number fluctuated whereas the number of $CD45^+$ cells decreased during the first 10 days of culture and remained low during the next 18 days (2x10^3 cells at day 28).

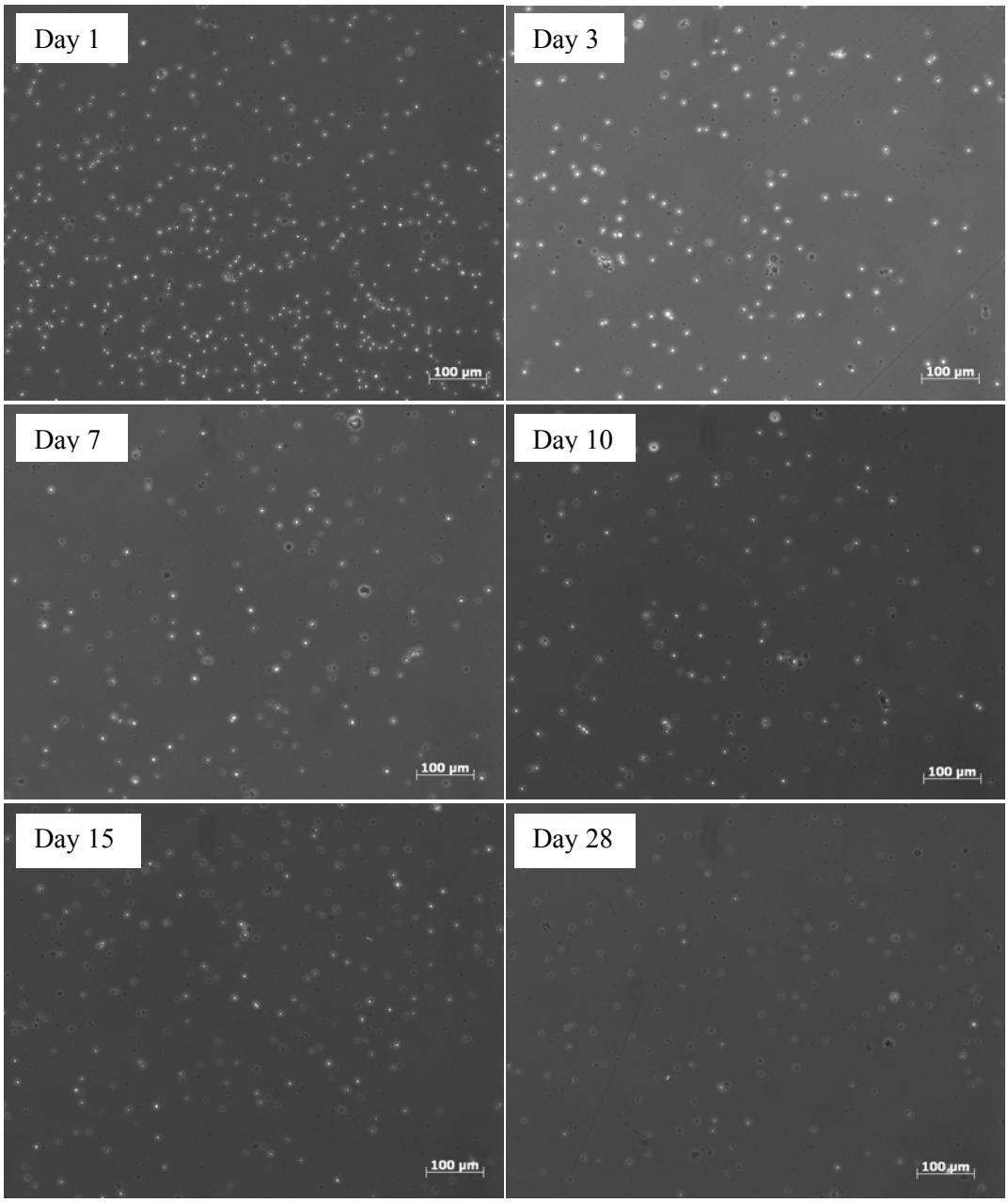

Fig. 3.30. *An overview of UBMCs cultured without a stromal layer and without cytokines - Day 1 and 3, Day 7 and 10, Day 15 and 28 (left to right, top to bottom)*

During the first 7 days of culture cell viability was relatively high for the UBMCs cultured without a stromal layer/cytokines (top images Figure 3.30). For the remaining three weeks of culture slightly bigger cells were observed when compared to days 1 and 3. More cell debris was present in the culture after day 10. At day 28, only a few viable cells were observed, the majority of the cells were not alive.

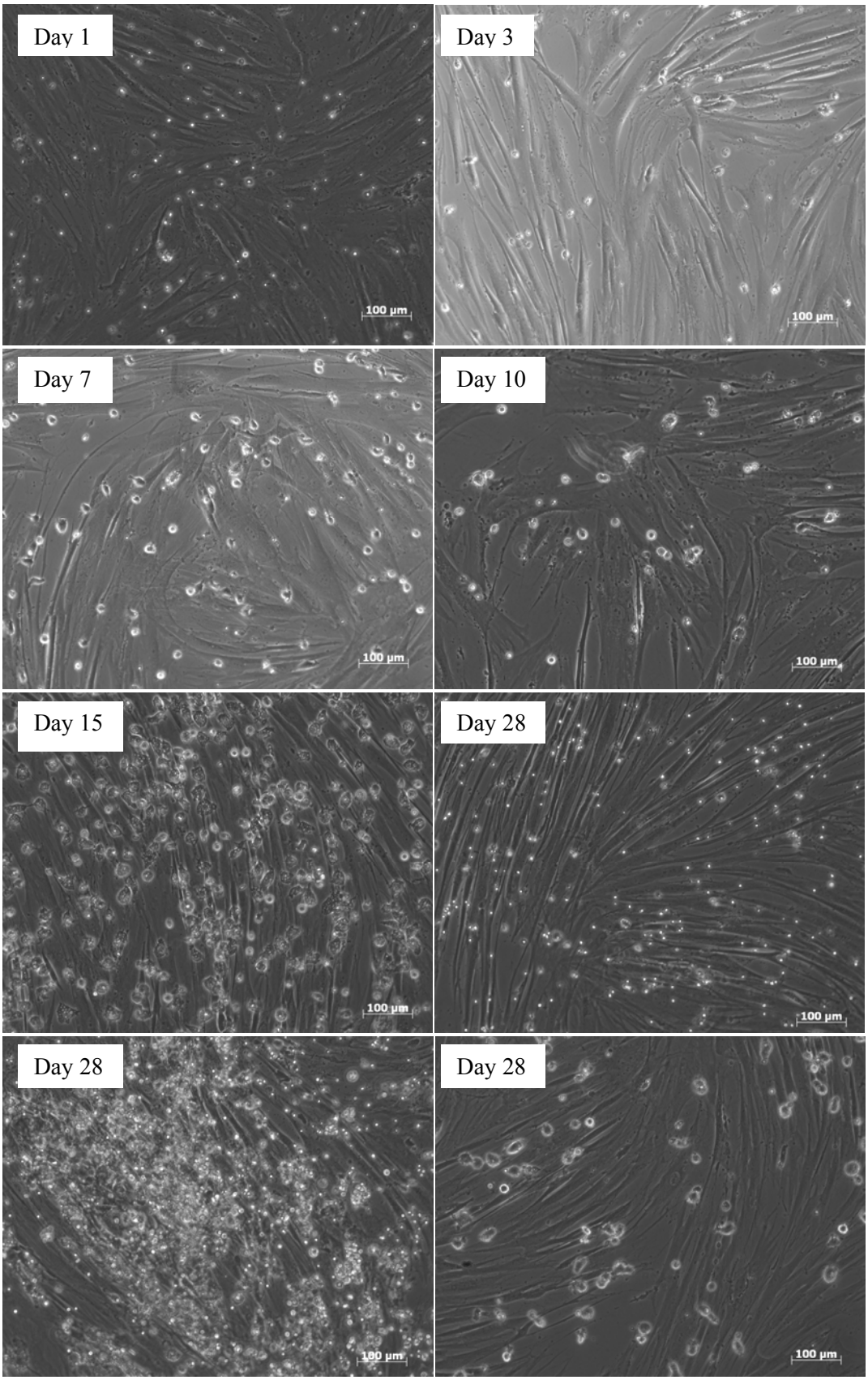

Fig. 3.31. *An overview of UBMCs cultured on a BMSC stromal layer - Day 1 and 3, Day 7 and 10, Day 15 and 28, Day 28 and 28 (left to right, top to bottom)*

For UBMCs cultured on a BMSC stromal layer, the cell number dropped during the first days of culture and this was reflected by the images shown in Figure 3.31. Few round shaped cells were observed on the elongated cells of the stromal layer. On day 15, an increase in cell number was observed. The three images representing the cultures on day 28 show various sections of the cultures with a high number of cells at the centre of the well and a less populated at the edge of the well.

When cells were cultured without a stromal layer in medium containing cytokines, cells with a round like morphology were observed (Figure 3.32). For this culture, there were fewer cells present on day 15 compared to the culture with the stromal layer. Cell debris was observed and in addition, the cells were smaller. On day 28, a higher number of cells was observed. These cells were smaller in size compared to the other culture conditions and cell debris was present.

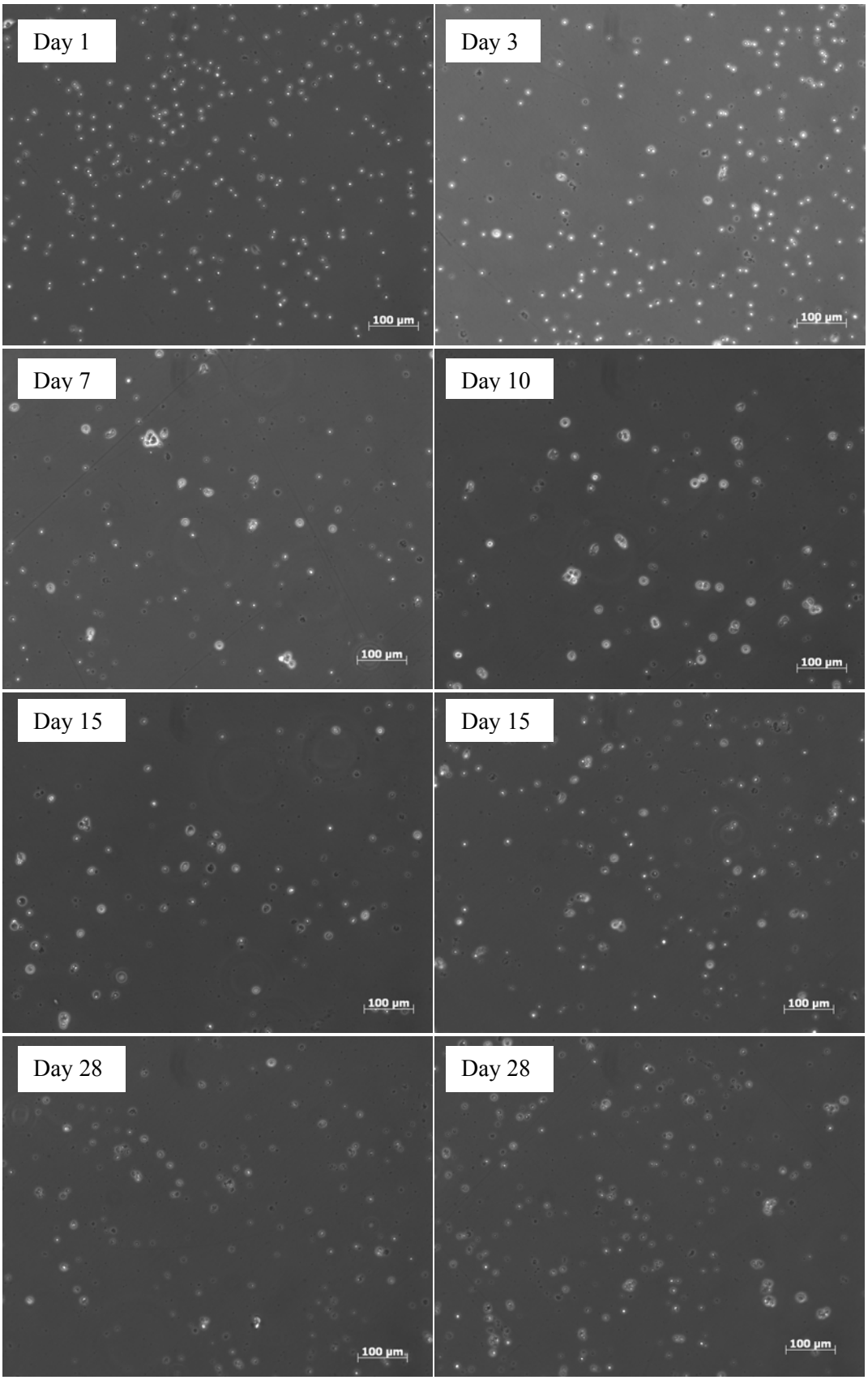

Fig. 3.32. *An overview of UBMCs cultured without a stroma and with cytokines - Day 1 and 3, Day 7 and 10, Day 15 and 15, Day 28 and 28 (left to right, top to bottom)*

The previous results showed that a BMSC stromal layer had a positive influence on the expansion of CD34 positive cells. In the following experiment cytokines were added to standard serum free I-medium where in one culture condition a BMSC stomal layer was present (w/ stroma) and in a second culture condition the stromal layer was not present (w/o stroma. This is different from previous experiments were either a stromal layer or cytokines were present. The results (Figure 3.33) showed that over 7 days, the expression of CD34 increased the most when the UBMCs were cultured in the presence of a BMSC stromal layer (30%). Table 3.3 shows that this percentage represented a total of ~440x10^3 CD34 positive cells. In the culture condition without an additional stromal cell layer the positive expression reached ~18% after 7 days. This represented ~16x10^3 cells with a CD34 phenotype (Table 3.3).

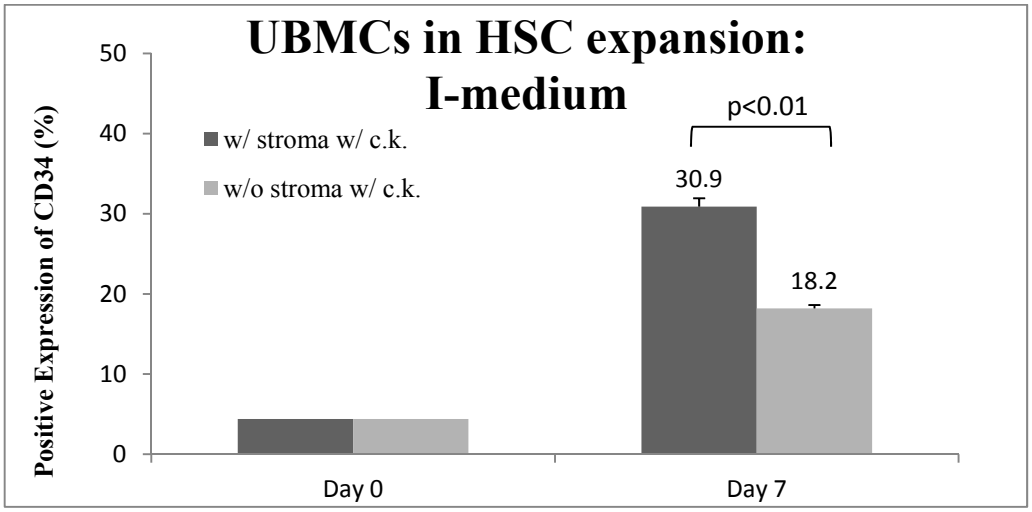

Fig. 3.33. *The relative positive expression of CD34 within each culture condition. The culture medium contained the cytokines SCF/TPO/Flt3-L. The UBMCs were cultured either with or without a BMSC stromal layer (n=3, error bars represent StDev).*
[w/ stroma w/ c.k = [with a BMSC stromal layer/with cytokines (SCF/TPO/Flt3-L)] - [w/o stroma w/ c.k = [without a stromal cell layer/with cytokines (SCF/TPO/Flt3-L)]

	Cell Number (#)	$CD34^+$ (%)	$CD34^+$ (#)
Day 0: after isolation	300*10^3	1.7	**~5.1*10^3**
Day 7: with BMSC stromal layer / with cytokines	*1,410*10^3*	*30.9*	**~440*10^3**
Day 7: without BMSCstromal layer / with cytokines	90*10^3	18.2	**~16*10^3**

Table 3.3. *The absolute number of total cells and $CD34^+$ cells from the UBMCs culture for HSC expansion*

The total number of cells obtained with the culture containing a BMSC stromal layer and medium containing cytokines was ~50 times higher than the cell number obtained at day 7 of a previous experiment with only a BMSC stromal layer to support the UBMCs for HSC expansion (Figure 3.25). In both the previous and current experiment the total number of cells for the culture condition containing only cytokines was $25x10^3$ cells and $90x10^3$ (~3.5 times higher). The UBMCs were cultured at the same cell density.

In an optimised repeat of this experiment with $6x10^3$ cells/mL, instead of $3x10^3$ cells/mL in a previous experiment, the percentage of positive CD34 expression was similar for the condition with medium containing cytokines and with a stromal layer, but the number of $CD34^+$ cells was more than twice as high. The efficiency for the culture condition containing cytokines only (without stromal layer) was not as high as in the previous experiment: respectively 8% vs. 18% (Figure 3.34).

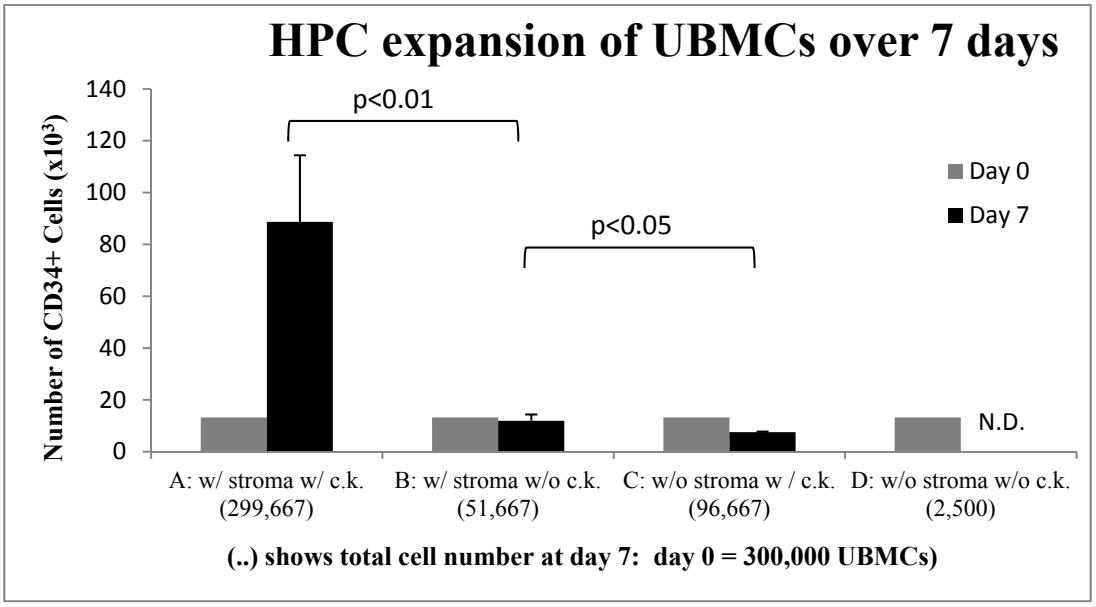

Fig. 3.34. *Expansion of $CD34^+$ cells within a population of UBMCs. For condition 'A' the percentage of $CD34^+$ cells rose from ~3% (day 0) to ~30%, for 'B' ~23%, and for 'C' ~8% at day 7(n=3, error bars represent StDev).*

3.5 Summary

HSCs were isolated from PB with purity rates over 80%. Expansion potential was minimal (< 7 days) with low numbers of $CD34^+$ cells: < 10^5 $CD34^+$ cells from ~7x10^7 PBMCs. Thawed UCB-$CD34^+$ cells were successfully expanded for over 10 days whereas $CD34^+$ cells isolated from thawed UBMCs showed low purity rates and expansion potential was minimal.

To improve prolonged cultures from fresh PB isolated cells and from thawed UCB isolated cells, $CD34^+$ cells were cultured in an environment including a stromal cell layer and MNCs. A number of cell populations in a variety of HSC expansion conditions contained over 30% $CD34^+$ cells up to 21 days. The culture condition with the highest expansion fold of $CD34^+$ cells, over 7 days, (~85x fold) contained UBMCs in I-medium including cytokines on a BMSC stromal layer.

The results obtained led to the optimisation of the stem cell environment to stimulate expansion output of CD34 positive cells. These data were subsequently used to create an environment to promote and sustain the differentiation of these $CD34^+$ HSCs into RBCs (Chapter 4).

3.6 Discussion

The establishment of HSC expansion for the first time in this laboratory was paramount for increasing the cell input during erythroid maturation. Therefore the question for this research topic was formulated as 'How to establish and optimise *in vitro* HSC expansion as a sufficient source of cells for the manufacturing RBCs?'.

The main challenge was to isolate and expand $CD34^+$ cells with their short-lived phenotype, compared to other cell types (*e.g.* MSCs, dental pulp stem cells (DPSCs)), which maintain long-term expansion cultures.

For the proliferation of HSCs a source of these $CD34^+$ cells was needed and both PB and UCB were either available directly or via research partners. These two sources were analysed for their $CD34^+$ isolation potential in this project. The stem cells were either isolated from fresh PB or frozen aliquots of UBMCs. The magnetic bead cell separation technique to isolate $CD34^+$ cells from fresh PB showed high efficiency rates with >80% of the cells showing positive for CD34. This technique is commonly used with comparable efficiency rates [91-93]. However, the number of $CD34^+$ isolated cells was too low (~10^4 cells from ~10^7 MNCs) to initiate larger scale expansion cultures. These numbers corresponds with figures stated in the literature but usually higher quantities of MNCs were obtained in those studies: ~10^8 MNCs from >250mL of blood [41].

In this project only 30-50mL of blood could be obtained per patient, which was restrictive for HSC isolation. An alternative cell source for PBMCs was buffy coat preparations provided by the NHS in a 10mL leukocyte cone. These cones contained a concentrated cell volume with the aim to obtain higher numbers of PBMCs for $CD34^+$ isolation. Due to the time intervals allowed for processing and travel of this tissue, only ~$6x10^7$ viable MNCs were present and subsequently ~$6x10^4$ cells were isolated utilising the CD34 magnetic bead separation technique. Although this cell population proved highly positive for CD34 after

several days of HSC expansion, only 10^4 viable cells could be obtained. Overall, these results together with the low number of MNCs and quality of the provided buffy coat resulted in the decision not to use these cells as a prime source in this project.

With limited quantities of PB available and low harvest rates from PBMCs, it was decided that UBMCs would be obtained from fellow research groups (*i.e.* project partners). UCB-$CD34^+$ were analysed for HSC expansion potential under various conditions to determine the optimal culture environment. All experimental setups aimed to generate as many progenitor cells as possible, eventually to be used for erythroid maturation.

UCB is known for containing a higher percentage of HSCs and a higher expansion potential of stem cells when compared to PB [56]. Due to the various geographical locations of collaboration research centres, UBMCs were aliquoted and processed for freezing on site, shipped frozen, stored in LN2 and thawed upon use in this laboratory (described in Chapter 2). The thawing protocols used were supplied by the same research centres. The aliquoted UBMCs were single donor instead of pooled donor aliquots, and therefore the quality varied between UBMC aliquots.

The thawed UCB-$CD34^+$ cells proved successful for HSC expansion for up to 10 days in commercially available medium (P-medium) with a 50x expansion fold over 10 days. This chemically defined medium, free of animal and human-derived components except Human Serum Albumin, required the addition of a commercially defined cytokine mix containing SCF, TPO, Flt3-L, and IL-3 for increased HSC expansion potential. Chemically-defined media without xenogeneic components and preferably allogeneic components is important for eventual use in human cell therapies [64, 94].

For all cell sources a culture density of $3x10^4$ cells/mL was used for HSC expansion. P-medium was also used for the expansion of PB-$CD34^+$ cells isolated from buffy coats. In

comparison, after 14 days of expansion these PB-CD34$^+$ cells did not express an HSC phenotype (Figure 3.6) whereas the population UCB-CD34$^+$ was still considered positive for CD34$^+$ after 10 days of culture (Figure 3.7). This data with this degree of expansion potential of UCB cells matched published data [95].

3.6.1 Media for HSC expansion

P-medium stimulated expansion of fresh PB-CD34$^+$ and thawed UCB-CD34$^+$ cells with rates similar to those found in the literature. UCB-CD34$^+$ cells were also used to analyse expansion, while aiming to maintain the CD34$^+$ phenotype, with basic medium tailored for erythroid maturation (D-medium) and HSC expansion (S-medium) supplemented with and without cytokines used in HSC expansion: SCF and IL-3. Whereas the D-medium was expected not to maintain the CD34$^+$ phenotype, it was hypothesised that the S-medium containing SCF/IL-3 would positively influence expansion of CD34$^+$ cells. This proved correct with this S-medium option resulting in a positive expression of ~24% whereas the results of the other three media options were regarded negatively with an expression lower than 6% (Figure 3.8). The expansion fold and viability were highest for S-medium (SCF/IL-3) however, the overall result being not as positive as hypothesised for HSC expansion and inferior in comparison to complete P-Medium. This could be the result of a different cytokine cocktail or due to a difference in the basal formulation of these two commercially available media. D-medium contained BSA whereas both P-medium and S-medium contained human serum albumin.

The research group in Leipzig, who specialised in HSC research, recommended the use of I-medium; optimised for HSC expansion and could either be used with FCS or BSA. The use of the cytokines SCF, TPO, and Flt3-L was advised for optimal HSC expansion with I-medium. The basic component of I-medium, IMDM, is widely used for the expansion of HSCs [55, 71, 96]. A range of cytokine concentrations is used in the literature and the

concentration used in this study was based on results provided by our research partners and published data [76, 96-98]. There was limited access to $CD34^+$ cells during this stage of the project which denied direct comparison of I-medium and P-medium for HSC expansion.

3.6.2 Isolation and culture of UCB-$CD34^+$ cells

The access to frozen aliquots of UCB-$CD34^+$ was restricted but the availability of frozen aliquots of UBMCs was ample for studying HSC expansion. Established protocols were used for thawing aliquoted UBMCs and subsequent $CD34^+$ magnetic cell separation. Cell separation was unsuccessful with extremely low $CD34^+$ positive expression of isolated cells. Insufficient availability of cell numbers allowed either in post-isolation cell analysis but not expansion, or expansion without post-isolation cell analysis. Complete P-medium was used for cell proliferation but due to low cell numbers available for culture expansion of thawed and isolated UCB-$CD34^+$, expansion fold rates were minimal over a 10 day culture period.
Published studies generally do not state whether fresh CB, frozen CB, or frozen UBMCs have been used. Researchers analysed various thawing methods demonstrating that a certain percentage of cell death is unavoidable within the first 48 hours after thawing [99, 100]. This was also observed for thawed aliquots, when UBMCs were used for either cell separation or cell culture.

In general, the cell cultures with magnetic bead isolated UCB-$CD34^+$ cells struggled to fully revive and expand after thawing. With the aim of solving this issue, the literature was analysed to optimise culture conditions for these cells. Previous studies reported on the support of UCB-$CD34^+$ cells by co-culture with MSCs to stimulate maintenance of HSCs over a prolonged period [48, 98]. In this project, these published methods were utilised: AMSCs and BMSCs were used in the co-culture to support prolonged HSCs expansion with FCS-containing complete I-medium. The use of an AMSC supportive layer indicates an advantage for cell expansion by maintaining cell morphology over 14 days with ~49% of the

population being positive for CD34 after 14 days. The BMSCs supported a higher cell expansion of UCB-CD34$^+$ over 14 days and around 44% of the population showed CD34$^+$ after 2 weeks. These results indicate a positive contribution of a stromal cell layer when compared to a non-co-culture over a prolonged period when fewer cells and a large quantity of cell debris were observed. In addition, the co-culture results are in keeping with data from the literature for the maintenance of the HSCs [48].

3.6.3 Culture conditions of PB-CD34$^+$ cells

The PB-CD34$^+$ isolated cells were cultured on a BMSC supportive cell layer to stimulate prolonged maintenance of HSCs as performed with UCB-CD34$^+$ cells. Whereas there was sufficient cell expansion with P-medium and D-medium, cell expansion was negligible in I-medium. However, the expression of CD34 was either lower than 40% (day 14) and 30% (day 21) for a number of culture conditions, or expression was barely detectable (Figure 3.16). Overall, both the expansion fold rate and percentage of CD34 cells were low for all culture conditions. AMSCs were used in a similar setup to UCB-CD34$^+$ previously discussed but due to low cell numbers this was done in series rather than in a parallel setup. However, these results led to a change of focus to use MNCs for HSC expansion and so PB-CD34$^+$ cells were not used anymore during this project.

3.6.4 PBMCs and UBMCs in HSC expansion conditions

With the ongoing challenge to culture sufficient $CD34^+$ cells over a prolonged period the project started the utilisation of MNCs for HSC expansion. The literature showed evidence of stimulation of $CD34^-$ PBMCs towards erythroid maturation [41]. The approach used for expanding UCB-$CD34^+$ cells was applied for the HSC expansion of MNCs; three types of media and stromal layers were analysed for optimal expansion potential.

In the first instance, PBMCs on a BMSC stromal layer cultured in I-medium containing cytokines showed the most promising results with a positive CD34 expression increasing from ~0% at day 0 to over 30% (day 14 and 21). The culture conditions having an AMSC stromal layer or no stromal layer, both containing the same cytokine cocktail, only showed a positive CD34 expression of ~20% or lower over 21 days. This culture environment with I-medium and a BMSC stromal layer was used to study the expansion of MNCs in more detail.

The UBMCs, all single donor, showed a drop in cell number during the first 3 to 7 days of culture as MNCs also contained non haematopoietic sensitive cells being exposed to a HSC optimised culture environment (Figure 3.25). This initial drop in cell viability was regarded as normal as comparable data was published [41]. The culture condition with only a stromal layer showed a higher increase in viable cells compared to medium with only cytokines, indicating that the stromal cells are important for cell viability and expansion. The number of $CD34^+$ cells increased during the first 7 days of culture and over 28 days a fold expansion of ~7 was recorded which was lower than the 52 fold increase for UCB-$CD34^+$ cells during a 10 day culture in P-medium.

UBMCs cultured at $3x10^4$ cells/mL in the presence of cytokines (no stromal layer) resulted in a minimal expansion of $CD34^+$ cells. When the cell density was increased to $6x10^4$ cells/mL, the expansion was negative (Figure 3.34). This could indicate that insufficient cytokines were available to fully stimulate the UBMCs, resulting in non-optimal culture

conditions. The highest expansion fold obtained for $CD34^+$ cells was ~85x for UBMCs supported by a BMSC stromal layer and cytokines, which is comparable to the values stated in the literature [101]. This was the 'richest support' for UBMCs in HSC expansion in terms of access to biological stimuli and at the same time this environment provided the best outcome for the proliferation of $CD34^+$ cells from MNCs.

As the BMSCs and AMSCs were harvested from a range of donors, differences were observed in the expansion rates of these MSCs from different donors. Some donors only produced cells with a very low expansion rate. During this project only MSCs were used with a normal expansion rate, but other possible differences between donors of MSCs were not investigated. This donor variability was also applicable to the single donor UBMCs and PBMCs used. Preferably, pooled donor MNCs should be used to minimise individual donor variability but due to the source of these cells the pooling of cells was not possible.

Chapter 4

Results - Manufacture of Red Blood Cells

4.1 Introduction

RBCs are produced in the human body and for decades scientists have focussed on translating this process into the laboratory. The *ex vivo* manufacture of RBCs has been achieved from HSCs [42]. Now, the focal point has shifted towards the optimisation of these cultures as part of a mission to find an alternative method for blood transfusion. As discussed in Chapter 1, at the current time, the supply of blood products is insufficient to keep up with the needs for medical treatments. Therefore, production and implementation of artificial blood products should, in turn, resolve the issues associated with conventional blood donation.

A key challenge is the creation of sufficient numbers of RBCs from a single HSC and the target is now to scale up the expansion of RBCs. In current blood transfusion practice the volume of a unit of blood is approximately 500mL, and contains ~2500 billion RBCs [102]. Therefore, in theory it is possible to produce a few units of RBCs *ex vivo* from one unit of UCB, but there are practical restrictions. For example, the manufacture of 1 unit of RBCs, with a working cell density of $5x10^6$ cells/mL, requires a culture surface the size of at least two tennis fields and ~40 litres of media (worth over $8000,-) [54]. In addition, upscaling from 2D culture flasks to 3D cultures in bioreactors is of paramount importance to achieve a higher cell density in order to reduce surface culture and medium volume. Other improvements can be achieved by creating a higher number of erythroid precursor cells during haematopoiesis (Chapter 3). Attempts to increase the output of RBCs will be described in this chapter.

A number of studies have abandoned the supportive cell layer as part of the cell differentiation process [49, 53]. One reason for such an alteration includes the possible upscaling to dynamic 3D cultures where a second, adherent, cell line will prove difficult. Another argument for feeder free cultures is the complexity of establishing and maintaining co-cultures of mixed cell populations. Feeder free cultures for erythroid maturation (EM)

proved to be challenging in efficient enucleation and Hb synthesis in terminal maturated cells [49]. But optimising the concentration of cytokines and additional medium stimuli may fine tune these processes to culture RBCs of similar quality when a feeder layer is available [43, 103].

The majority of research has focussed on the erythroid maturation of $CD34^+$ HSCs which have been isolated from BM, PB, and UCB. For years a supportive stromal layer has been used during the erythroid maturation of HSCs [42] and during this research project this particular method was utilised as outlined in Chapter 2. Here, the starting cell density was 10^4 cells/mL for phase I using IMDM medium (Invitrogen), named D-Medium during this project, containing 100 ng/mL SCF, 5 ng/mL IL-3, 5 U/mL EPO and 10^{-4} M hydrocortisone (Day 0-8). During phase II, day 8-11, the cell density was 10^4 (PB), $4x10^4$ (UCB), or $6x10^4$ cells/cm^2 on a confluent BMSC stromal layer (feeder layer) and D-medium was used containing 5 U/mL EPO. At day 11, the cells were washed to remove any residual EPO and transferred back to the cultures containing the stromal layer and D-medium. Cells were maintained up to an additional two weeks.

The decision to use not only PB-$CD34^+$ and UCB-$CD34^+$ cells in this study, but also to include MNCs was based on data published by Van den Akker *et al* (2010) who compared $CD34^+$ cells, $CD34^-$ cells, and PBMCs for erythroid maturation, in combination with the limited access to PB and UBMCs for the isolation of sufficient numbers of $CD34^+$ cells. Van den Akker *et al* also reported on an alternative approach to boost the erythroblast yield from PB samples. They isolated $CD34^-$ cells and $CD34^+$ cells from PBMCs and showed that the output for $CD34^-$ cells contained the most significantly number of early erythroid progenitors [41]. Another study reported on the depletion of $CD3^+$ and/or $CD14^+$ cells from UBMCs to stimulate the *ex vivo* expansion potential of $CD34^+$ cells [59]. These studies showed that the $CD34^+$ HSCs within the heterogeneous MNC population (UCB, PB) were

affected by surrounding cells and that the research area which includes $CD34^-$ MNCs for the manufacture of RBCs requires further in depth analysis.

In short, the first aim of this study was to produce RBCs from PB and foremost UCB isolated cells. The second aim, as outlined in this chapter, was to research methods that allowed for improving the expansion rate of these cells and subsequently advance the erythroid maturation rate.

The hypothesis of this chapter is: Can the erythroid maturation be maximised by optimising the input of cells during the differentiation process?

4.2 Erythroid maturation of PB-CD34$^+$ cells

After successful isolation of CD34$^+$ cells from PBMCs, including the expansion of these cells, we used this source of cells to introduce a culture to an environment for erythroid maturation and analyse cell behaviour. 4.92x10^7 PBMCs were used for the magnetic isolation of CD34$^+$ cells. Approximately 1.5x10^5 cells were isolated and cultured at 3x10^3 cells/mL in a 12 well plate containing (serum free) D-medium including EPO, SCF and IL-3. At the end of a 7 day culture period, phase I of erythroid maturation, a total of 1.6x10^5 cells were collected and transferred to wells containing a BMSC stromal layer and D-medium containing EPO. Figure 4.1 shows the cells on a stromal layer. A low cell number was observed during this second phase of erythroid maturation. Minimal cell proliferation in combination with a disrupted stromal cell layer resulted in a non-viable culture.

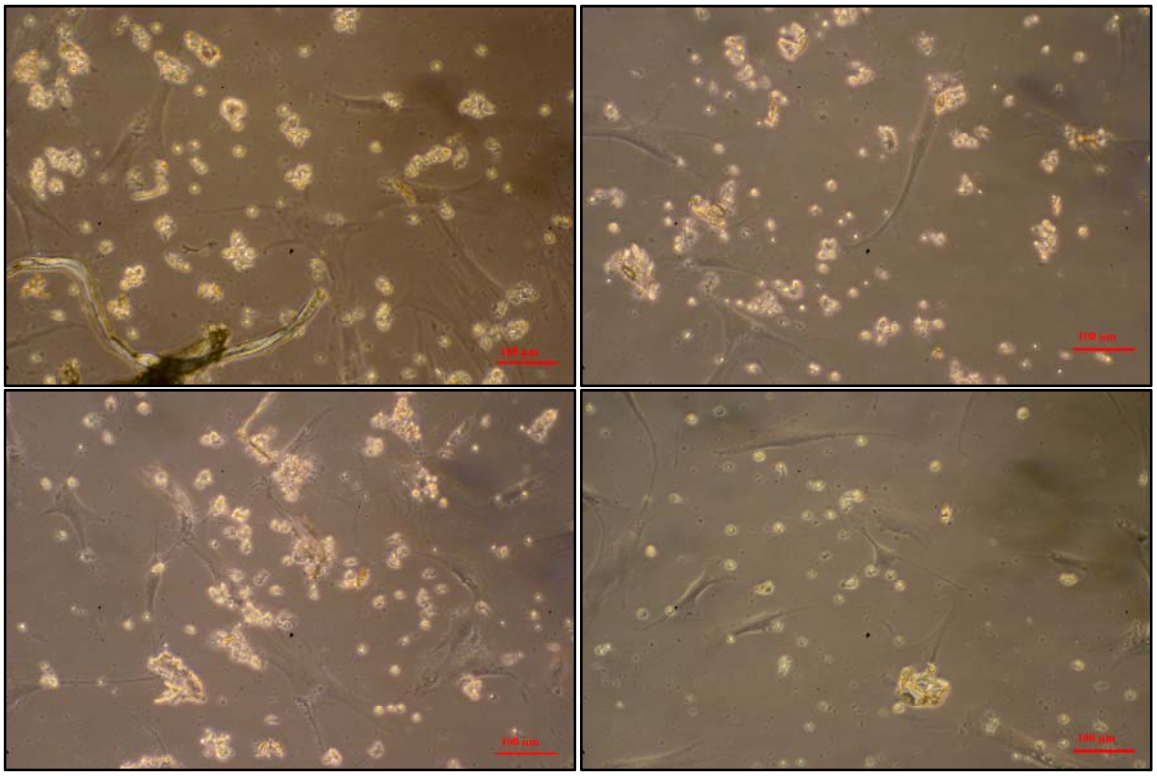

Fig. 4.1. *Morphology on day 9 of erythroid maturation; a co-culture of HSCs and BMSCs in EPO containing D-medium*

4.3 Erythroid maturation of UCB-CD34$^+$ cells

Unsuccessful erythroid maturation of PB-CD34$^+$ cells resulted in a process to optimise CD34$^+$ expansion and differentiation methods. UBMCs were previously used for the optimisation of HSC cultures and in this experiment UCB-CD34$^+$ cells were analysed for their maturation into RBCs. UCB-CD34$^+$ cells were thawed and ~3x10^5 cells were cultured at 3x10^4 cells/mL in D-medium containing SCF, EPO, and IL-3. When cells were subcultured at day 4, the cell density was increased to 6x10^4 cells/mL.

At the start of the second phase of erythroid maturation (day 10), ~2x10^6 cells were collected and 5x10^5 of these cells were subcultured in a co-culture with confluent BMSCs at 5x10^4 cells/mL in D-medium containing EPO. The remaining cells were analysed by flow cytometry for the surface markers CD34 (HSC marker [104]) and CD44 (found on HPC subpopulation of MNCs [105] and erythroid cells [4, 106]) in addition to the presence of Hb-β (Figure 4.2). Surface molecule CD44 also participates in the adhesion of HSC to stromal cells [48, 107]. The cell population was 92.9% positive for CD34 whereas this was 82.8% positive for CD44. When the cells were analysed for Hb-β using a cell permeability kit and flow cytometry 90.9% of the cells showed a positive expression.

Cell morphology was monitored during the third phase of the erythroid maturation culture. An overview of the culture at day 12 (Figure 4.3A) showed cells in suspension on top of a stromal cell layer (elongated, adherent cells). The cells in suspension varied in size with the biggest cells of a similar size as UCB isolated cells (Figure 1.3). The smallest cells had approximately half the diameter of the largest cells. The higher magnification image (Figure 4.3B) provides a clear picture with the suspension cells on top of the stromal cell layer. At day 12, a pellet of these cells showed for the first time a red colour similar to a pellet of RBCs and this was maintained throughout the remaining culture period (data not shown). After 20 days of culture, an overview image (Figure 4.4A) showed a dense populated cell culture. During 20 days of differentiation, the majority of the cells changed morphology and

became smaller in size compared to UCB-CD34^{+}. The cells had a round like morphology (Figure 4.4B).

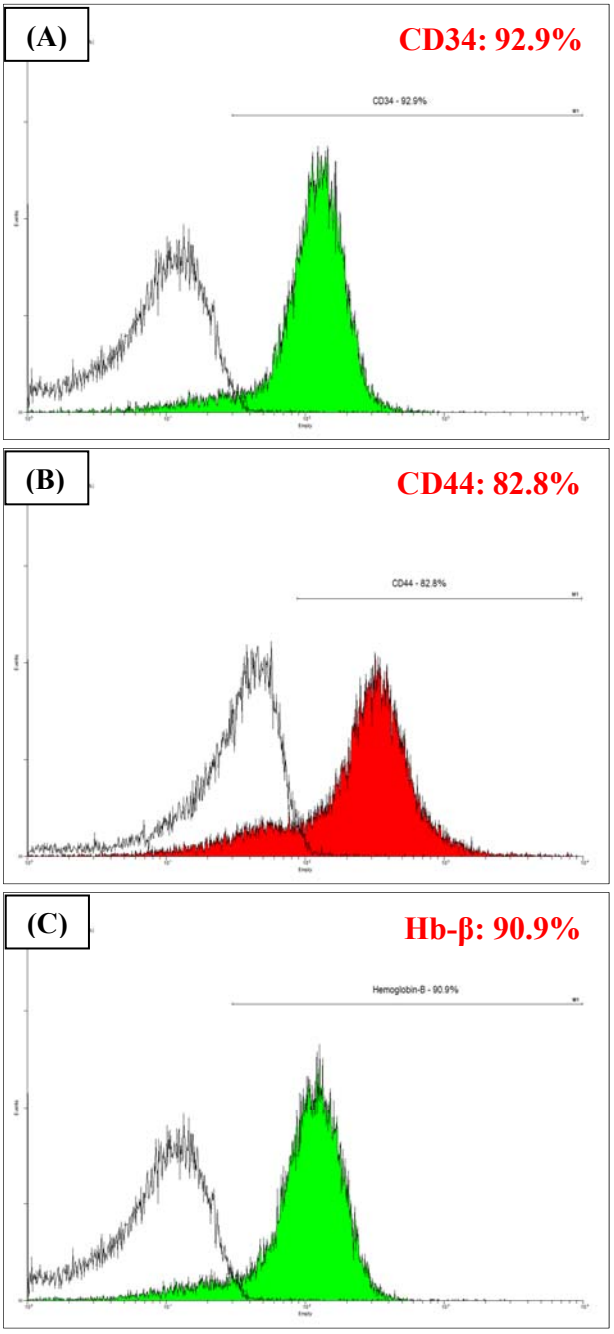

Fig. 4.2. *After 10 days of* erythroid maturation*, day 2 of phase II, the cell population was analysed for the presence of CD34 (A), CD44 (B) and the molecule Hb-β*

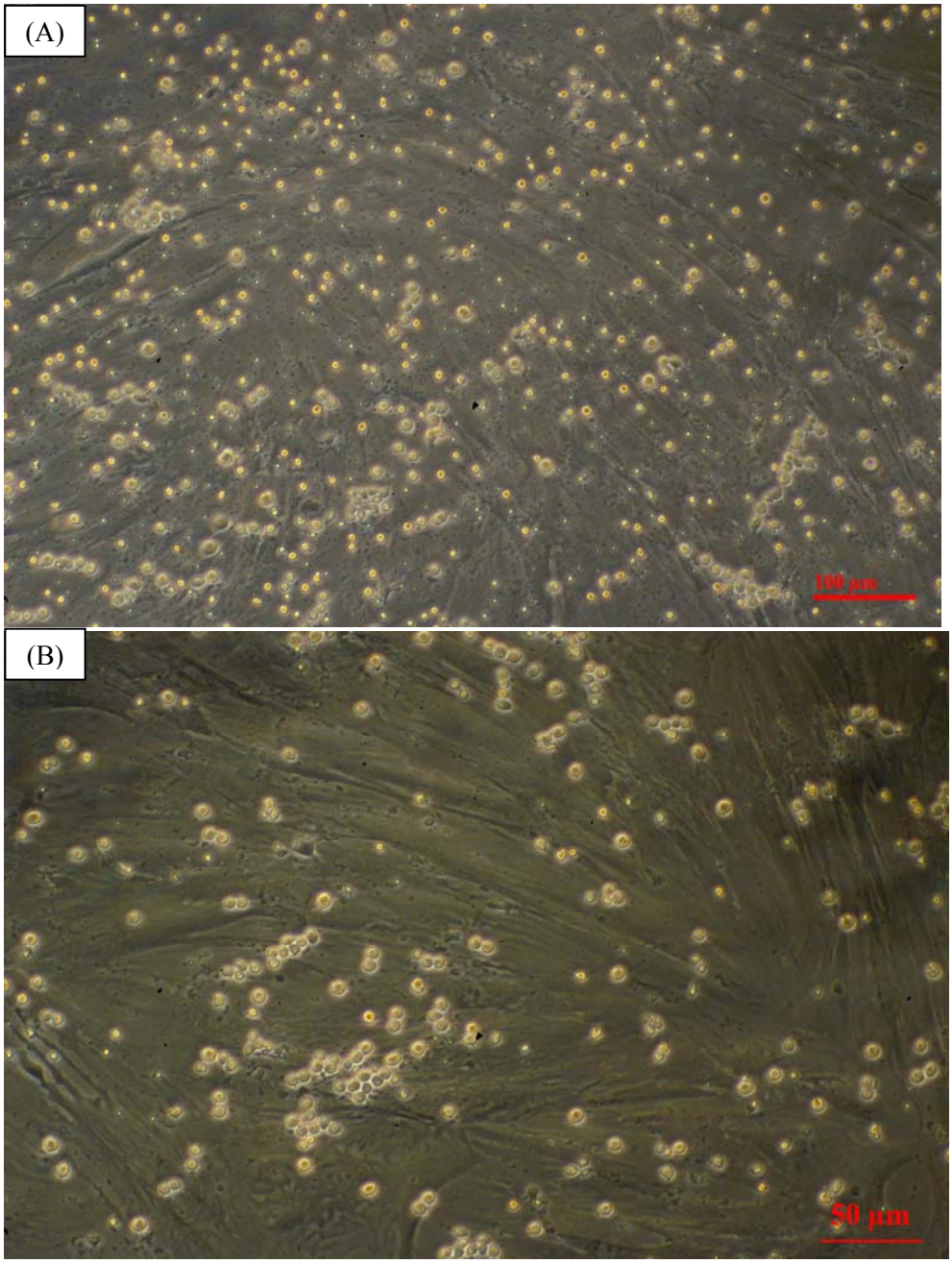

Fig. 4.3. *Morphology of UCB-CD34$^+$ cells on a stromal cell layer after 12 days* of erythroid maturation: overview *(A) and higher magnification (B)*

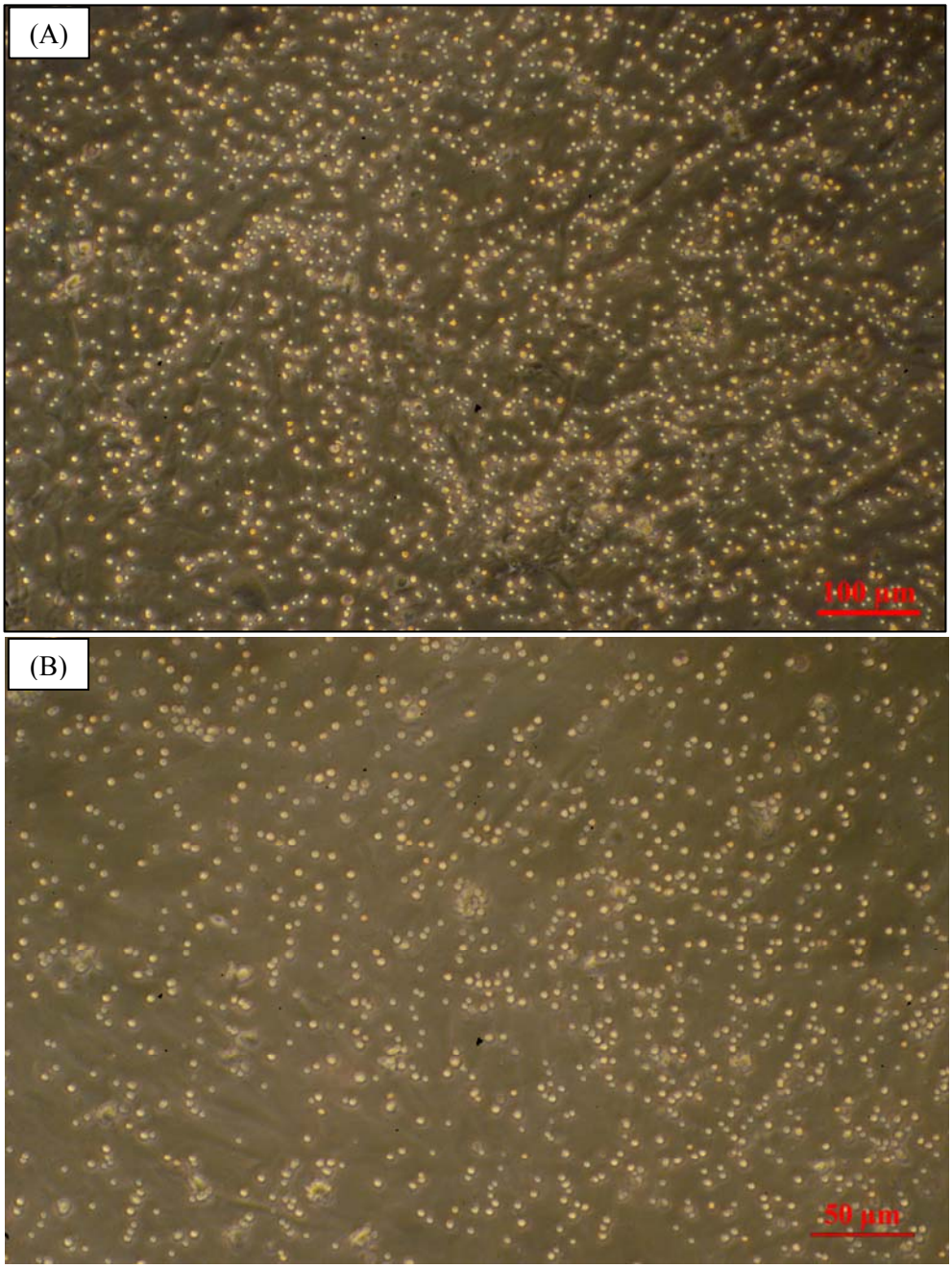

Fig. 4.4. *Morphology of UCB-CD34^{+} cells on a stromal cell layer after 20 days of erythroid maturation: overview (A) and higher magnification (B)*

At day 24, cells were examined after staining with the histology stain BCB as shown in Figure 4.5. This histological examination is a method to visualise erythrocyte generation by the presence of reticuloctyes. After staining with BCB, the reticular filaments should appear deep blue against a pale blue background. In this figure, cells marked with a black arrow have a dark pink colour and pale grey cells with a number of darker dots were marked with a red arrow. The cells with a black arrow were ~5-7μm in diameter whereas the red arrow marked cells were slightly larger in size. Larger cells (>20μm in diameter) with a purple-colour and a dark pink stained nucleus were also observed.

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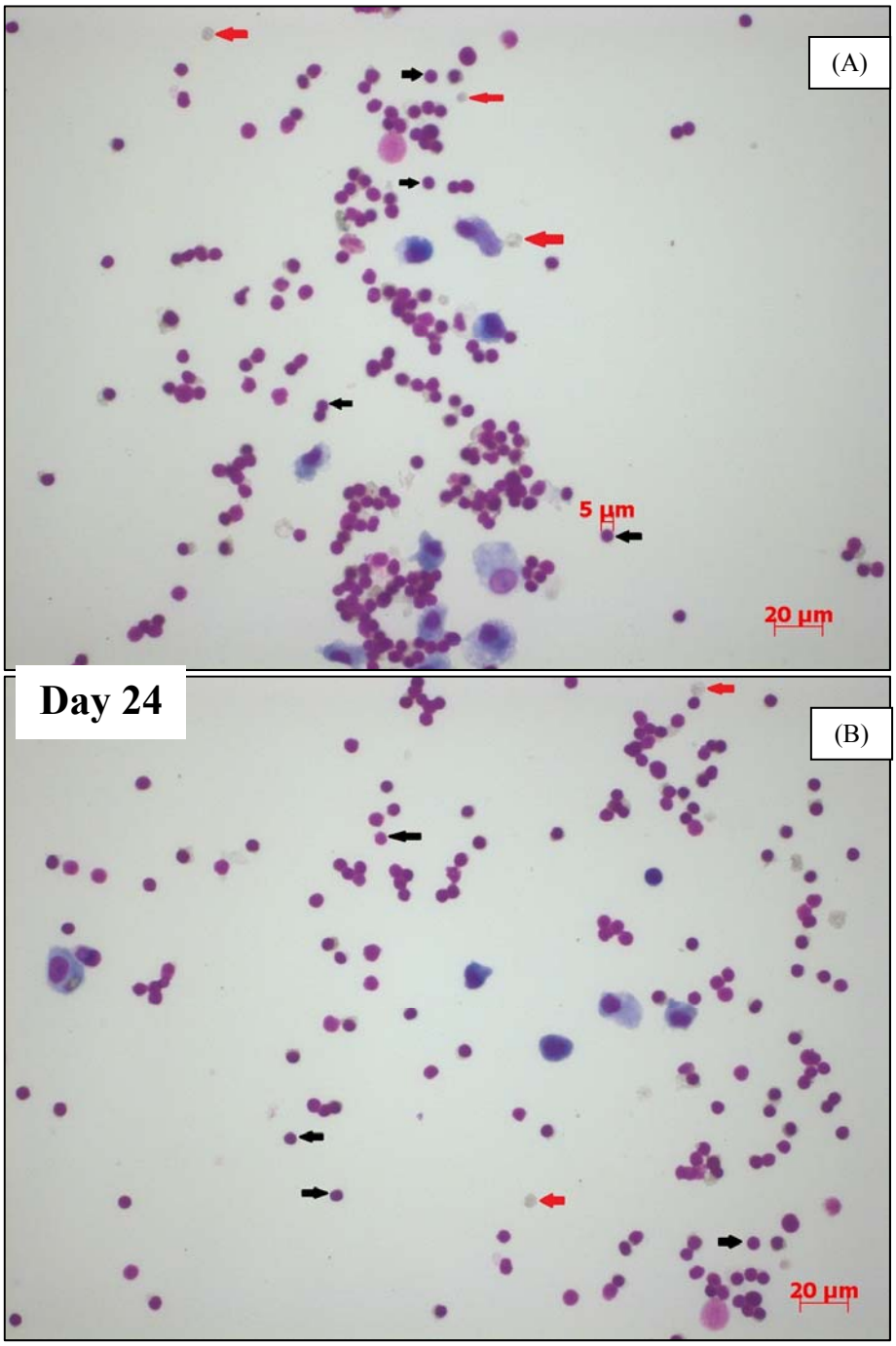

Fig. 4.5. *Cells stained with BCB after 24 days of erythroid maturation*

At days 18, 21, and 24 cells were analysed for three different antibodies using flow cytometry (CD34, CD44, and Hb-β). In this particular experiment, it was found that the $CD34^+$ expression remained high during the initial 10 days of culture. After an additional culture period of 8 days (day 18) the percentage of cells containing the $CD34^+$ marker stayed above 10%. After an additional 7 days of erythroid maturation of this cell population, an overall lowest percentage of positive CD34 expression was recorded: 11.7% at day 25 (Figure 4.6A). This same graph showed how the expression of CD44, dropped when compared to previously collected data (Figure 4.2B). From ~80% positive at day 10, the percentage of cells with a CD44 antibody reached ~55% at day 18 and was ~15% after 21 and 25 days (Figure 4.6A).

During the same culture period as stated above, the highest percentage of cells containing this oxygen carrying molecule Hb-β was ~95% at day 25 (Figure 4.6A). There was an increase in cells containing Hb-β during the last week of culture with ~50% of the cell population on day 18 and ~80% on day 21. Figure 4.8B shows the change in marker expression between day 10 and day 25 of erythroid maturation. This graph shows clearly how the expression of CD34 and CD44 diminished whereas the percentage of cells containing Hb-β increased.

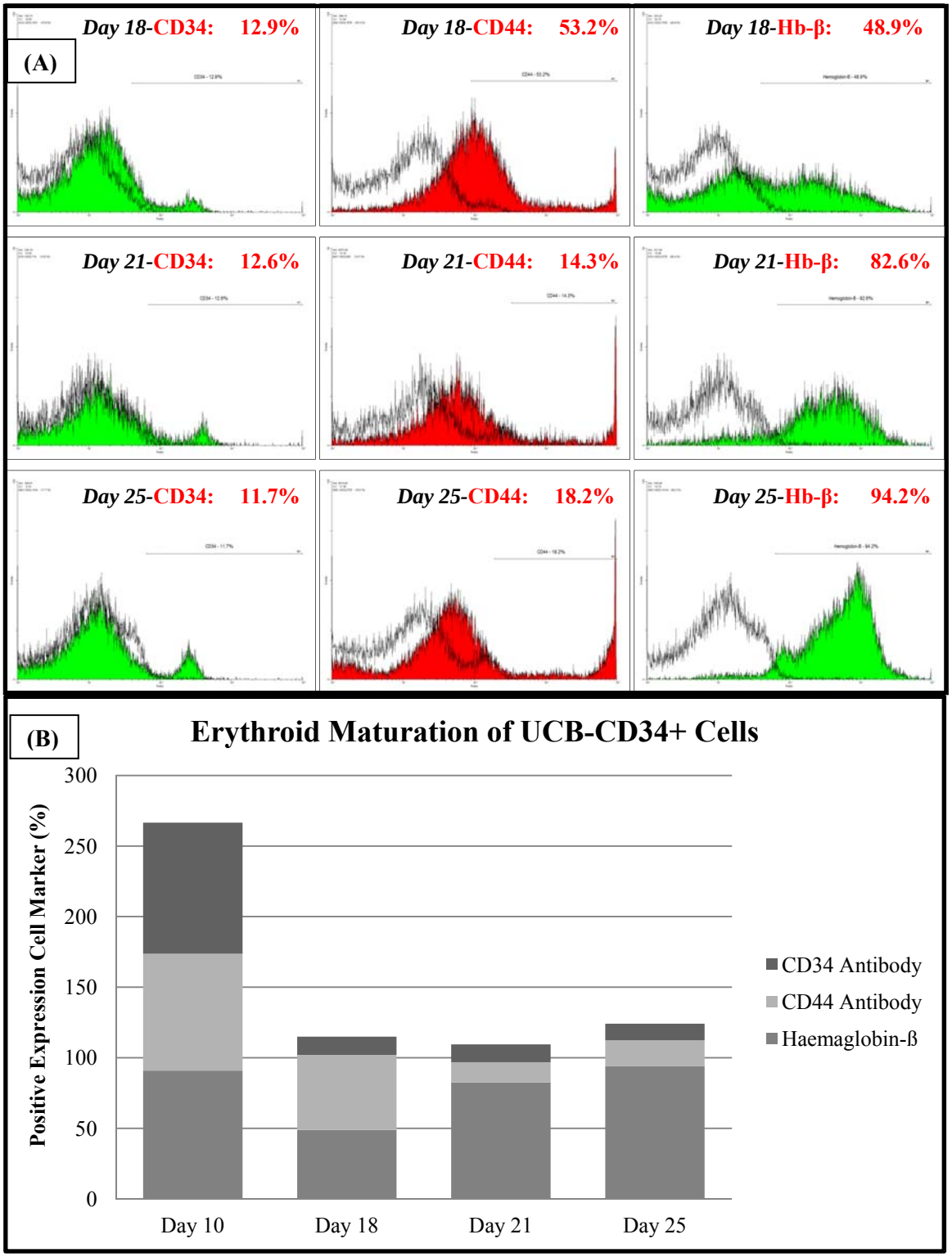

Fig. 4.6. *Flow Cytometry graphs (A) and accompanying overview (B) of cell markers important in the erythroid maturation of HSCs*

4.4 Erythroid maturation of UBMCs

In the previous chapter, HSC expansion of PBMCs and UBMCs including the growth of $CD34^+$ cells with a heterogeneous population was documented. These results combined with data published [41] resulted in the use of UBMCs for the manufacture of RBCs.

To analyse the erythroid potential of UCB-isolated MNCs, including a majority of $CD34^-$ cells, UBMCs were first expanded under different HSC expansion conditions before the cells were introduced to the three phases of erythroid maturation as described before [42] and used in previous experiments (Chapter 3).

We defined three HSC culture conditions to pre-treat UBMCs from day -7 (start expansion) up to day 0 (start erythroid maturation):

- (A) = UBMCs on a MSC stroma using I-medium containing cytokines (50 ng/mL of SCF/TPO/Flt3-L);
- (B) = UBMCs using I-medium containing cytokines (50 ng/mL of SCF/TPO/Flt3-L);
- (C) = UBMCs on a MSC stroma using I-medium without cytokines

In the subsequent phase, during erythroid maturation, cells from conditions (A), (B), and (C) were used and compared with UBMCs without pre-culture treatment (D). All erythroid maturation cultures used D-medium containing 100 ng/mL SCF, 50 ng/mL IL-3 (A, B, and C) or 5 ng/mL IL-3 (D), 5 U/mL EPO and 10^{-4} M hydrocortisone for 8 days. Next, the cells were transferred to cultures containing a BMSC stromal layer and D-medium containing 5 U/mL EPO. After 2 days the cells were washed to remove any residual EPO and placed back in to the cultures containing a BMSC stromal layer and D-medium without cytokines for up to 14 days. During this culture period the cell numbers and erythroid maturation markers were analysed.

For the first 7 days, pre-erythroid maturation at day 0, the cell number decreased for (B) and (C), but when UBMCs were cultured in medium containing cytokines and in the presence of a stromal layer the cell number increased 5 fold (Figure 4.7). At day 0, culture (A) contained ~440x10^3 $CD34^+$ cells (30.9%), culture (B) contained ~20x10^3 $CD34^+$ cells (18.2%) and culture (D) contained ~13x10^3 $CD34^+$ cells (4.4%). No cell count was performed for culture (C) due to insufficient cell numbers. For all cultures, the highest cell number during the erythroid maturation was recorded at day 15. At day 10, a high number of suspension cells adhered to the stromal layer and this explained the low recorded cell number for culture (C).

Overall, the culture without any pre-HSC expansion (D) showed the highest cell number throughout the entire culture period and at day 24 a total of ~1.2x10^6 cells were harvested. From the cultures (B), (C) and (D) at day 24, only 50% of the cells was alive.

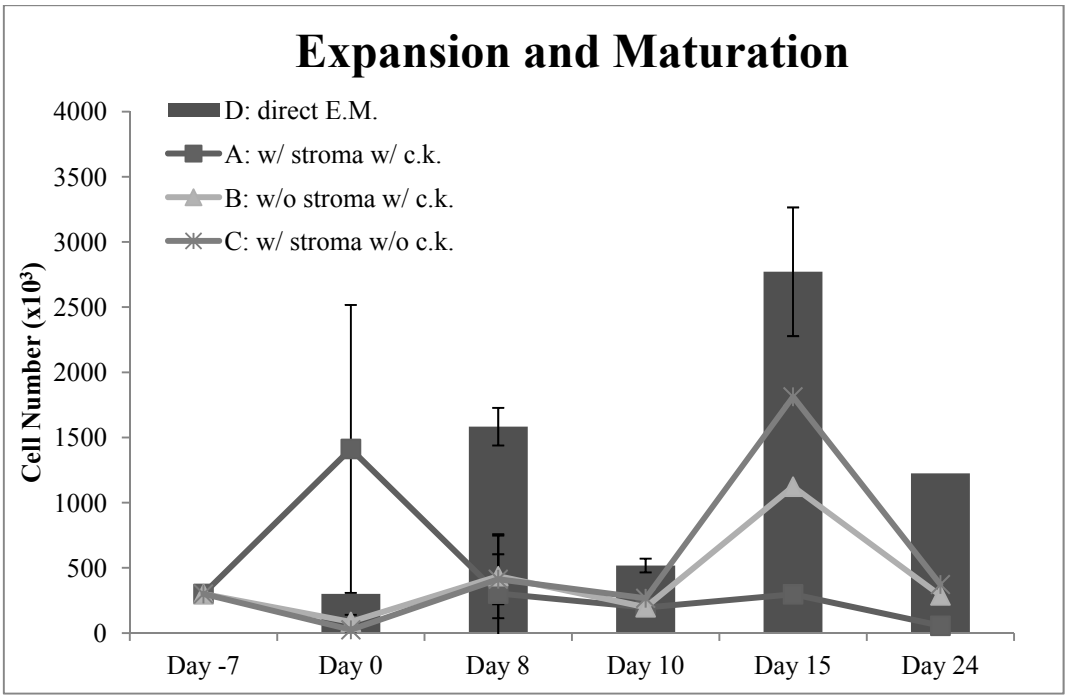

Fig. 4.7. *Overview of viable cell numbers during initial expansion of UBMCs; day -7 – 0 (A-C) and during erythroid maturation; day 0 – 24 (A-D). The bars represent data from condition D, the lines represent data from the conditions undergoing expansion and subsequent maturation (A-C) (n=3, error bars represent StDev).*

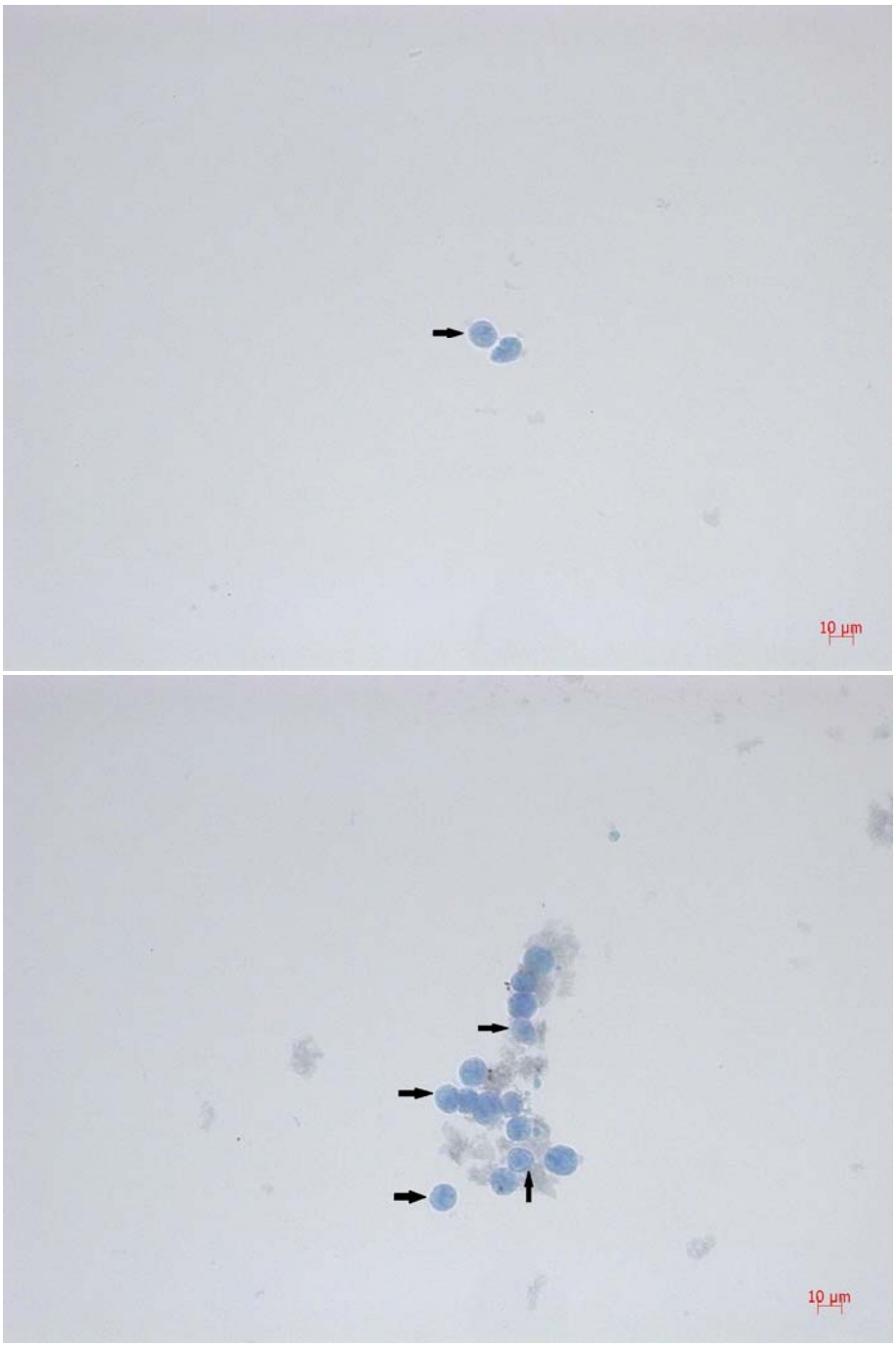

Fig. 4.8. *Cells stained with BCB at day 9 of direct EM (Condition D)*

The cells depicted in Figure 4.8 show BCB-stained MNCs at day 9 of erythroid maturation. Cells marked by a black arrow showed a pale blue background containing deeper blue filaments.

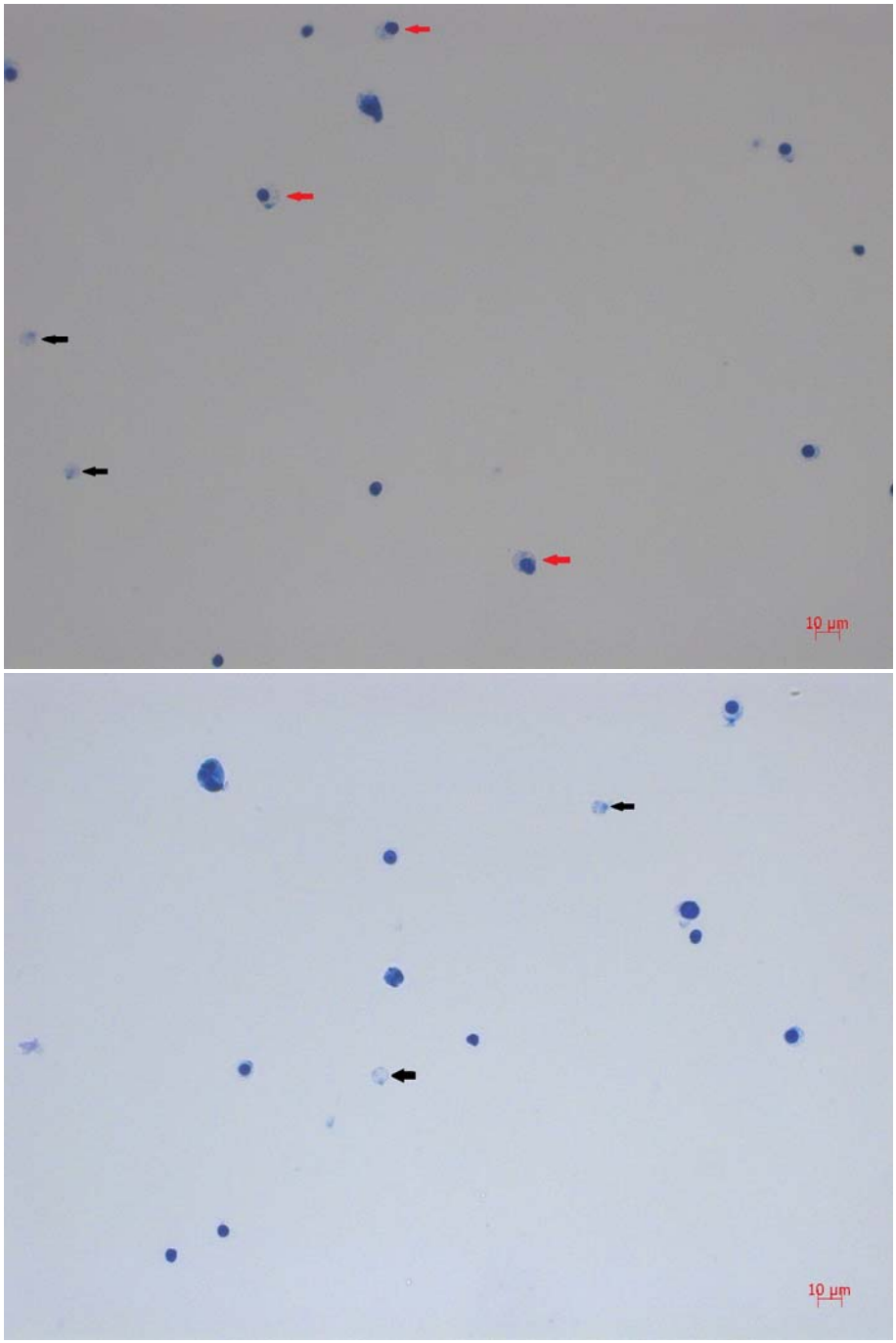

Fig. 4.9. *Cells stained with Methylene Blue at day 16 of direct EM (Condition D)*

At day 16, MNCs in erythroid maturation were stained with Methylene Blue to distinguish cells with a nucleus from enucleated cells (Figure 4.9). The nucleus appears dark blue against a pale coloured cytoplasm with this stain. Cells marked with a black arrow showed pale blue coloured cells with deeper blue filaments. These cells are approximately 10µm in diameter. Cells marked with a red arrow appeared to extrude the nucleus.

The cell marker profiles for all cultures (A-D) during erythroid maturation have been recorded with flow cytometry at day 15 and day 24 (Figure 4.10). These data have been set out against the same cell marker profile of untreated UBMCs (day 0). Cells from culture condition (A) at day 24 were only analysed for Hb-β due to insufficient cell numbers (Figure 4.7). Data for day 15 and day 24 was compared using statistics (Chapter 2.6.4) and for all cell markers the p-value was calculated.

At day 15 and day 24, between 1% and 6% of each cell population was positive for CD34 (CD34: $p<0.5$). When CD45 and CD44 were analysed, it was observed that a high percentage of the UBMC population was positive (CD45: ~96% and CD44: ~99%). During erythroid maturation the expression of these markers decreased below 15% for CD45 at day 15 and below 10% at day 24 (CD45: $p<0.5$). It was recorded that for each cell populations between 30% and 55% were positive for CD44 at day 15. At day 24, between 22% and ~42% of the cells were positive (CD44: $p<0.2$). CD44 can be detected on the surface of many cells including those found within the haematopoietic cell lineages [108] and on red cell membrane [109]. The marker CD36 can be found on colony forming unit erythrocytes (CFU-E's) and was used to detect cells that were in the intermediate stage between HSC and RBC. At day 15, a cell population of ~40% was positive for CD36 (D). The cell population with the lowest positive expression was observed in culture (B): ~18%. At day 24 of the culture period conditions (B) and (C) the recorded positive expression for CD36 was ~38% whereas this was ~24% for culture (D) (CD36: $p=0.5$).

CD71 is a marker that can be found on erythroid precursors as reported by [42, 110]. On day 15, more than 40% (A, B) and more than 80% (C, D) of the cell populations were positive for CD71. On day 24 the recorded expression was ~30% for (B) and (C) and the expression was ~50% for (D) (CD71: $p<0.1$). The expression for UBMCs was ~5% on day 0.

Cell markers which are commonly associated with erythrocytes (RBC) are Glycophorin A (CD235a) and adult Hb-β [110]. The UBMC population at day 0 was 5% positive for

CD235a and ~45% positive for Hb-β. At day 15, a 95% (B, C, D) and 80% (A) positive cell population was recorded for CD235a. At day 24 this was ~95% for the cultures (B), (C), and (D) (CD235a: $p<0.5$). When the expression of Hb-β was analysed at day 15, a positive cell population of ~13% was recorded for all culture conditions (A, B, C, and D). At day 24, the flow cytometry results showed that ~75% of the cultures (B) and (C) were positive. For culture (A), ~40% of the cells contained Hb-β whereas this was 60% of the cells from culture (D) (Hb-β: $p<0.5$).

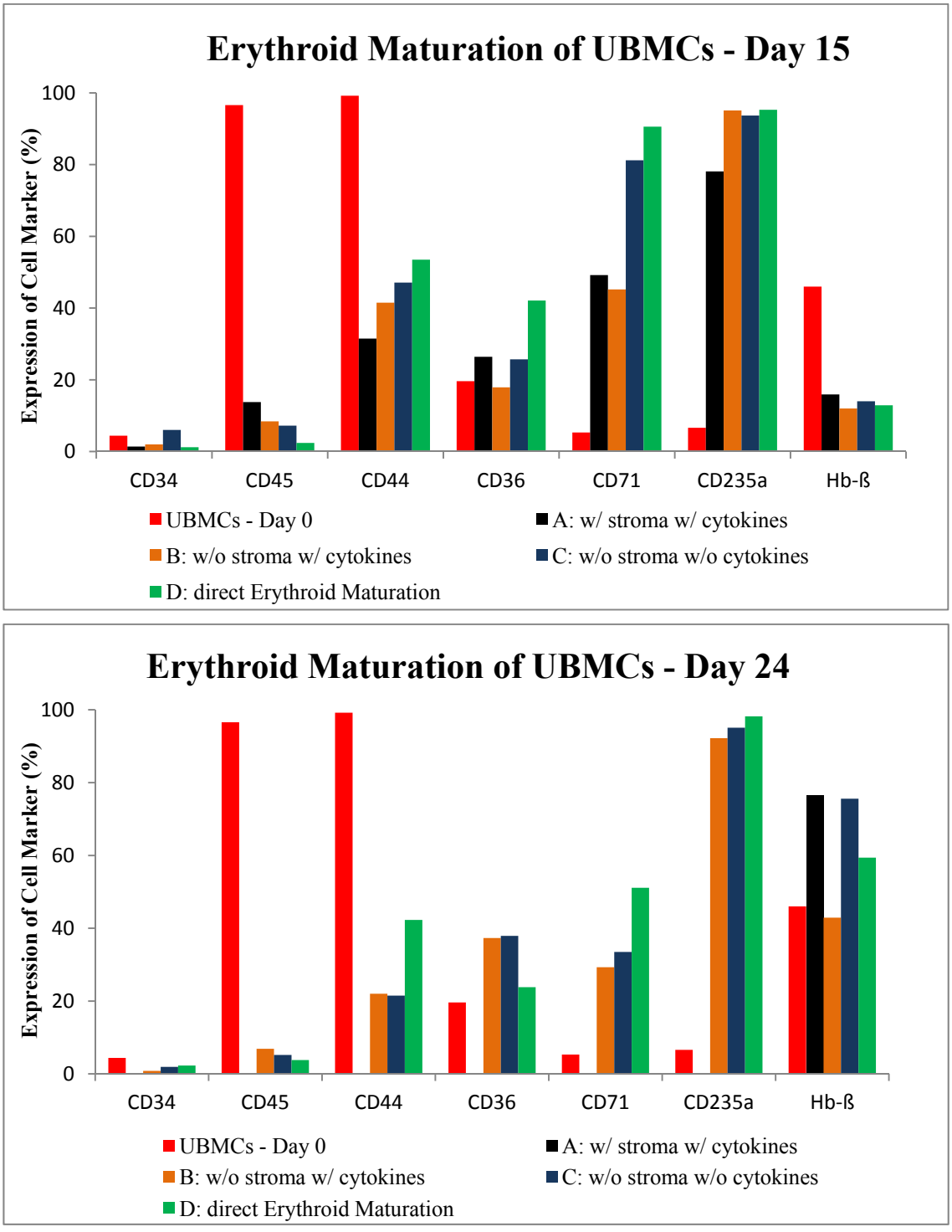

Fig. 4.10. *UBMCs were cultured for 7 days in HSC expansion medium prior to erythroid maturation under different conditions:* ***(A)*** *= 'with stroma/with cytokines';* ***(B)*** *= 'without stroma/with cytokines';* ***(C)*** *= 'with stroma/without cytokines'.* ***(D)*** *= UBMCs which have not been cultured in HSC expansion medium prior to erythroid maturation. The red bar represents the marker profile of UBMCs after isolation whereas the other bars represent the conditions A-C at the day 15 (above) and day 24 (under) of the differentiation period. The green bar represents the marker profile for condition D. The horizontal axis shows the haematopoietic and erythroid markers (n=1 due to limited cell numbers after isolation).*

The erythroid maturation of UBMCs has been analysed in more detail during a period of 21 days. The culture conditions were similar to the conditions as stated in the previous experiment including pre-treatment by HSC expansion of UBMCs in I-medium during 7 days: condition (A), (B), and (C). No pre-treatment was applied to the cells of condition (D). After the pre-treatment of (A), (B), and (C), all culture conditions were introduced to erythroid maturation using D-medium. During phase I (Day 0-8) the medium was supplemented with 100 ng/mL SCF, 5 ng/mL IL-3, 5 U/mL EPO, and 10^{-4} M hydrocortisone. Phase II (day 8-12) and phase III (day 12-21) were treated similar as stated in a previous experiment (Chapter 4). Data recorded during this experiment was based on cell numbers and flow cytometric analysis (CD117, CD34, CD45, CD36, CD71, CD235a, Hb-β).

The expansion of HSCs, a pre-treatment of UBMCs, over 7 days showed that the culture 'with stroma/with cytokines' showed the most dramatic increase in CD34 positive cells: ~$1.3x10^4$ cells (day -7) to ~$8.9x10^4$ cells (day 0). The total number of cells recorded at day -7 (start pre-treatment) and at day 0 (end pre-treatment) is similar: ~$3x10^5$ cells (Figure 4.11).

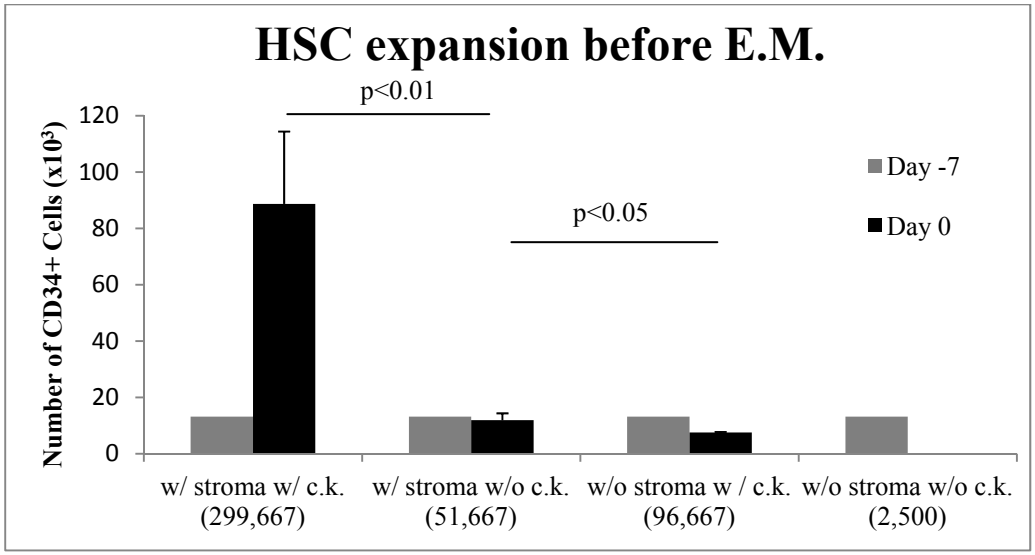

Fig. 4.11. *HSC expansion of UBMCs over 7 days to increase the number of $CD34^+$ cells before the start of erythroid maturation (day 0). The horizontal axis shows the various culture conditions and the number in parentheses states the total cell number at day 0. All cultures started with $3x10^5$ UBMCs at day -7 (n=3, error bars represent StDev).*

For the culture conditions 'with stroma/without cytokines' (C) and 'without stroma/with cytokines' (B), the number of $CD34^+$ cells dropped from ~$1.3x10^4$ (day -7) to ~$1.2x10^4$ (C) and ~$0.8x10^4$ (B) respectively. The total number of cells was ~$0.5x10^5$ (C) and ~$1x10^5$ (B) after 7 days of HSC expansion respectively. The culture condition of UBMCS 'without stroma/without cytokines' was not viable over 7 days. Cell morphology was observed for the culture conditions (A), (B), and (C) at the end of HSC expansion and for condition (D) at the start of erythroid maturation (day 0) as is shown in Figure 4.12

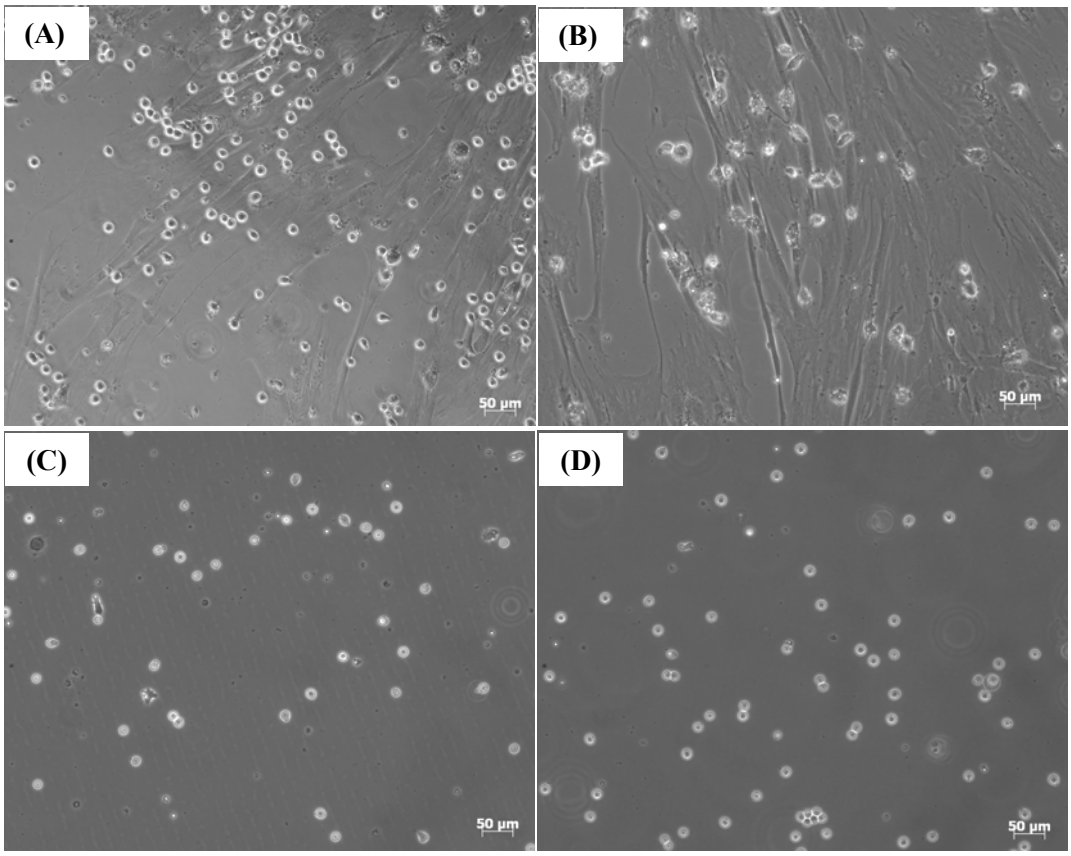

Fig. 4.12. *Morphology of cells on day 0, prior to erythroid maturation (D) and after the HSC expansion treatment (A, B, C)*

The results from flow cytometric analysis showed the pre-treated UBMCs (A, B, C) compared against the not pre-untreated UBMCs (D) during erythroid maturation (Figure 4.13). For a range of cell markers, the expression on cells for pre-treated versus non pre-treated cultures were similar: CD117 (12-16% versus, ~17%, CD34 (0-4% versus ~1%), CD45 (6-15% versus ~2%), CD36 (20-30% versus ~32%), and CD235a (90-97% versus ~98%).

When the three remaining markers were analysed (CD44, CD71, and Hb-β), it was observed that culture (D) showed a higher positive expression over all other culture conditions (A, B, C). At day 21, ~50% of the cell population in culture (D) was positive for CD44 whereas this was ~34% for (A) and ~22% for (B) and (C). For CD71, ~27% of the cell population was positive (B) and this was ~39% for (C), ~52% for (A), and ~59% for (D). From the cells in culture (B), ~46% contained Hb-β. For culture (A) and (C), ~73% of the cells contained Hb-β whereas this was ~82% of the cells for culture (D).

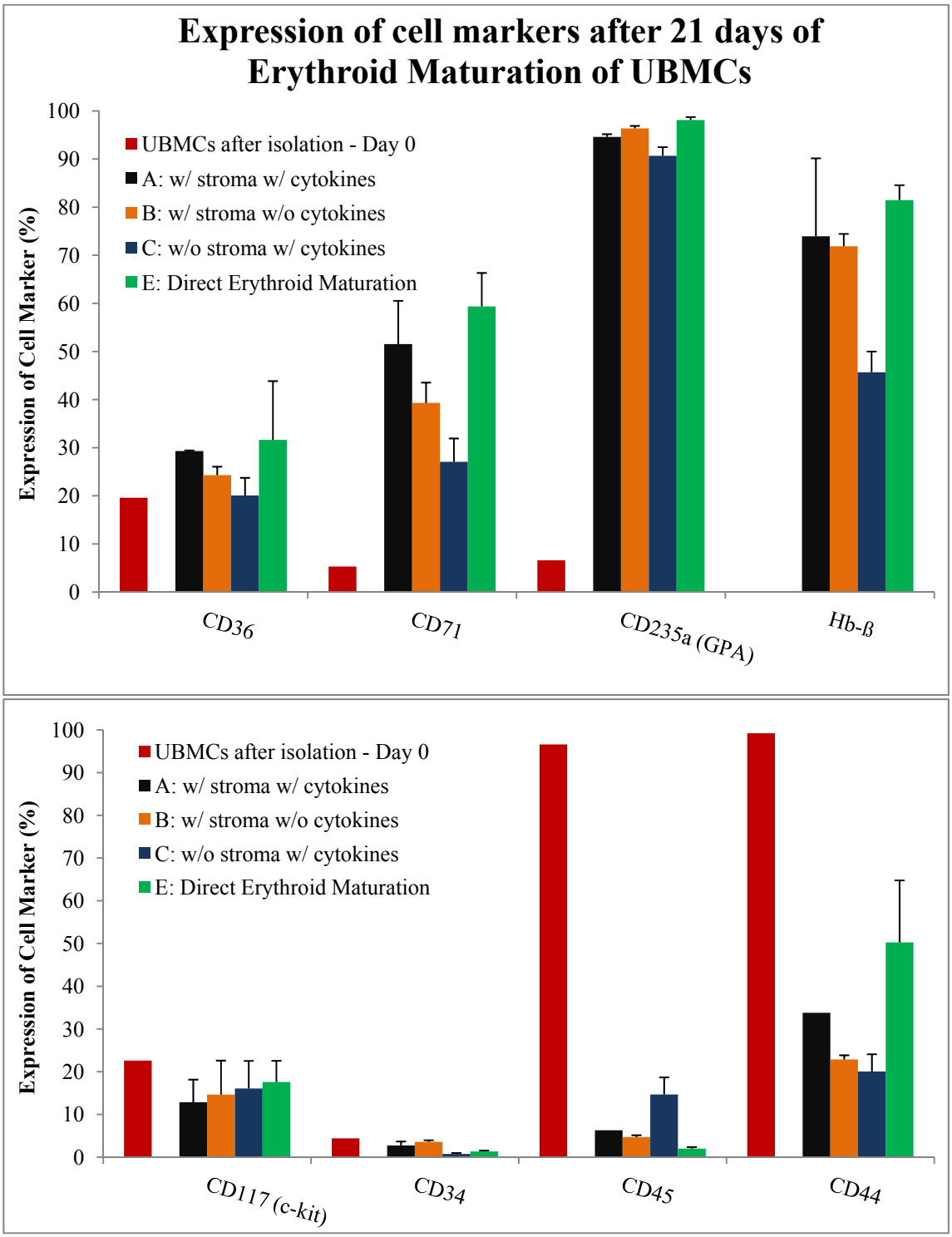

Fig. 4.13. *The marker profile of cultures (A-D) after 21 days of erythroid maturation. UBMCs were cultured for 7 days in HSC expansion conditions,* ***(A)*** *= 'with stroma/with cytokines';* ***(B)*** *= 'without stroma/ with cytokines';* ***(C)*** *= 'with stroma/ without cytokines', prior to erythroid maturation.* ***(D)*** *= UBMCs which have not been cultured in HSC expansion medium prior to erythroid maturation. The red bar represents the marker profile of UBMCs after isolation whereas the other bars represent the conditions A-C at the end of the differentiation period. The green bar represents the marker profile for condition D at day 21. The horizontal axis shows the haematopoietic and erythroid markers (n=3, error bars represent StDev).*

An overview with the number of cells at various time points during the erythroid maturation showed which culture conditions had a steady growth of cell numbers and which culture conditions had a different growth pattern (Figure 4.14). The untreated UBMCs (D) and the pre-treated UBMCs on a stromal layer (C) have a similar growth curve and almost $4x10^6$ cells were harvested at day 21. The pre-treated UBMCs with only cytokines showed an increase in cell number over the first 8 days of erythroid maturation before maintaining a cell number of ~$1.4x10^6$ cells. The pre-treated UBMCs (A), 'with stroma/with cytokines', had a different growth curve with a dramatic increase in cell number over the first 8 days ($6x10^6$ cells) before decreasing over ~14 days: ~$2.5x10^6$ cells at day 12 and ~$0.9x10^6$ cells at day 21.

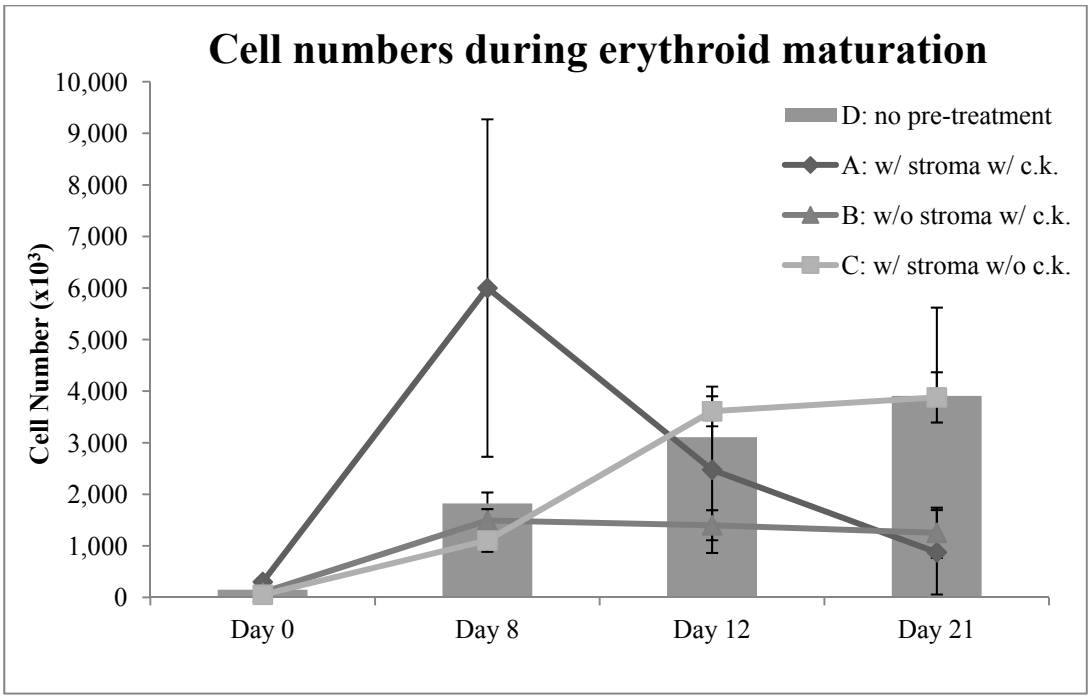

Fig. 4.14. *The fluctuation in cell number during 21 days of erythroid maturation.* ***(A)*** *= 'with stroma/with cytokines';* ***(B)*** *= 'without stroma/with cytokines';* ***(C)*** *= 'with stroma/without cytokines';* ***(D)*** *= 'without pre-treatment' (n=3, error bars represent StDev).*

The cell pellets were analysed for each culture condition after two and three weeks of erythroid maturation (Figure 4.15). The cell pellets of (A), (B), and (C) had a light bright red colour after 14 days of culture. Culture (D) showed a slightly darker red colour after 12 days of culture whereas the cell pellet of UBMCs at day 0 was white (data not shown). After 21 days of erythroid maturation the cell pellets of (A), (B), (C), and (D) were slightly larger when compared to previous time points. All cell pellets showed a darker red colour at day 21, except for condition (C) where cells were a slightly brighter red colour.

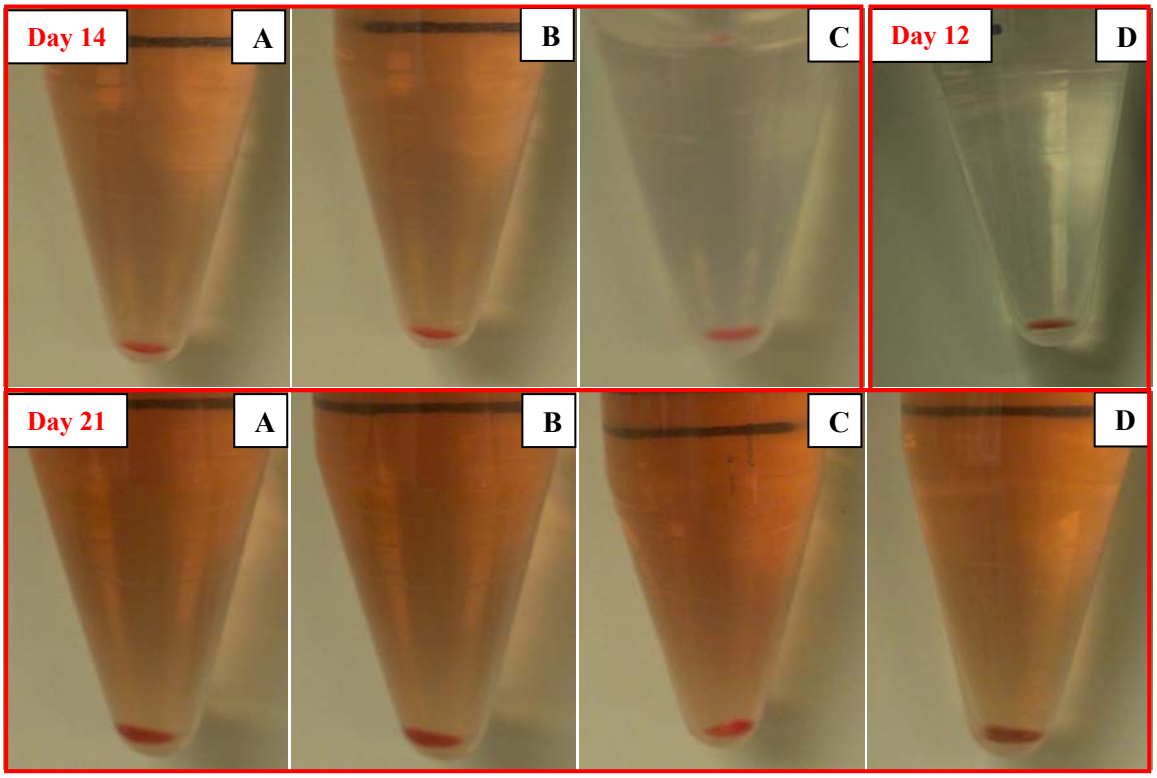

Fig. 4.15. *The cell pellets of each culture condition after approximately 2 weeks (top) and 3 weeks (bottom).* ***(A)*** *= 'with stroma/with cytokines';* ***(B)*** *= 'without stroma/with cytokines';* ***(C)*** *= 'with stroma/without cytokines';* ***(D)*** *= 'without pre-treatment'*

The previous experiments analysed the potential of UBMCs for erythroid maturation. During a subsequent experiment the UBMCs were separated into a CD34 negative cell population ($CD34^-$) and a CD34 positive cell population ($CD34^+$), using the magnetic bead cell isolation method. After cell separation, cells were transferred directly into medium for Phase I of erythroid maturation. Phase II (day 8) and Phase III (day 11) were executed subsequently as stated previously. For both cell populations, the cell numbers were recorded and cell pellets were observed. The $CD34^-$ cell population was analysed for a series of cell markers (CD34, CD45, CD36, CD71, CD235a) at days 0 and 17.

The cell numbers showed that for the $CD34^+$ cell population the increase between day 0 and day 23 is ~10 fold whereas this is ~45 fold between day 0 and day 17 (Figure 4.15). The culture started with ~$4x10^3$ cells and ~$3.2x10^4$ cells were harvested at the end of culture which is an increase in cell number (Figure 4.16). For the $CD34^-$ cell population, the increase is ~0.04 fold over 23 days and ~0.95 fold over 17 days. The overall cell number decreased during erythroid maturation from ~$2.0x10^6$ cells at day 0 to $7.0x10^5$ cells at day 17. When cell pellets were observed at day 11, the pellet originating from $CD34^-$ cells was red whereas the pellet with cells originating from the $CD34^+$ cells was white. At day 17 both cell pellets were bright red and at day 23 the pellets were red but smaller in size when compared to the cell pellets from day 17 (no data shown).

When the cell cultures were analysed with an inverted light microscope the culture of $CD34^-$ cells showed a high quantity of dead cells and cell debris with a high number of cells attached to the stromal layer (day 17). For the cell cultures of $CD34^+$ cells at day 17, there were only a small number of dead cells present and a high number of cells were attached to the stromal layer (data not shown). At day 23, the $CD34^-$ cell culture showed a large number of cells attached to the stromal cell layer. The cells were smaller in size compared to those in the $CD34^+$ cell culture.

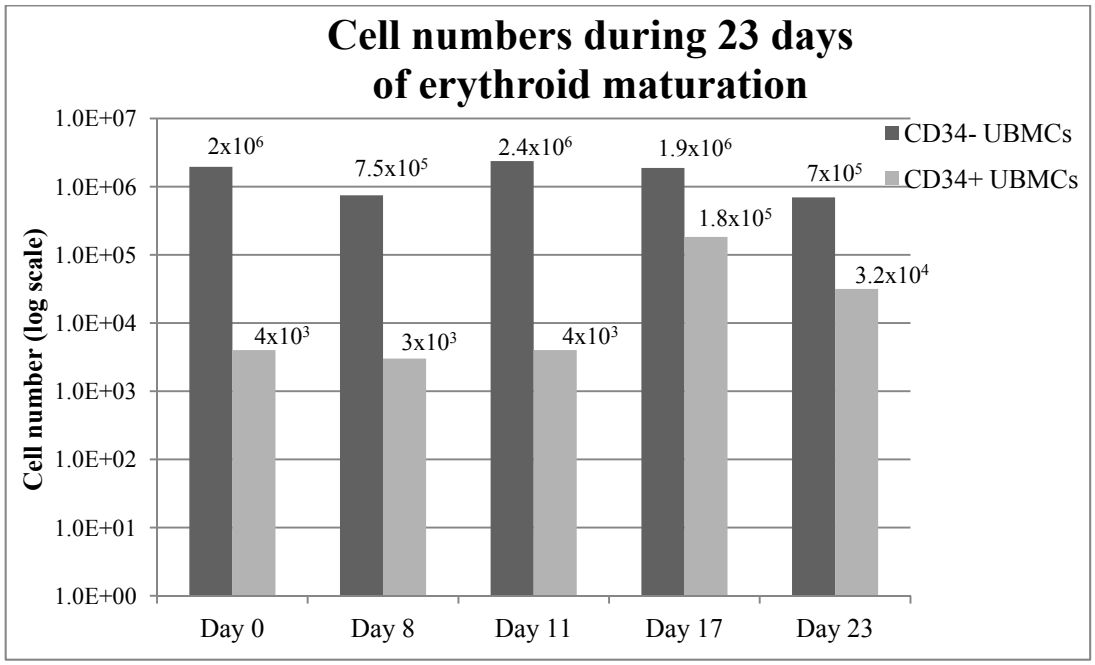

Fig. 4.16. *Development of cell numbers during 23 days of erythroid maturation*

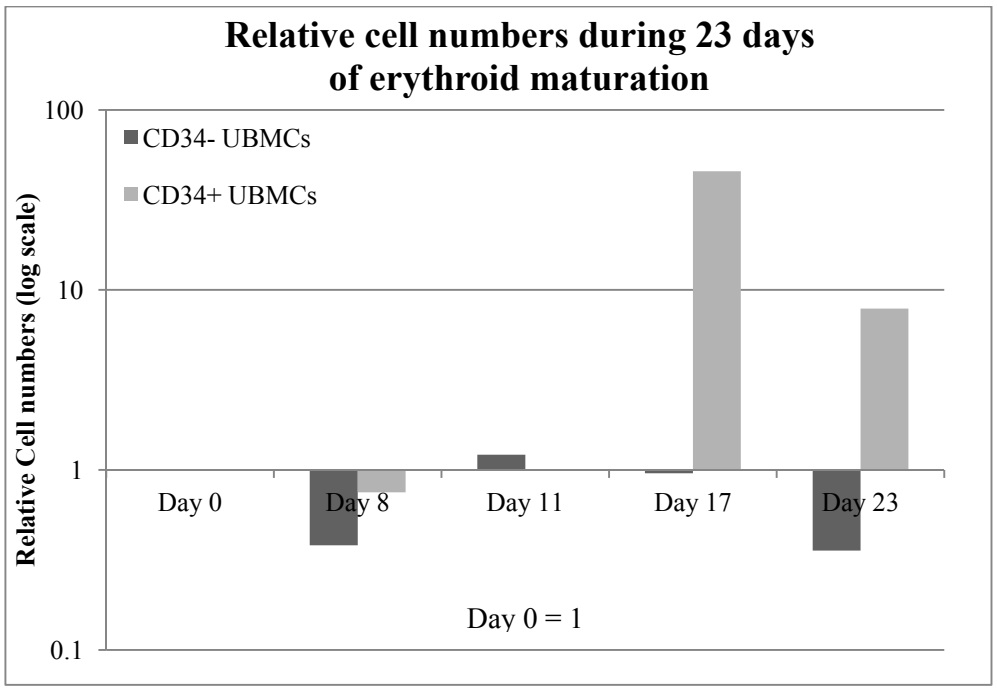

Fig. 4.17. *Relative cell numbers during 23 days of erythroid maturation*

The data collected with flow cytometry showed the change in cell marker expression for CD34$^-$ cells cultured for 23 days under erythroid maturation conditions (Figure 4.17). The expression of CD34 stayed below 6% and the expression of CD45 dropped from ~97% to ~3% over 17 days. A marker for the intermediate stage of erythroid maturation (CFU-E's) in the differentiation process, CD36, showed an increase in expression resulting in a positive cell population of ~70%. One of the erythroid lineage markers, CD71, was found on ~84% of the cell population at day 17. The RBC surface marker CD235a was observed on ~92% of the cell population at this timepoint.

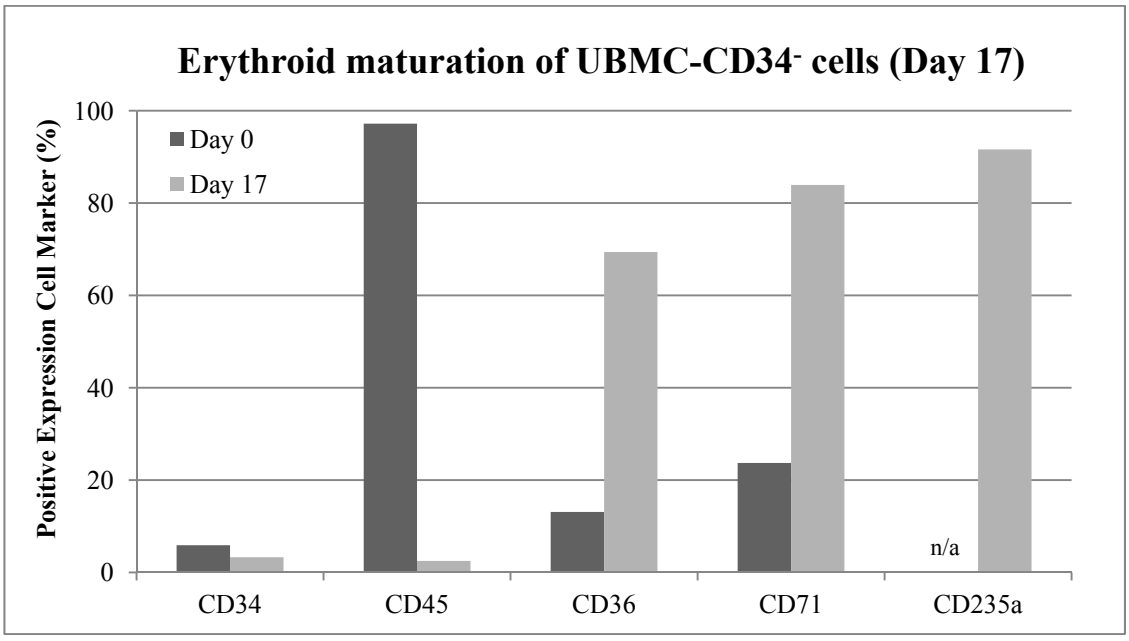

Fig. 4.18. *Cell markers in erythroid maturation of UBMC-CD34$^-$ cells after 17 days of culture*

4.5 Summary

This chapter focussed on the erythroid maturation of cells isolated from PB and UCB. The erythroid maturation of PB-CD34$^+$ cells was challenging due to low cell numbers available from isolated PBMCs. Erythroid maturation of UCB-CD34$^+$ cells was more successful with viable cells throughout the entire culture period. At the end of the maturation stage, cells contained Hb and showed similar cell morphology as immature RBCs.

Differentiation of UBMCs proved successful too with relatively high cell numbers compared to UCB-CD34$^+$ cells. Cell pellets after 21 days, red in colour, were similar to human isolated RBCs. At the end of the differentiation period, more than 80% of the cell population contained Hb-β and over 95% of the cells were positive for the red cell membrane antibody CD235a. Erythroid maturation of thawed UCB-CD34$^-$ cells showed promising results compared to CD34$^+$ cells isolated from thawed UBMCs but needs further optimising when compared to differentiated UBMCs. In summary, Figure 4.19 shows the distinctive different antibody profiles for differentiated UBMCs at day 1 (black bars) and after 2 weeks (blue bars) and 3 weeks (brown bars).

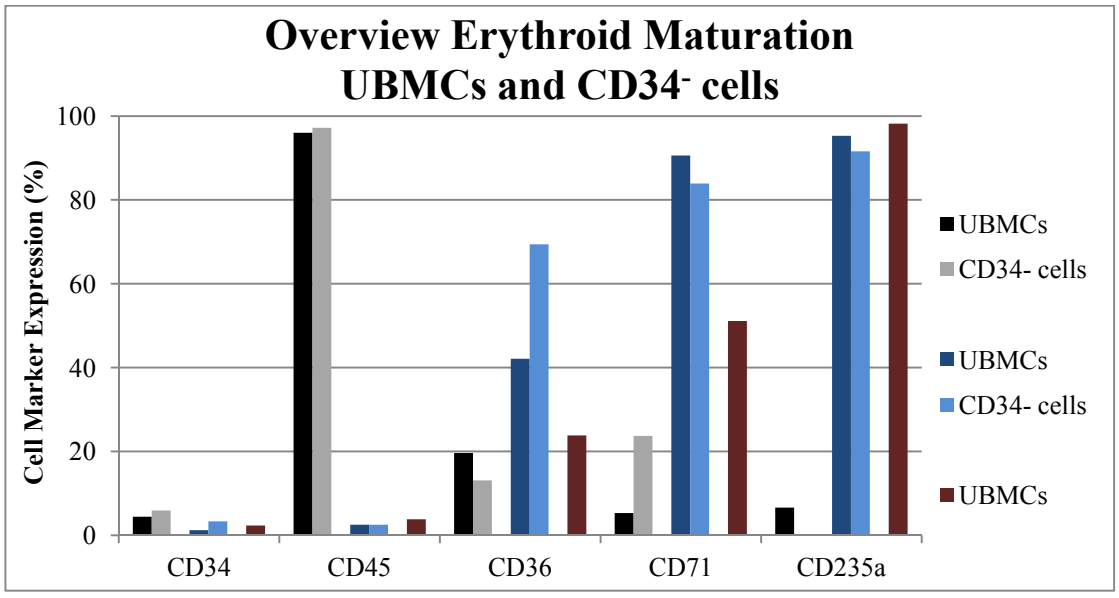

Fig. 4.19. *Collection of erythroid maturation cell marker profiles. Cells in differentiation cultures for approximately 3 weeks showed lower expression of CD36 and CD71 compared to cells in differentiation for 2 weeks.* ***Black/grey bars*** *show data collected at day 0.* ***Blue bars*** *show data collected after 2 weeks and* ***brown bars*** *show data collected after 3 weeks. Data is a collection of data as shown in Figures 4.10 and 4.13.*

4.6 Discussion

At the start of the main project to differentiate stem cells into RBCs, this research focussed initially on $CD34^+$ cell isolation from PB and UCB. As these cell sources are commonly used in the literature these were the point of interest for optimising the erythroid maturation culture conditions in this project. The use of PB-$CD34^+$ and UCB-$CD34^+$ cells proved challenging as shown by the results concerning the haematopoietic expansion of these cells (Chapter 3).

The number of isolated PB-$CD34^+$ cells, combined with their low proliferation rate, was insufficient to study erythroid maturation in depth. When comparing these data to published research the encountered issue appeared to be limited access to this cell source. Therefore the research focus shifted to the use of UCB-$CD34^+$ cells which were supplied by project partners.

The method used to differentiate HSCs into RBCs was based on a study by Giarratana *et al* (2005) where they published data describing the *ex vivo* generation of fully mature human RBCs from HSCs [42]. This research group was amongst the first groups to publish the successful generation of mature RBCs and also succeeded in testing *ex vivo* generated RBCs in a human during a clinical study [53]. Other studies published have used comparable methods to generate RBCs *in vitro*, with different success rates regarding the generation of fully mature RBCs [44, 45, 63, 111].

As the method in this highly cited publication proved successful [42], this technique was implemented for all the erythroid maturation cultures during this project. A human MSC stromal layer was introduced, as preferred over a murine stromal layer, during phase II and III. Giarratana *et al* mimicked the marrow microenvironment by application of cytokines and stromal cells and expansion folds up to $1.95x10^6$ and $1.22x10^5$ were achieved for cord blood and PB cells respectively, with functional reticulocytes and RBCs as a result [42].

When the same research group successfully attempted the transfusion of autologous cultured RBCs into a human, they expanded 10^6 PB-CD34$^+$ cells into $3.7x10^{10}$ cells with an enucleation rate of 68% without the use of stromal cells [Giarratana *et al*, 2011]. This is an expansion fold of $2.5x10^4$ for enucleated cells and these two studies demonstrate the effect of stromal cells on the enucleation efficiency.

4.6.1 Erythroid maturation of UCB-CD34$^+$ cells

Section 4.3 shows the results of erythroid maturation with cell size decreasing over 24 days. The viable cells were analysed for the cell markers CD34 and CD44 and the Hb-β molecule. Whereas all markers were expressed at over 80% at day 10, the acquired data on day 18, 21, and 25 showed a different, but more expected story. The expression of CD34 decreased whereas the percentage of cells containing Hb-β increased to almost 95%.

The CD44 marker decreased to below 20% over 25 days. CD44 is highly expressed on HPCs and facilitates cell-cell adhesion on MSCs [105]. The results shown in Figure 4.12 show that this surface protein is profoundly expressed on UMBCs and this matches published data [105]. In addition to this, a study by Chen *et al* (2009) showed a decrease in CD44 expression during the cell transition stage from proerythroblast to orthochromatic erythroblast/reticuloctyes. They also showed a progressive decrease in CD44 expression with decreased cell size [106]. Histological analysis did not provide conclusive data about the erythroid maturation stage of differentiated stem cells, but it showed a decrease in cell size and cell enucleation. Pellets of these cells had a red colour after 12 days of maturation, comparable to published data [4].

The use of thawed UCB-CD34$^+$ cells proved more successful for erythroid maturation when compared to the cells obtained with the CD34 magnetic cell separation of thawed UBMCs,

partly a result of the availability of larger cell numbers. These latter cells proved difficult to expand in an HSC culture environment due to insufficient cell numbers, as shown in Chapter 3, and therefore the proposed subsequent erythroid maturation step proved difficult. The majority of studies do not state whether fresh MNCs, thawed blood, or thawed MNCs were used. It is believed that the recovery of cells from fresh blood is superior to the recovery of cells from cryopreserved blood and aliquoted MNCs but improvements have been made regarding the cryopreservation and recovery of these cells [99].

As a result of the limited supply of UCB-$CD34^+$ cells, the variety in quality of supplied UBMCs, and the limited success of isolating $CD34^+$ cells from thawed UBMCs, the focus shifted towards the utilisation of UBMCs for erythroid maturation and was based on a study by Van den Akker *et al* (2010) [42].

4.6.2 Erythroid maturation of UBMCs

The UBMCs were expanded using three different haematopoietic culture conditions (condition (A), B), and (C)) prior to erythroid maturation and results were compared with directly erythroid-maturated UBMCs (condition (D)). For these experiments it was hypothesised that UBMCs under HSC expansion conditions would prove beneficial for the manufacture of RBCs when compared to non-HSC expanded UBMCs.

The results showed that the increase in number of $CD34^+$ cells during expansion of UBMCs did not necessarily resulted in a higher cell output during erythroid maturation (Figure 4.7). However, during the 7 day haematopoietic expansion culture a higher cell density with $6x10^4$ cells/mL (Figure 4.14) could be linked to a higher output of red cells when compared to a starting cell density of $3x10^4$ cells/mL (Figure 4.7).

Histological analysis of cells during erythroid maturation (condition (D)) showed a number of cells without a nucleus (cell diameter < 10μm) at day 16. At the same time point a number of cells appeared to extrude the nucleus and nucleated cells were still present as well (Figure 4.9). The enucleated cells showed similarities with data published by Giarratana *et al* (2005) [42], although the overall success rate appeared to be lower during this experiment. When these cells were analysed for the presence of haematopoietic and erythroid surface markers the data suggest that at day 15 the cells possessed the erythroid precursor marker CD71 and RBC marker CD235a. After 24 days of erythroid maturation, the same cell population showed higher levels of CD235a and Hb-β and lower levels of the erythroid precursors CD36 and CD71 indicating successful maturation (Figure 4.10). For confirmation of successful production of cRBC, an aliquot of the cultured cells was tested with hospital equipment used for analysing blood samples: UniCel DxH 800 (Beckman Coulter). Due to insufficient cell numbers this test could not be completed.

Culture conditions (A), (B), and (C) were compared with condition (D) and flow cytometric data indicated that on day 24 all the all surface markers showed a high degree of similarity compared to their expression on day 15. However, the expression of Hb-β was comparable for the four culture conditions after 15 days of erythroid maturation but at day 24 the expression varied tremendously. Cells cultured in the presence of a stroma during pre-HSC expansion (condition (A) and (C)) showed the highest expression of Hb-β (~75%) whereas condition (B) only showed a positive expression of 40%. With high levels of CD235a (>90%) for condition (B) and (C) this indicates that cultured cells were in the final stage of erythroid maturation as the expression of CD71 had dropped during the final 9 days of culture.

During the flow cytometric analysis of Hb, adult Hb-β was used and therefore only conclusions can be drawn regarding the adult Hb content of differentiated cells. As is shown by Giarratana et al (2005 and 2011) [42, 53], cultured RBCs contain a high percentage of

adult Hb (over 85%). During this research cells were not analysed for fetal Hb, but the literature showed that a minor fraction of the cultured RBCs contained this type of Hb (~10%). However, these cells originated from adult $CD34^+$ stem cells (*e.g.* PB, BM) whereas cultured RBCs originating from neonatal stem cells (*e.g.* CB) mainly contained fetal Hb: ~64% [42].

To study the erythroid maturation further, this experiment was repeated with an extended culture Phase II: 4 days instead of 2 days. This resulted in a shorter culture Phase III: 9 days instead of 14 days. The duration of culture Phase I was still 8 days and the duration of the three Phases were now comparable to the described erythroid maturation method [42]. Not only could flow cytometric data now be obtained for culture condition (A), cell pellets were also analysed and showed a red colour from day 12 (condition (D)) and day 14 (condition (A), (B), and (C)) of erythroid maturation (Figure 4.15). The bright red colour indicates the presence of oxygen bound to the haem group of Hb (oxyhaemoglobin) [3].

The surface marker profiles were of a similar pattern to thoseobtained during the previous experiment, with condition (A) having a comparable profile to data obtained for condition (D) at day 21 of erythroid maturation. The only major differences between condition (A) and (D) can be found in a lower expression of CD44 (~30% and ~50% respectively) and a lower cell number (~$4x10^6$ and ~$1x10^6$ respectively). Condition (C) showed a lower surface expression for most markers of the erythroid lineage, except CD235a, when compared to the profile of condition (D), but the cell number during erythroid maturation was similar. Condition (B) showed a lower surface expression for all markers of the erythroid lineage and the cell number was also lower when compared with condition (D) (Figure 4.14).

These data indicated that direct erythroid maturation of UBMCs had a positive effect on the manufacture of RBCs, including higher cell numbers, when compared to pre-HSC expanded UBMCs.

This study also analysed the potential benefit of using UCB-CD34$^-$ cells for erythroid maturation in relation to published data about the benefit of using PB-CD34$^-$ cells to increase the erythroblast yield [41]. Similar differentiation methods were used as was previously stated and resulted in lower expansion for UCB-CD34$^-$ cells (0.04 fold). However, during differentiation the number of UCB-CD34$^+$ cells increased 10 fold but this was still regarded as a low fold increase. The cell surface marker profile showed an indication of transition of UCB-CD34$^-$ cells into the erythroid cell lineage and cell pellets indicated the presence of Hb from day 11 (red coloured pellet). The small number of UCB-CD34$^+$ cells only allowed for analysis of the cell pellet and from day 17 this was coloured red, indicating later erythroid maturation compared to UCB-CD34$^-$ cells.

Overall, the erythroid maturation of pre-HSC-expanded UBMCs was not as successful as direct maturation of UBMCs (Figure 4.13) and results were different to data published by Van den Akker *et al* (2010). A possible explanation could be the difference in differentiation protocol including a different cytokine cocktail and absence of stromal layer. In this project, the differentiation of UBMCs was successful compared to the differentiation of CD34$^+$ cells and CD34$^-$ cells but further characterisation of these *ex vivo* generated cells would be needed for full comparison analysis with published data. This would require higher cell numbers at the start of culture to obtain sufficient cells during the end stage of erythroid maturation.

During the experiments described in chapter 4 the difference between non-haematopoietic stimulated and haematopoietic stimulated UBMCs for the differentiation towards the erythroid lineage was assessed. There was no clear indication that HSC expansion of UBMCs prior to erythroid maturation would benefit the manufacture of RBCs with the currently used method. The differentiation of UBMCs for the erythroid cell lineage would need further optimisation to increase cell output and this should allow for full cell characterisation with hospital accredited equipment for RBC analysis as well.

Chapter 5

Results – Development of the Bioreactor

5.1 Introduction

Cell cultures in a 2D environment (*e.g.* flasks, wells plates) have been highly beneficial for cell expansion. It allows for a simplified approach to study cells, whether human or animal derived. This is ideal for basic research but it is restricted in terms of creating large numbers of cells. In contrast, use of bioreactors can create a dynamic 3D culture which in turn is paramount for upscaling and producing sufficient cell numbers. These bioreactors have been developed in various formats and sizes over the past decades. Large numbers are used in laboratories as part of cell culture development processes.

Figure 5.1A shows a schematic overview of a bioreactor. The bioreactor shows the addition of medium to provide nutrients (fed) to the cell culture. This type of culture is called a fed-batch process as the cell product is harvested batch wise at the end of the culture run. Figure 5.1B shows a schematic overview of a bioreactor where the cell culture process is perfusion based. In this system nutrients are continuously refreshed but where the cells are also retained in the bioreactor to prevent loss of product. This system is more complex and this type of bioreactor will be the intended product of this research.

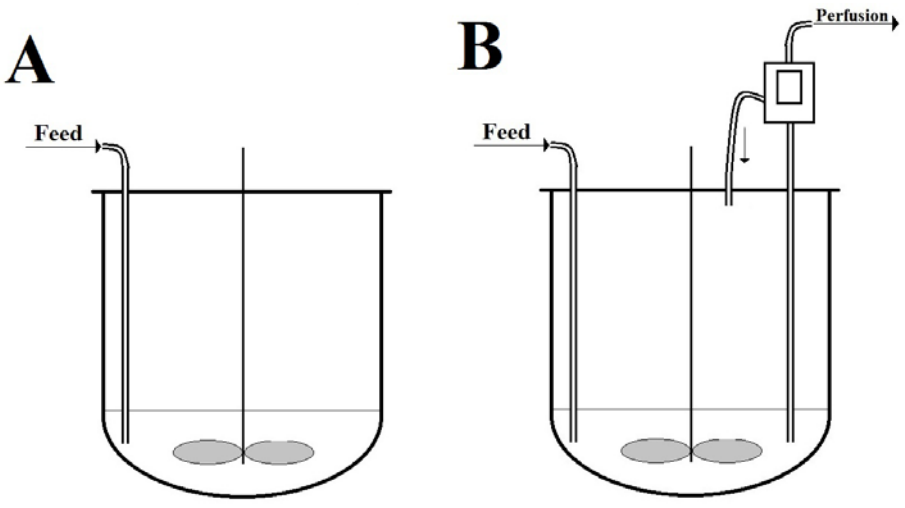

Fig. 5.1. *A schematic overview of a fed-batch bioreactor* ***(A)****. A schematic overview of a bioreactor with a perfusion system* ***(B)****.*

In this project, commercially available bioreactors were used to develop, improve and create a system for the culture of HSCs. The aims were to expand the HSC population and subsequently differentiate these cells into RBCs. To setup a system capable of achieving this, bioreactors of various volumes were analysed and during the developmental stages a range of devices and adaptations (*e.g.* cell retention devices, cell sampling) were tested.

Of particular interest is the BioSep, a cell retention device used in this research, and is part of the bioreactor perfusion system (Figure 5.1B). A cell retention device is an useful device to create high cell density cultures similar to those of red blood cells. This device will be tested thoroughly and compared to another cell retention device. The BioSep is an acoustic-cell separator where acoustic waves in the resonator chamber are used to separate the cells from the medium. The benefit of this system is the lack of moving mechanical parts and therefore the BioSep is less prone to fouling and mechanical failure. During separation in the resonator chamber, a part of the liquid is pumped through and removed as a cell-free perfusion flow. The other liquid part is returned to the bioreactor and contains the cells that have been retained in the chamber as a result of the acoustic standing waves.

During this research, the human U937 suspension cell line was used as a model for rapid growing cells as HSCs and MNCs were limited in supply. These U937 cells allowed for extensive testing to create a bioreactor environment ready for the development of haematopoietic and erythroid cultures. Figure 5.2 shows a schematic overview of the bioreactor design, with an overview and explanation of each of the three different stages of the bioreactor. This bioreactor design was the basis for this project.

The hypothesis of this chapter is: Can the process of manufacturing red blood cells be upscaled to a bioreactor process by improving the culture environment for this type of culture?

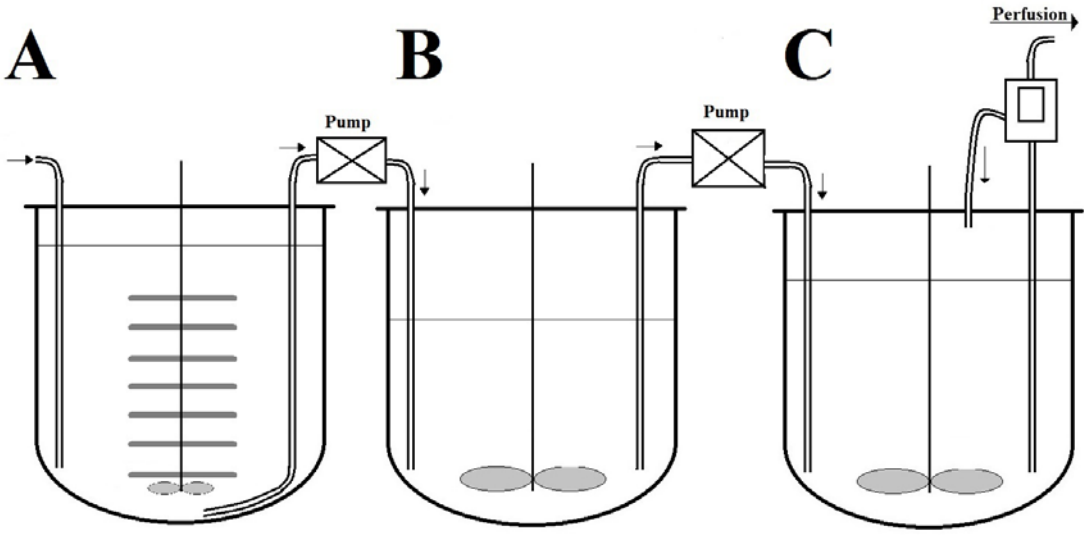

Fig. 5.2. *A schematic design showing the three different stages of the bioreactor, which could potentially be used to produce red blood cells. Part* ***(A)*** *shows the initial stage where the haematopoietic stem cells divide on slow rotating discs (grey bars); with the 'mother' cells adhering to the surface, whereas the 'daughter' cells go into the suspension culture before being transferred to the second stage of the bioreactor. Part* ***(B)*** *allows for the cells to differentiate towards the final stages of the erythroid lineage after which they are transferred to the third and last stage of the bioreactor. In this part* ***(C)****, the erythroid cells are allowed to fully mature into fully functional red blood cells and an integrated perfusion system will ensure a high cell density product.*

5.2 Initial Bioreactor Tests

In an approach to initiate bigger scale cultures for HSC expansion various sizes of bioreactors, a variety of other bioreactor equipment, was analysed. For this experiment a 2L bioreactor vessel, with a maximum working volume of 1500 mL, was used with an acoustic cell retention device (BioSep, 10L/day) attached. No stirrer device was used and there was no pH/DO control. The entire setup was placed in a closed room with an ambient temperature of 37 °C (Figure 5.3).

Fig. 5.3. *Setup 2L bioreactor and BioSepon top of the head plate.*

The bioreactor contained 500 mL of DMEM medium and the Feed Vessel contained 800 mL of fresh DMEM medium. The Feed Vessel with medium contained ~$9x10^6$ MSCs (~$11x10^3$ cells/mL) and was placed on a shaking device. After 1 day, ~$1x10^6$ cells were harvested from the bioreactor vessel. The Feed Vessel without medium contained ~$4.3x10^5$ cells (>95% alive). The Harvest Vessel was connected to the output of the BioSep and contained 900mL of medium and ~$5x10^5$ cells. Both the Bioreactor Vessel and the Harvest Vessel contained a large quantity of cell debris. From the cell number at the start of culture (~$9x10^6$), only ~$2x10^6$ viable cells were detected at the end of culture (1 day). The BioSep Control Unit was set to Run for ‘5 minutes’, Stop for ‘10 seconds’, and Powered at ‘5 Watts’.

DPSCs were also used to test the bioreactor in a different setup, maintained in a closed room with an ambient temperature of 37 °C (Figure 5.4). The 2L bioreactor vessel with the BioSep (10L/day) mounted on the head plate contained ~750mL DMEM medium. The connected Feed/Harvest Vessel (one container) contained ~250 mL DMEM medium. The BioSep Control Unit was set at Run for '5 minutes', Stop for '3 seconds', and Power at '3 Watt'.

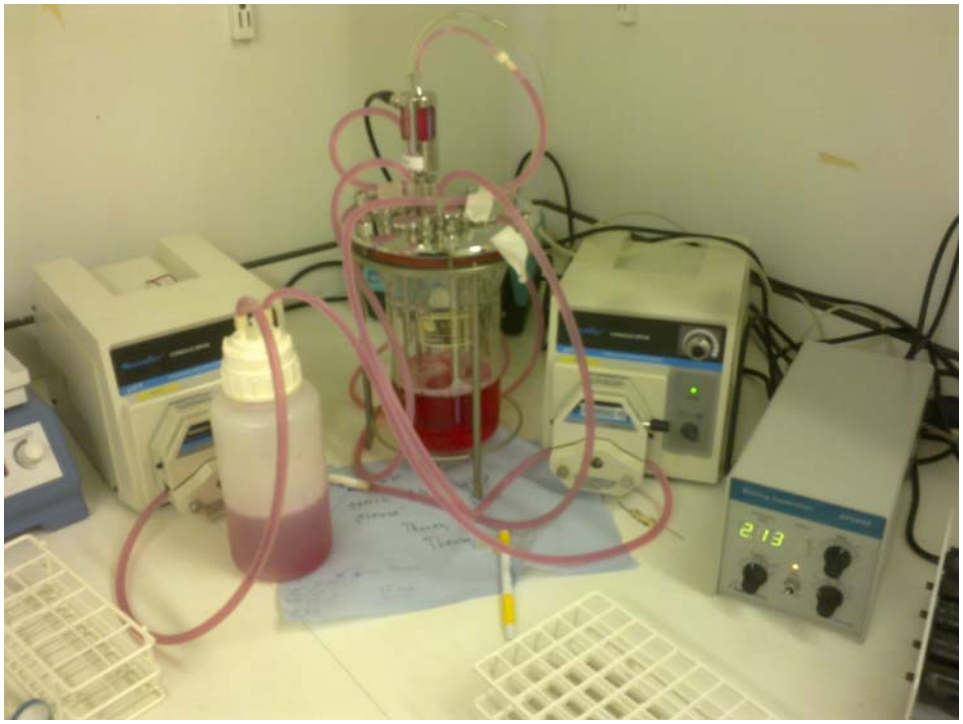

Fig. 5.4. *Continued testing of initial bioreactor cultures (including BioSep)*

At day 0, ~100x10^6 cells were transferred to the Bioreactor Vessel under aseptic conditions and cells were cultured for 2 days. No stirrer device was used and no pH and no DO control was available. A layer of cells on the bottom of the bioreactor vessel was observed at day 2. At the end of the culture 5.4x10^7 alive cells and 2.6x10^7 dead cells were harvested from the Bioreactor Vessel (275mL). For the Feed/Harvest Vessel containing 750 mL medium, the cell count was 1.2x10^7 alive cells and ~5x10^6 dead cells. The pH of the medium in both the Bioreactor and Harvest Vessels was 8.5 (37 °C) at day 2 whereas a pH of ~7.4 was measured at the start of culture.

The previous experiments with dynamic cultures (2L) showed that without precise control of the cell environment the cell viability was compromised. Therefore control of the bioreactor culture was required. To log data and control culture parameters (*e.g.* pH, dO_2) a My-Control Unit and BioXpert software (Applikon Biotechnology, Delft, NL) was used as described in Chapter 2. A typical bioreactor culture used calibrated sensors. The pH sensor was calibrated pre-sterilisation as guided by the My-Control UI, using two solutions, one with a pH of 4 and the other pH of 7. The dissolved oxygen (DO) sensor was calibrated post-sterilisation, guided by the My-Control UI and used maximum air influx to adjust set point to 100% under aseptic conditions (Figure 5.5).

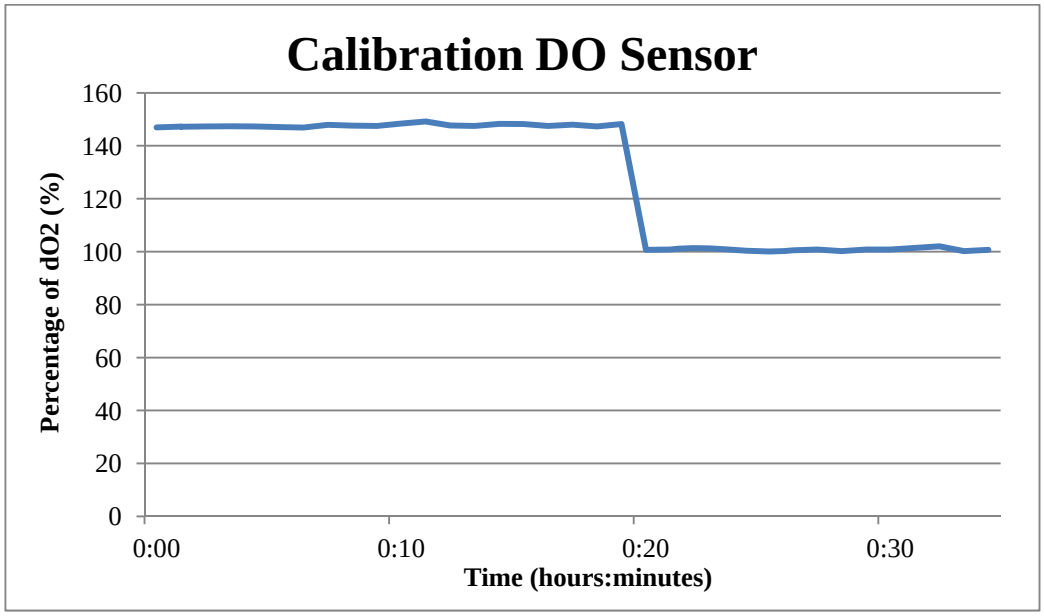

Fig. 5.5. *Calibration of DO sensor at maximum air influx; set point at 100%*

5.3 Small Scale Bioreactor

A system with a 250 mL MiniBioreactor (working volume 200 mL) was tested. The pH was controlled by the addition of CO_2 and an alkali solution. The DO concentration was controlled by the addition of air only (set point 50%). The temperature was maintained at 37°C using a My-Control powered heating blanket. To allow for a homogeneous environment and cell distribution a marine impeller was used with a stirrer speed of 50rpm. As a control, a stirrer flask without sensors and without pH and DO control was used. For both vessels, the starting volume of medium was 50mL and at each subsequent day 50mL of medium was slowly added, up to a total volume of 200mL.

In this experiment, a cell line for suspension cultures was used. The basic culture of this human lymphoblastic cell line, U937, was described in Chapter 2. Both vessels contained $7x10^6$ cells (140,000 cells/mL) at day 0. After 3 days of culture, the MiniBioreactor contained ~$38x10^6$ alive cells (~190,000 cells/mL). Over 95% of the total cell population was alive. The stirrer flask contained ~$3.7x10^6$ alive cells (~187,000 cells/mL) which was less than 15% of the total alive cell population.

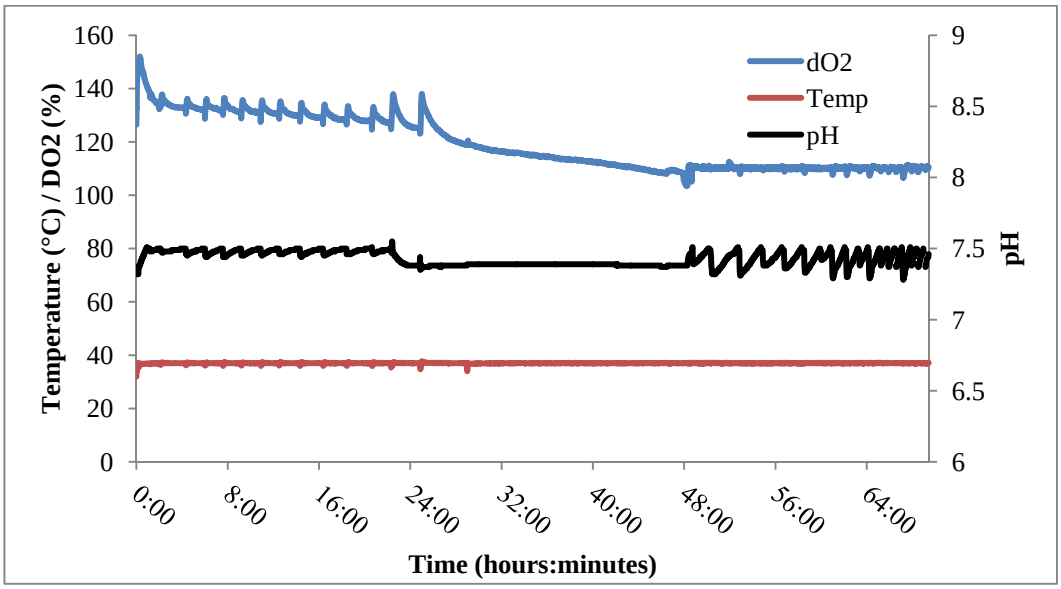

Fig. 5.6. *Parameters recorded during a dynamic culture using a MiniBioreactor (250mL) and U937 cells. Stirrer speed was set at 75rpm, pH was set at 7.4, and DO was set at 50%*

The sensor readings showed a stable temperature and pH range of 7.3-7.5 (Figure 5.6). The DO curve showed an initial steep rise before gradually decreasing to ~110% after 48 hours of culture. This concentration of DO was maintained for the next 24 hours.

The cell viability and expansion rate of U937 cells was analysed in a controlled bioreactor and compared with cell cultures in an uncontrolled bioreactor and a static culture flask. This determined in what way the correct pH and oxygen levels would influence these cell parameters and what the possible shortcomings for the control of cell environment would be. It was hypothesised that an accurate controlled culture environment would stimulate cell growth. The sensors of the two identical bioreactors were calibrated as stated in Chapter 2. At day 0, both bioreactors and the culture flask contained 42 mL of medium and ~$10x10^6$ cells (~$2.4x10^5$ cells/mL). The temperature was maintained at 37°C for all three culture vessels. Bioreactor 'C1' was controlled for both the pH and DO whereas bioreactor 'M1' was not controlled for these parameters. Both bioreactors had a set stirrer speed of 75rpm.

The standard tissue culture flask was placed in a 5% CO_2 and 37°C controlled, humidified incubator. The total culture period for all three vessels was 9 days with the addition of 50 mL fresh medium on days 1, 2, and 5. The cell viability was determined at the start and end of the culture period (Table 5.1). The Controlled Bioreactor (C1) showed 97.8% viability and ~11.1x fold amplification of viable U937 cells. For the Monitored Bioreactor (M1) this was 68.1% and a fold amplification of ~10.6x. The result for the static flask showed a cell viability of 57.2% and a fold amplification of 4.7x.

After the cell suspension was removed from the glass bioreactor vessel, it was found that less than 0.05% of all cells adhered to the glass vessel wall of the controlled bioreactor (C1). This was ~0% for the monitored bioreactor (M1).

	Day 0		Day 9		
	Alive Cells (#)	Alive	Alive Cells (#)	Alive	Dead (#)
Monitored Bioreactor	10x10^6	>95%	106.5x10^6	68.1%	49.9x10^6
Controlled Bioreactor	10x10^6	>95%	111x10^6	97.8%	2.5x10^6
Static Flask	10x10^6	>95%	47x10^6	57.2%	35.2x10^6

Table 5.1. *The U937 cell viability shown for all three culture vessels at day 0 and day 9*

The sensor registration profile showed the instability of the pH and the DO during the first 48 hours of culture before dropping to 7.3 (pH) and 10% (DO) respectively after ~96 hours. The time points when medium was added can be confirmed from distinctive peaks in pH and DO registration profile: after 24 hours, 48 hours, and 120 hours (Figure 5.6). The temperature was stable at 37ºC throughout the cell culture, with a slight drop after 24 hours, 48 hours, and 120 hours when medium was added.

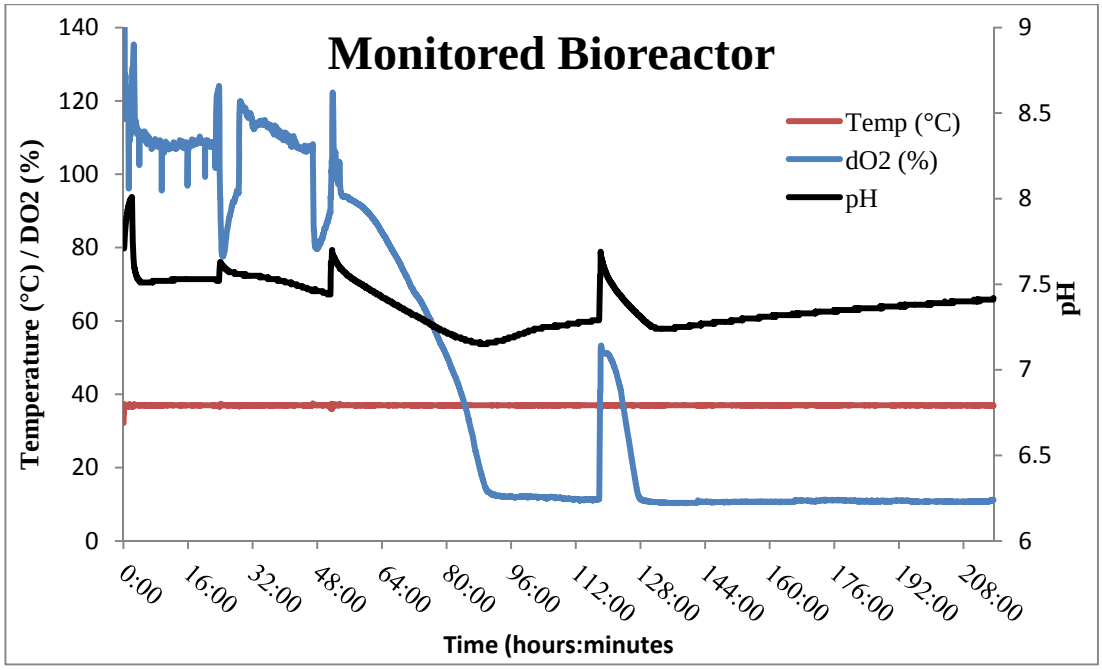

Fig. 5.7. *The sensor registration profile (pH/DO/Temp) of the monitored MiniBioreactor (250mL) containing U937 cells over a 9 day culture period. Stirrer speed was set at 75rpm*

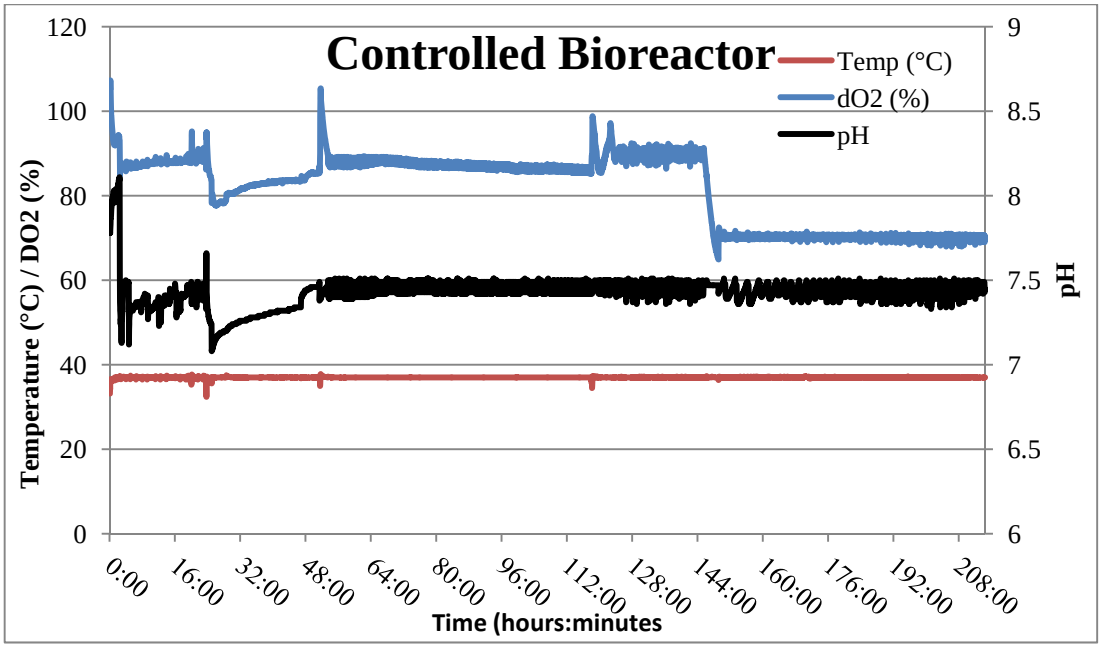

Fig. 5.8. *The sensor registration profile (pH/DO/Temp) of the controlled MiniBioreactor (250mL) containing U937 cells over a 9 day culture period. Stirrer speed was set at 75rpm*

The sensor registration profile shows the instability of the pH during the first 48 hours of culture before it stabilised ~7.4 for the remainder of the culture. The timepoint of when medium was added could be observed from the peaks within the DO profile after 24, 48 and 120 hours (Figure 5.8). Because the DO during this experiment was controlled by the addition of air only it was not possible for the bioreactor to lower the DO level to 50%. The temperature was stable at 37ºC with a minor drop (1ºC-3 ºC) when fresh medium (50mL) was added.

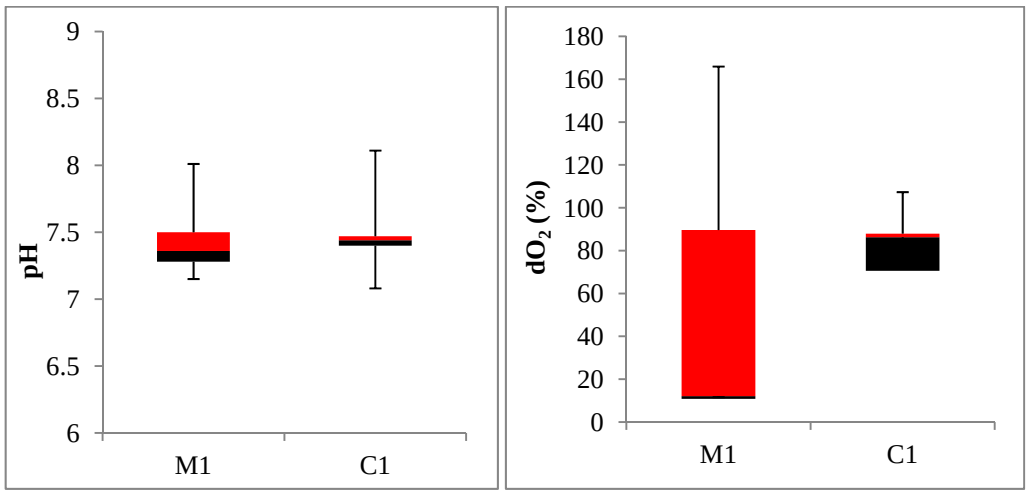

Fig. 5.9. *Box plot of the pH and DO values for the monitored (M1) and the controlled (C1) bioreactor shows the distribution of values over 9 days. Each box plot is divided in 4 quarters, each containing 25% of the recorded values with the mean as the value between Quarter 2 (black bar) and Quarter 3 (red bar)*

The box plots in Figure 5.9 show the difference for the pH and DO values and how these values were distributed for both the Monitored and the Controlled bioreactors. There was a wider distribution of values for Q2 and Q3 around the mean for the Monitored bioreactor.

5.4 Cell Retention

To allow for a carefully orchestrated scale up culture using bioreactors, a multi-step culture protocol was implemented. During the first phase of this culture a static culture flask was used and a 3L capacity bioreactor was used during the third phase. The MiniBioreactor (250mL) was responsible for the second, and intermediate, phase to scale up to a dynamic culture. The DO was controlled by the addition of air and the pH was controlled with CO_2 and an alkali solution.

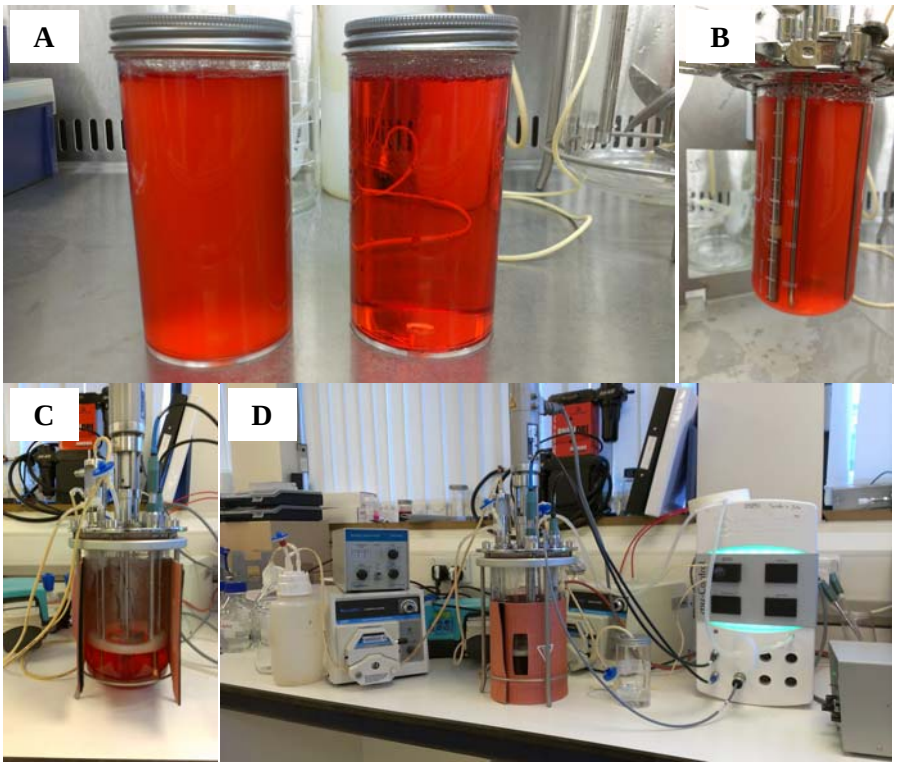

Fig. 5.10. *An overview of bioreactors. A) Harvested cell suspension from the MiniBioreactor (B) next to fresh medium without any U937 cells. C) 3L bioreactor with a stirrer and BioSep (10L), thermo blanket open. D) My-Control Unit and Bioreactor with pumps and medium/waste vessels*

During the first phase of this scale up process, a T75 with 30 mL of medium was used to culture U937 cells for 3 days before transferring the cell suspension to the MiniBioreactor containing 210 mL of fresh medium. After a 4 day culture period ~$280x10^6$ cells (>95% alive) were harvested. During the third, and final, phase the cell suspension was transferred to the 3L Bioreactor containing 260 mL of fresh medium (Figure 5.10) under aseptic conditions.

The BioSep and a stirrer were connected to the 3L Bioreactor to allow for medium refreshment and a homogeneous distribution of nutrition and oxygen. The BioSep Control Unit was set to run for '10 minutes', Stop for '3 seconds', and Powered at '2 Watts'. Attached to the bioreactor was a 'Feed' vessel containing 2000 mL of fresh medium to be gradually added to the bioreactor over a 7 day period. At the end of the third phase, the bioreactor contained 1400 mL of medium containing ~$1.4x10^9$ alive and ~$600x10^6$ dead cells (~70% alive cells). Approximately 1300 mL medium was collected in the 'waste vessel' containing ~$92x10^6$ alive cells (6.57% of all viable cells).

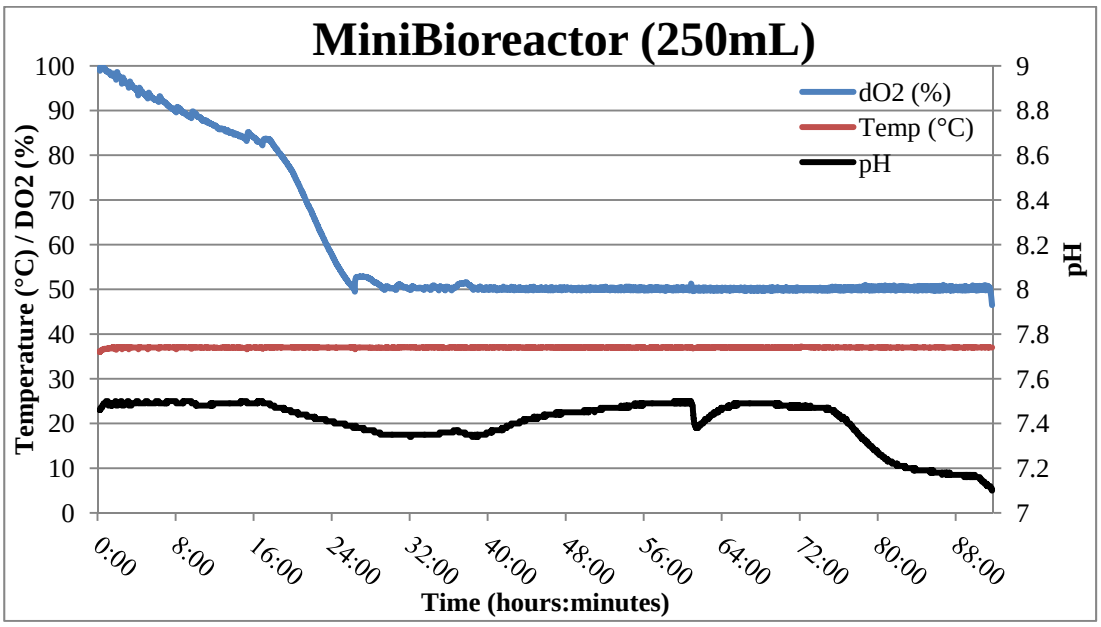

Fig. 5.11. *The sensor registration profile (pH/DO/Temp) of the controlled MiniBioreactor (250mL) containing U937 cells over a 4 day culture period (part 1). Stirrer speed was set at 100rpm, DO was set at 50%, and pH was set at 7.4*

The sensor registration profiles were recorded for both bioreactors (Figures 5.11 and 5.12). The set point for DO was 50% and the pH was set at 7.4. Both graphs show a temperature registration of 37ºC for the entire culture period and the DO dropped to 50% (set point) after approximately 24 hours of culture. The full culture period was approximately 88 hours for the 3L Bioreactor. The registration profile for the MiniBioreactor showed a pH between 7.3 and 7.5 during the first 76 hours before the pH dropped to 7.1 after 91 hours of culture. The pH for the 3L Bioreactor was 7.1 for the first few minutes whilst the culture was initiated

before it reached a stable 7.3 for 128 hours. The pH then rose to 7.5 for the remainder of the culture period.

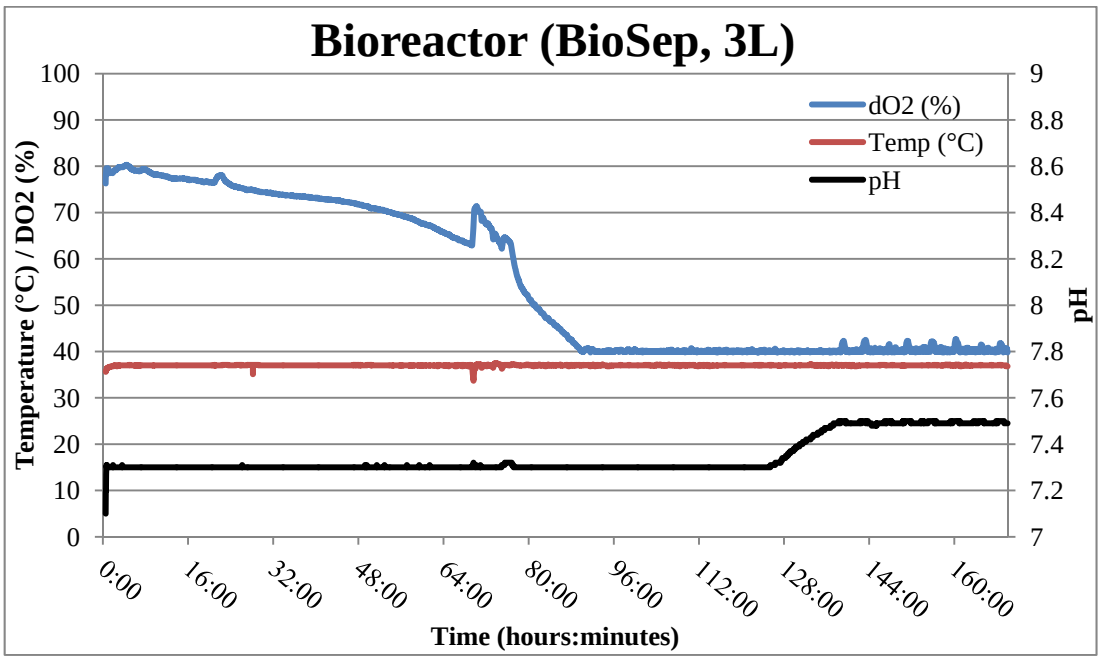

Fig. 5.12. *The sensor registration profile (pH/DO/Temp) of the controlled Bioreactor (3L) containing U937 cells over another 4 day culture period (part 2). Stirrer speed was set at 100rpm, DO was set at 50%, and pH was set at 7.4*

The BioSep was compared with another cell retention device to analyse cell retention efficiency and cell viability. This device, a Spinfilter, was used in a similar Bioreactor as was used for the Biosep (Figure 5.13). To control the DO and the pH the addition of air (DO), and CO_2 and an alkali solution (pH) was added. 80 mL of U937 cell suspension was added to the bioreactor which contained 300 mL fresh medium.

The stirrer speed of the marine impeller, and Spinfilter, was set at 100rpm. At regular intervals fresh medium was added to the vessel and used medium was harvested via the Spinfilter to allow for optimal cell growth conditions. Fresh medium (~1750mL) in a separate vessel was available at the start of the culture. After ~120 hours of culture, all fresh medium was transferred to the bioreactor vessel. At day 6, the culture was terminated and all vessels were analysed for cell numbers, cell viability, and medium volume.

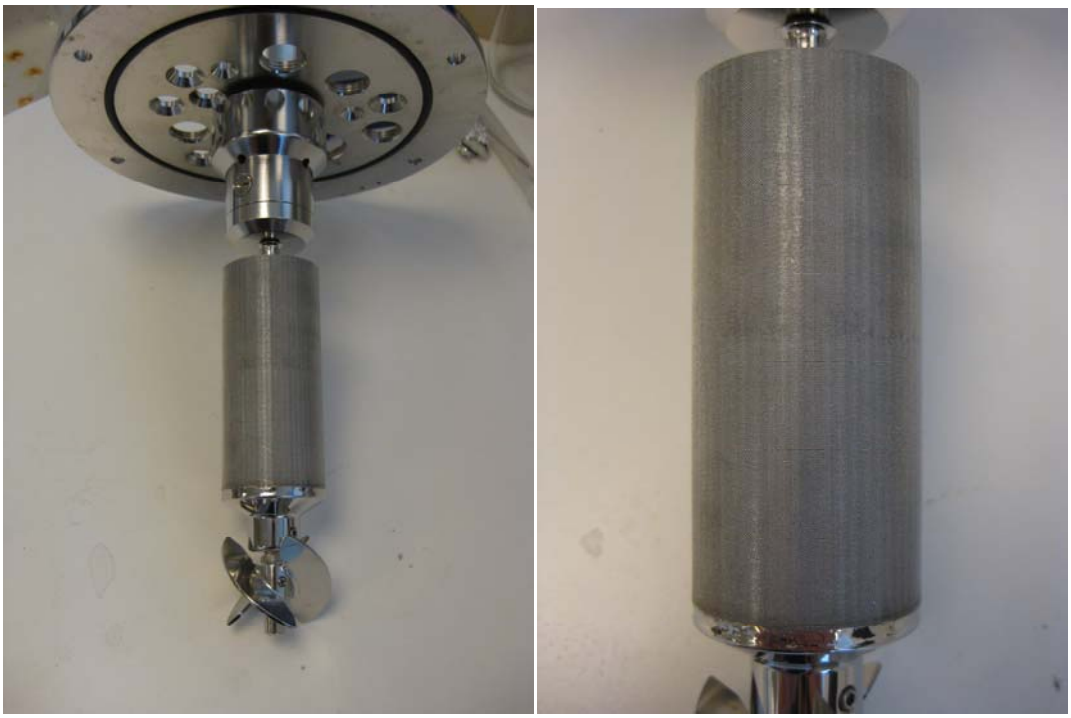

Figure 5.13. *Spinfilter and impeller (left) and Spinfilter close up (right)*

The sensor registration profile (Figure 5.14) showed the influence of the addition of medium on the pH and DO during the first 120 hours of culture. Profile peaks were noticeable and only when fresh medium was no longer added at day 6, were the profile stable. The pH sensor registered a stable value of below 7.5 and a temperature of ~37ºC was registered throughout the entire culture. The DO was ~100% at the start of culture and decreased gradually during the first 96 hours before stabilising at 30% after 120 hours of culture.

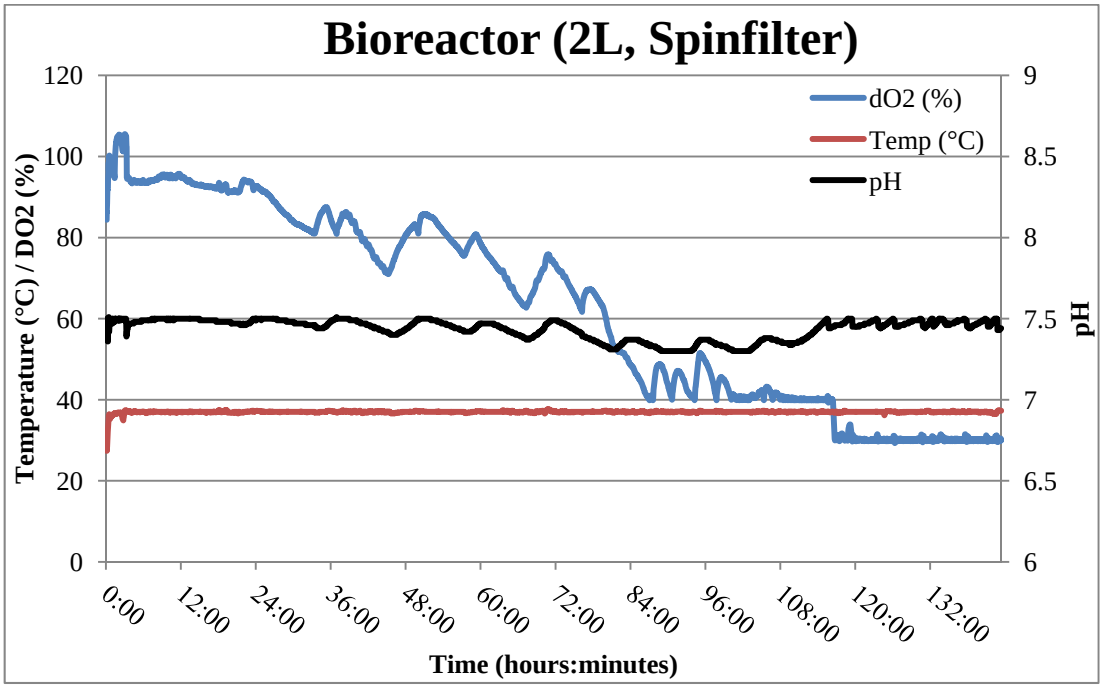

Fig 5.14. *Sensor registration profile (pH/DO/Temp) of the controlled Bioreactor (2L) with a spin filter containing U937 cells over a 6 day culture period. Stirrer speed was set at 100rpm, DO was set at 40% (0-120 hours) and 30% (after 120 hours), and pH was set at 7.4*

The cell numbers in Table 5.2 showed how many viable cells had been retained in the bioreactor vessel. Approximately 90% of all cells were retained when the spinfilter was used as a cell retention device.

Day 6	Bioreactor vessel with spinfilter	Harvest vessel
Medium Volume	837 mL	1315 mL
Alive Cells	~$1.5x10^9$	~$0.084x10^9$
Cell Density (Alive)	~$1.8x10^6$ cells/mL	~$0.06x10^6$ cells/mL
Cell Viability (Alive)	89.5%	46.4%
Notes	The viable cell population was 81.3% of all cells within this system.	High amount of debris present.

Table 5.2. *Cell analysis of vessels used for the bioreactor with spinfilter*

The cell data is depicted in Figure 5.15 and showed the difference in cell number between the bioreactor vessel and the harvest vessel and the increase in cell density over 6 days of culture. The total number of cells counted in the bioreactor vessel was $1.7x10^9$ and this was

~10 fold greater than the number obtained from the harvest vessel. The increase in cell density of viable cells in the bioreactor vessel was ~1x10^6 cells/mL compared to ~1.8x10^6 cells/mL. The optimal cell density for these cells is 2-9x10^5 cells/mL in normal static culture.

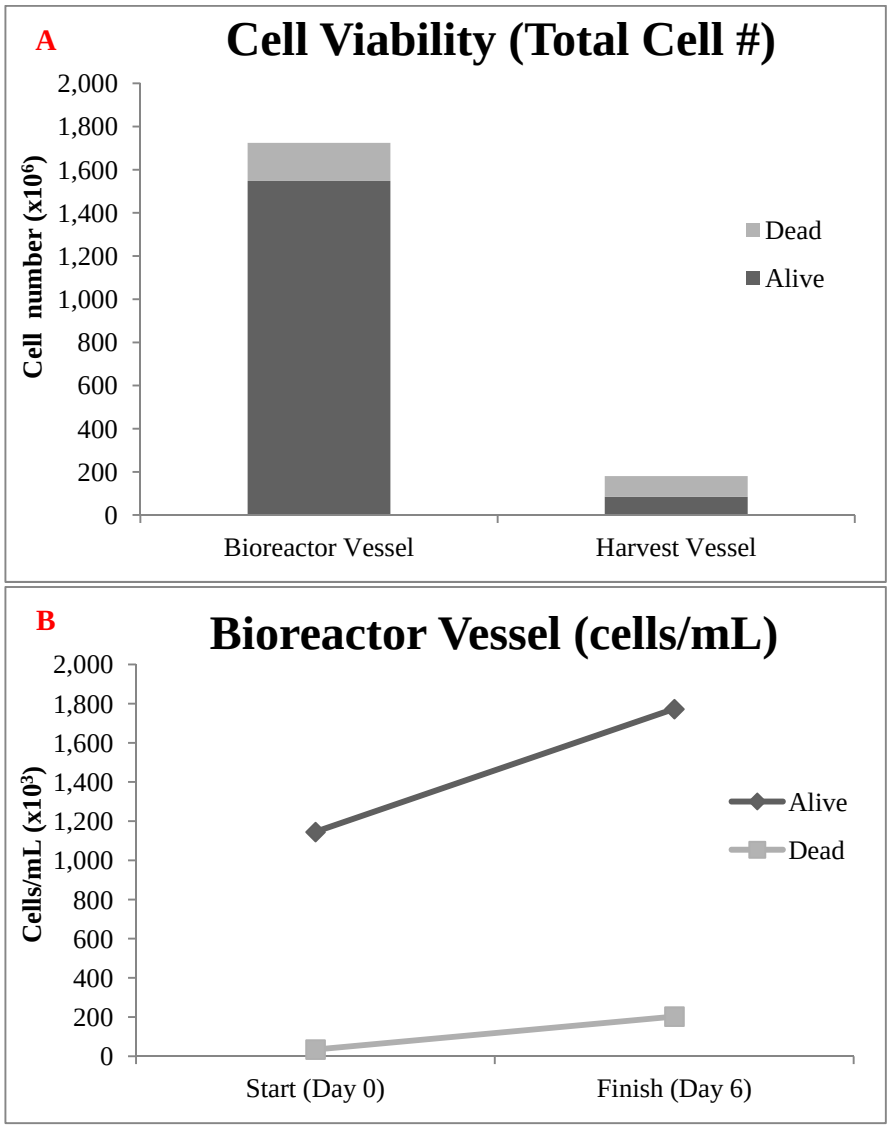

Fig. 5.15. *A) Cell viability graph for the Bioreactor with Spinfilter, and associated Medium Waste Vessel. B) Cell density graph over 6 days for the Bioreactor Vessel*

5.5 Upscaling and Cell Viability

The previous experiments showed the improvement in the upscaling of cell cultures. Cell viabilities of more than 95% were achieved for the MiniBioreactor but the cell viability for the 2-3L bioreactors was only ~75%. Therefore the 2-3L bioreactor needed more research in optimising cell viability. A more careful upscaling could allow for a better controlled cell expansion resulting in higher cell viability.

Another challenge during medium refreshment, with the use of a cell retention device, was to minimise the transfer of viable cells from the bioreactor vessel to the harvest vessel. The BioSep has advanced control settings and was therefore a suitable device for cell retention to optimise cell cultures. The BioSep Control Unit was set to Run for ’10 minutes’, Stop for ‘3 seconds’ and Powered at ‘2 Watts’ as was recommended by the suppliers guide for a cell concentration of $2x10^6$ cells/mL and a medium net flow rate of 2L/day.

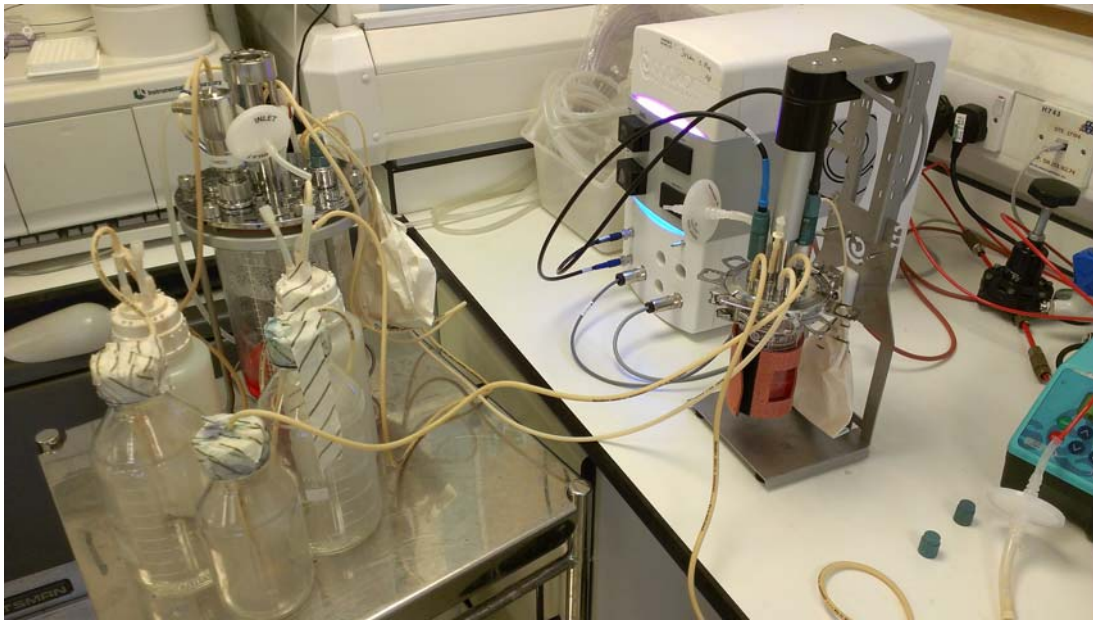

Fig. 5.16. *Setup of a closed system with the MiniBioreactor in progress (right) and connected Bioreactor (3L) with BioSep (left) for the last stage of culture*

A MiniBioreactor and a connected 3L Bioreactor Vessel with BioSep and a My-Control Unit was used (Figure 5.16). At day 0, 50mL of cell suspension ($6.5x10^5$ cells/mL) was added to the MiniBioreactor and incrementally medium was added up to 200 mL over 3 days. Next, the cell suspension was pumped to the 3L Bioreactor Vessel containing 800 mL of medium; the total volume of cell suspension was 1000 mL. Fresh medium (1700 mL) was available in a connected vessel to supply fresh medium to the Bioreactor Vessel. The pH was controlled by the addition of CO_2 and the DO level was controlled by the addition of air.

The sensor registration profile showed the time points of medium addition to the MiniBioreactor after 8 hours, 24 hours, and 48 hours. The time point of medium additions can be observed by the slight rise in DO (5-10%) and pH (~0.1) and a minor drop in temperature (1-2ºC). The temperature recorded was stable at 37ºC and the pH was kept between 7.3 and 7.5. The DO dropped gradually from 100% to 20% over ~66 hours (Figure 5.17A).

The addition and harvest of medium for the 3L Bioreactor Vessel was more gradual and this was reflected in the sensor registration profile (Figure 5.17B). The pH was high (7.7) upon initial start-up but was stable between 7.3 and 7.5 for the remainder of the culture period. The temperature was low (~25ºC) at the start of the culture, but rose to 37ºC within 1 hour and remained stable until the end of the culture. The DO level dropped over a ~96 hour period to the setpoint of 20%; a slow decrease during the first 48 hours of culture and a rapid decrease during the last 30 hours of this ~96 hour culture period. For the remaining culture period the DO level remained stable at 20%.

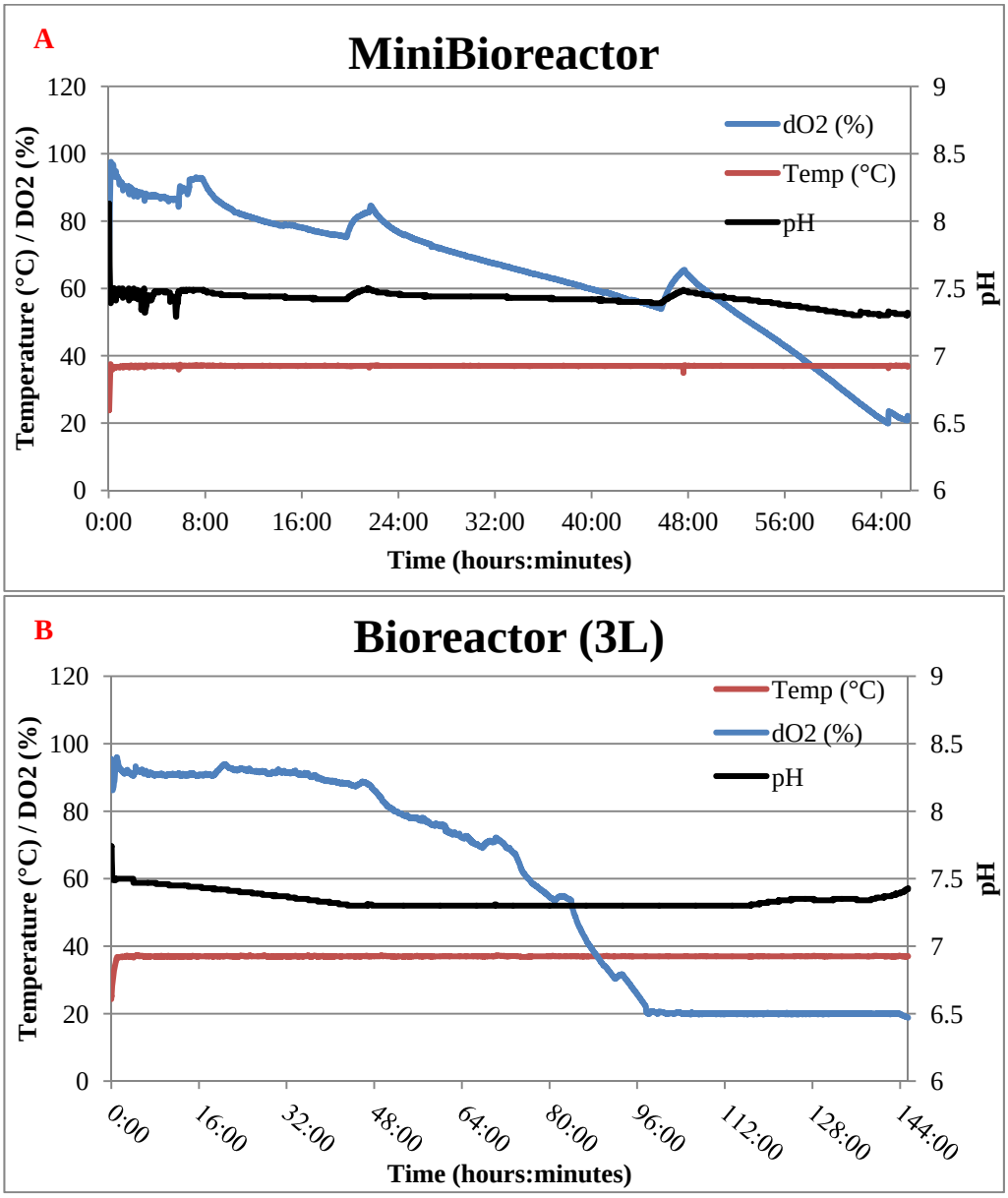

Fig. 5.17. *The sensor registration profile (pH/DO/Temp) of the MiniBioreactor (A) and the 3L Bioreactor with BioSep (B) filter containing U937 cells over respectively a ~2 day and 6 day culture period. Stirrer speed was set at 75rpm, DO was set at 20%, and pH was set at 7.4*

The cell number and viability is depicted in Figure 5.18 and shows the total number of cells at the end of the overall culture period and the increase in cell density during culture. Analysis of the 3L Bioreactor Vessel showed that $2.4x10^9$ alive cells were harvested (~95.5% cell viability) and that ~2.5% of all the cells was transferred to the Medium Harvest Vessel during culture. The strategically controlled approach of scaling up a dynamic culture resulted in a steep increase in cell viability/mL while medium volume was expanded as well. The relatively low number of dead cells, compared with previous experiments confirmed the benefit of this approach.

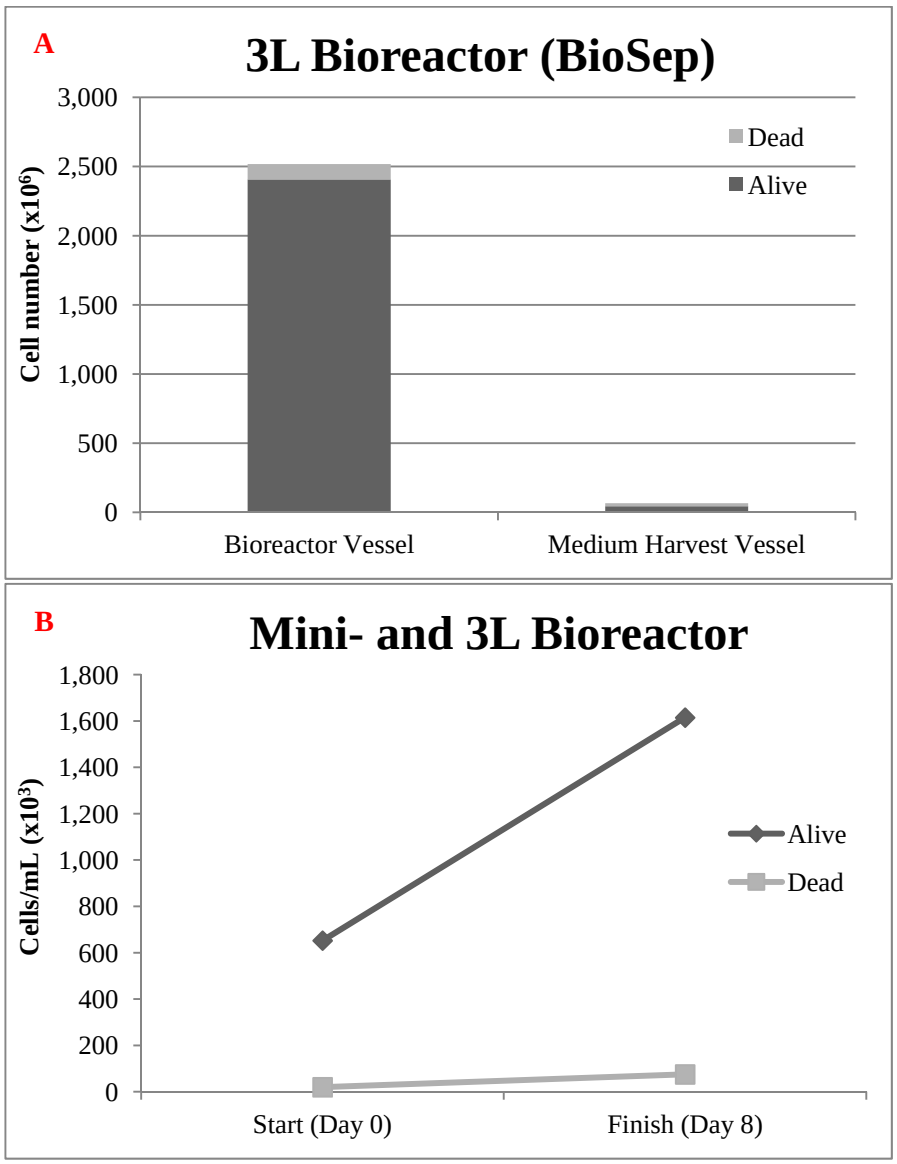

Fig. 5.18. *A) Cell viability graph for the 3L Bioreactor with BioSep, and associated Medium Waste Vessel at the end of culture. B) Cell density graph over 8 days for the subsequent culture period of the MiniBioreactor and the 3L Bioreactor*

In the previous experiments cell numbers were analysed at the start and end of a dynamic culture, not during culture. In order to gain better insight into cell expansion and viability continuous access to the culture was required. Therefore, access ports were designed and implemented into the MiniBioreactor to obtain cell samples using a 1 mL syringe (Figure 5.19). One port was created with access to the bottom level of the vessel (I) and another port had access to the middle level of the vessel (II) as shown in the image. During system testing, the stirrer speed was set at 75rpm. The dynamic culture was not pH or DO controlled; DO and pH values were profiled. Data recorded is shown in Figure 5.21.

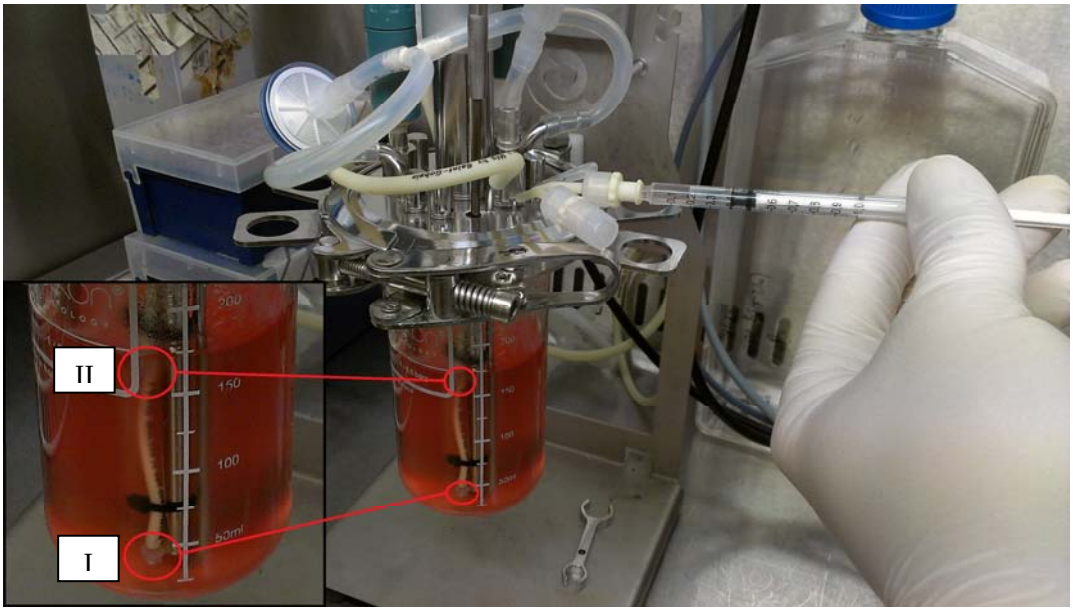

Fig. 5.19. *Manual cell sampling system for the MiniBioreactor in an aseptic environment with the 'low' (I) and 'high' (II) cell sample height marked*

The objective of the first tests was to obtain representative cell samples from the culture (Figure 5.20A) and in an additional test cell samples from high and low access ports in the MiniBioreactor were compared (Figure 5.20B). The obtained cell samples, 0.2mL in volume, were analysed using Trypan Blue to determine cell viability. A haemocytomer was used to count cell numbers.

The cell samples taken during the first stage were similar in number at set intervals. This similarity in cell number was also applicable to second stage testing. Graph 'A' shows the increase in viable cells over 24 hours. The steepness of the slope of dead cells indicated that the increase in viable cells was higher than that of dead cells in this bioreactor.

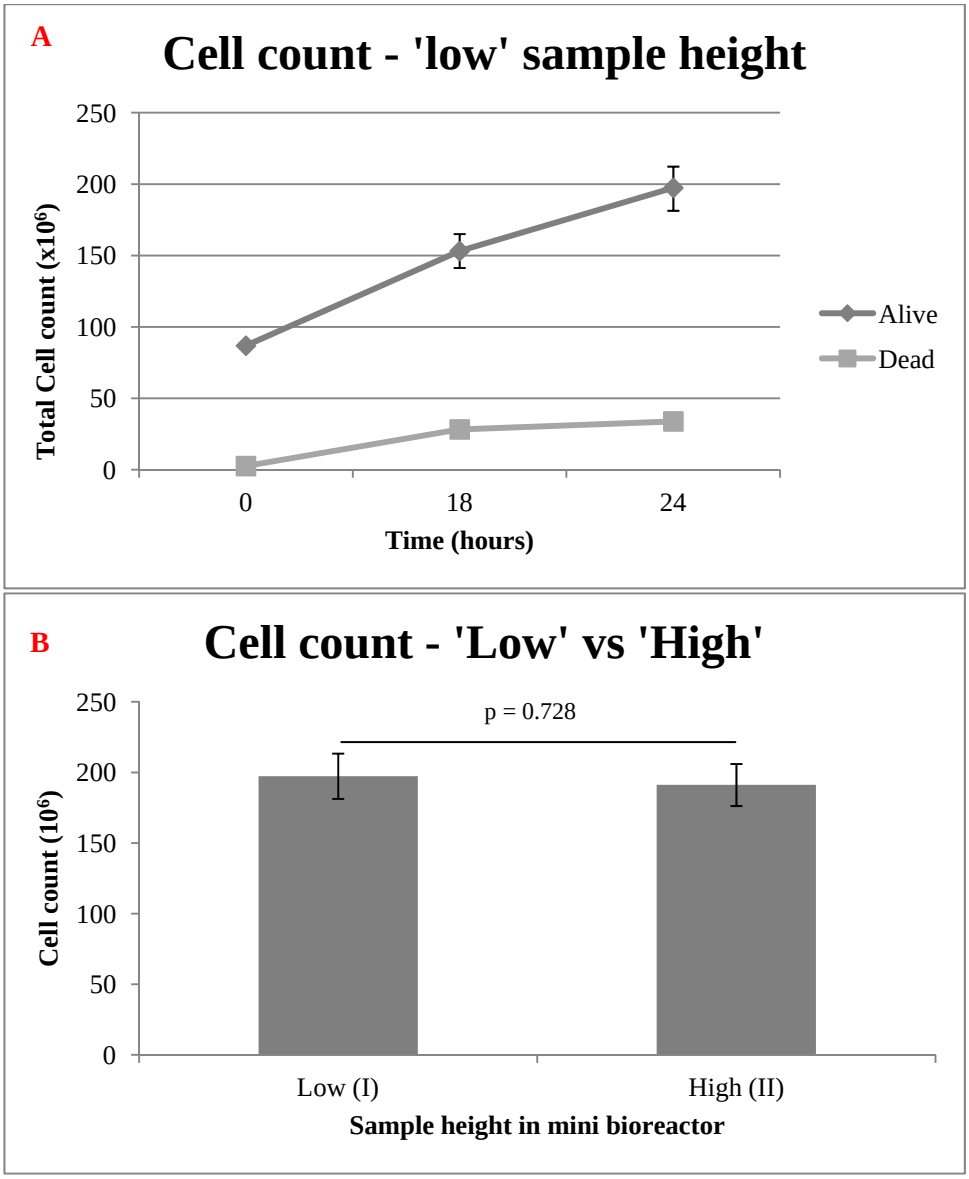

Fig. 5.20. *A) Cell number and viability data as acquired by the Cell Sampling system in the MiniBioreactor. B) Comparison of cell numbers acquired via the 'low' and 'high' cell sampling system after 24 hours (Student T-Test: p > 0.5, n=3, error bars represent StDev).*

When cell samples from the lower (I) and the higher (II) access ports were compared it was observed that the samples from each port contained similar numbers of cells and this correlation was confirmed by a Student T-Test. Therefore the hypothesis stating that acquiring cells from two different sample access ports heights results in a difference in cell number was rejected.

The sensor registration profile showed a stable temperature of 37ºC. The pH recorded was 8.5 during the first hour before it dropped to 7.5. Next, the pH decreased to 7.3 over 19 hours before it rose again to 7.5. During the last 4 hours of culture the pH dropped to ~7.4. In the same graph (Figure 5.21) the DO percentage was plotted. After an initial rise to ~110% DO level dropped to 20% before it rose rapidly to ~50% before it decreased again.

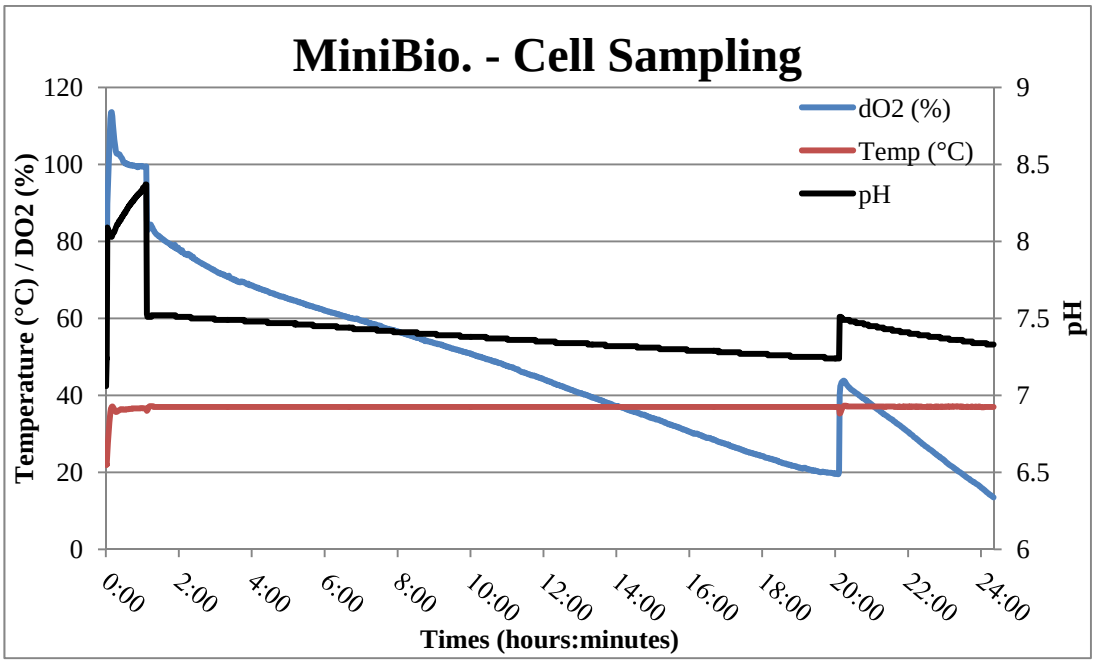

Fig. 5.21. *The sensor registration profile (pH/DO/Temp) of the MiniBioreactor with a manual cell sampling system containing U937 cells over a 24hour period. Stirrer speed was set at 75rpm, while DO and pH were not controlled*

5.6 Addition of air, nitrogen and CO_2

During the previous experiments there was a long delay for the DO levels to drop from a high level to the desired setpoint (e.g. 20%). In order to gain accurate control of the DO level, a module was installed to the My-Control Unit to allow for the addition of nitrogen to the bioreactor. Nitrogen is an inert gas which is widely used to remove oxygen from cell environments [112]. The purpose was to achieve a controlled lower DO level in the medium. This more precise control resulted in the graph depicted in Figure 5.22.

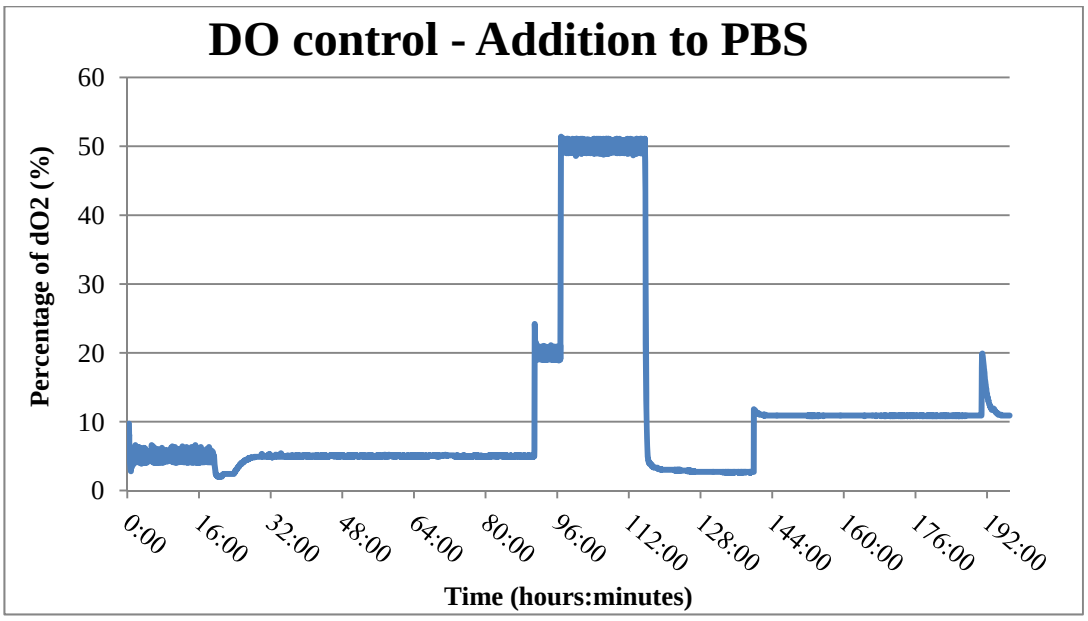

Fig. 5.22. *Improved control of DO levels within PBS by addition of air and nitrogen into the MiniBioreactor. The setpoints were changed during testing and were respectively 5%, 2%, 5%, 20%, 50%, 5%, 10%, 20% and 10%*

In another experiment of similar setup, the responsiveness of the DO levels was tested by the addition of air and nitrogen above the medium instead of addition from within the medium. The results showed that the level control was less accurate and lacked responsiveness as it took approximately 8 hours to adjust the DO level and in addition the fluctuation was higher as well (Figure 5.23).

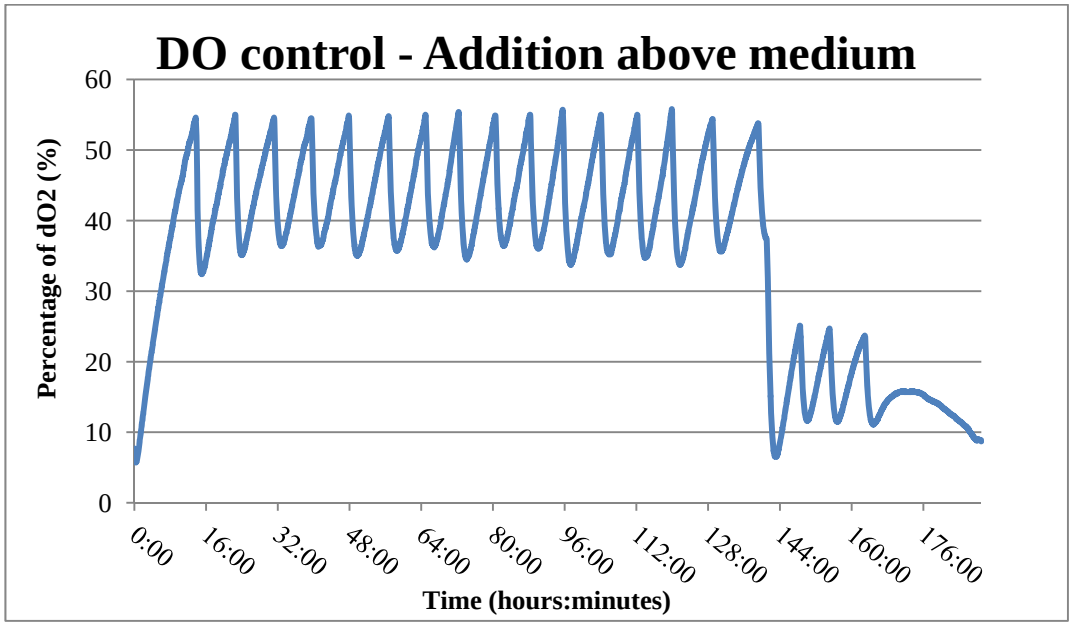

Fig. 5.23. *Control of dO_2 levels above the medium by addition of the inert N_2 and air into the MiniBioreactor. The setpoints have been changed during culture and are respectively 50% and 20%*

An important aspect of this bioreactor is the addition of air, nitrogen, CO_2 to control the pH and DO. The basic method is the addition of gases via a normal sparger, but this creates large air bubbles which could negatively affect the cell viability [113-115]. A commercial product (pore size: 15µm) was tested to produce smaller bubbles and is shown in Figure 5.24: This commercial porous sparger was screwed into the normal sparger. The bubbles released from each sparger were rated for bubble size and the results were listed in Table 5.3; a video of each sparger in use was created to observe and compare the bubbles. The flow rate of supplied air was 5 mL/min.

The commercially available option was compared with a range of experimental spargers (e-spargers). These e-spargers varied in 'pore size' (200µm, 300µm, 400µm, 500µm) and 'Wall Depth' (1mm, 1.5mm, 2mm). The Wall Depth is the length of the access hole (*i.e.* depth) into the sparger. All e-spargers with a 200µm pore size had the front side unfinished as can be observed from the images. All e-spargers did not contain a rubber gasket o-ring whereas the porous sparger contained this o-ring to seal the connection with the normal sparger port. The size of the bubbles were analysed for each sparger and were listed in Table 5.3.

The first series of e-spargers (1mm Wall) were tested and only the 300µm version produced smaller sized bubbles than the Applikon Porous Sparger. The 400µm and 500µm spargers showed no leakage but the bubbles were bigger in diameter than the bubbles produced by the commercially available sparger. The 200µm sparger did not produce any bubbles via the sparger head as shown in Figure 5.25.

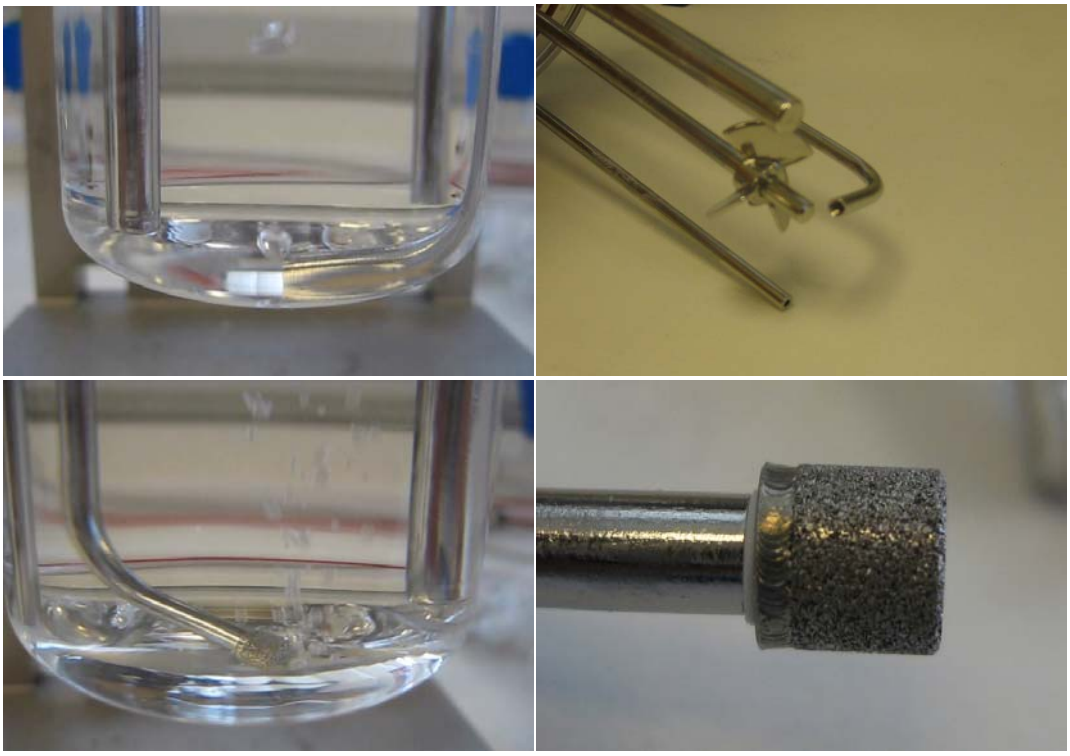

Fig. 5.24. *The standard sparger produced large size bubbles (top) whereas the Applikon Porous Sparger produced smaller sized bubbles (bottom)*

The second series of e-spargers (1.5mm Wall) were tested and the 300µm version produced the smallest sized bubbles of all tested spargers. All 1.5mm Wall spargers, except the 500µm version, showed a leak at the connection side. The 400µm and 500µm versions produced bubbles which were bigger than the commercially available porous version, but these bubbles were smaller than the bubbles produced by the standard sparger. The 200µm sparger did not produce any bubbles other than via a leak (Figure 5.26).

The third series of e-spargers (2mm Wall) were tested and the 300µm version produced bubbles similar inside when compared to the 1mm Wall 300µm type sparger. All 2mm Wall spargers, except the 500µm version, showed a leak at the connection side. The 400µm

version produced bubbles of similar size as the Applikon Porous Sparger and the 500µm version produced bubbles which were only slightly smaller in than those produced by the standard sparger. The 200µm sparger did not produce any bubbles other than via a leak (Figure 5.27).

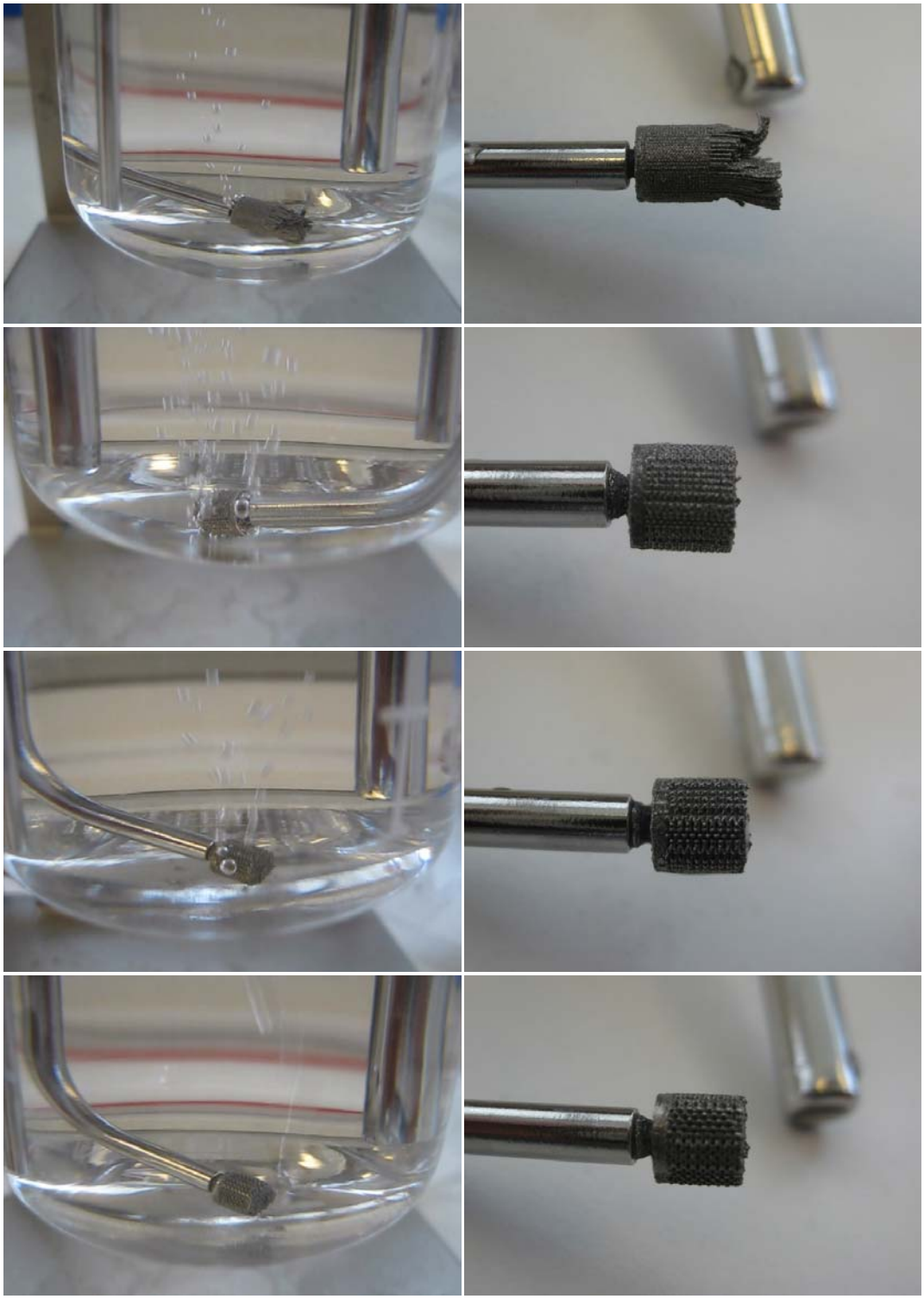

Fig. 5.25. *Experimental porous spargers with a 1mm Wall and with various pore sizes: 200µm, 300µm 400µm, 500µm (from top to bottom)*

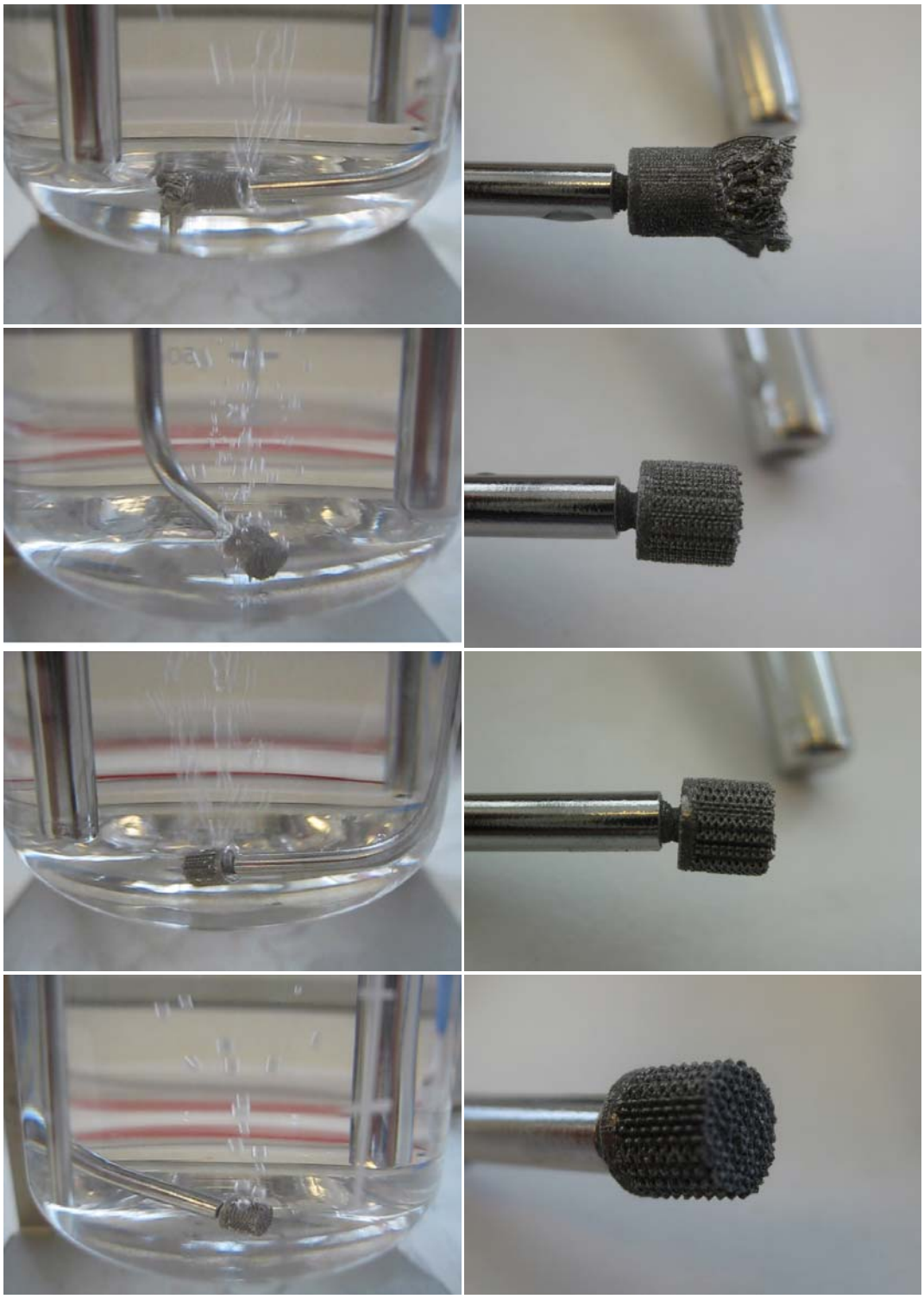

Fig. 5.26. *Experimental porous spargers with a 1.5mm Wall and with various pore sizes: 200µm, 300µm 400µm, 500µm (from top to bottom)*

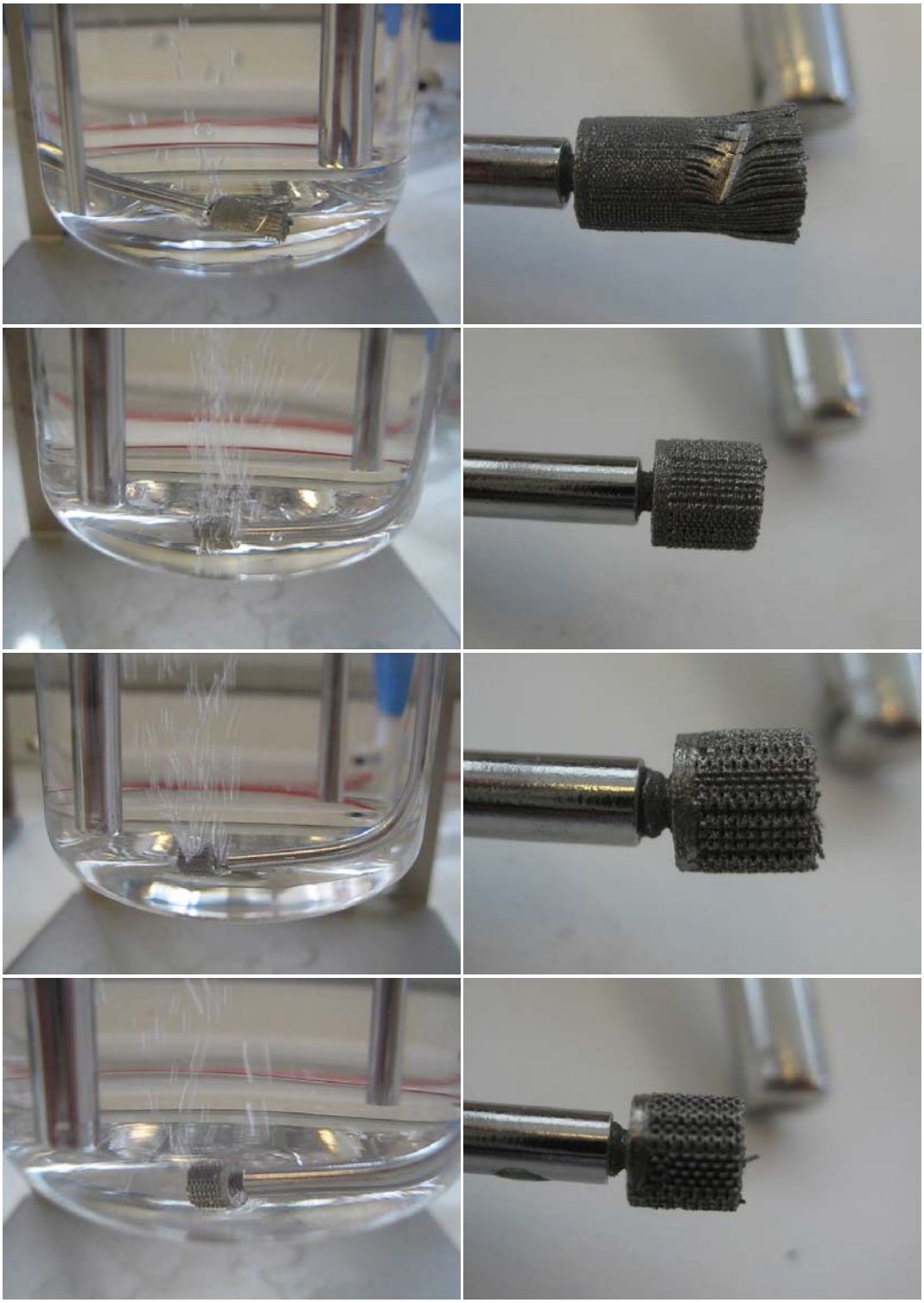

Fig. 5.27. *Experimental porous spargers with a 2mm Wall and with various pore sizes: 200µm, 300µm 400µm, 500µm (from top to bottom)*

#		Bubble size (Scale 1 to 10 - small to big)	Leakage (YES/NO)	Finished (YES/NO)	Homogenous Air Bubbles	Comments
1	no porous sparger	10	NO	YES	*NA*	Benchmark for 'big' bubbles
2	porous sparger MiniBio	3, 4, 5	NO	YES	NO	Mixture of bubble sizes
3	1 mm Wall - 200 µm UC	NO	YES	NO	*NA*	No bubbles from the porous head
4	1 mm Wall - 300 µm UC	2, 3	YES	YES	YES	Slightly smaller bubbles than the MiniBio sparger
5	1 mm Wall - 400 µm UC	5 and 6	NO	YES	NO	
6	1 mm Wall - 500 µm UC	8	NO	YES	NO	
7	1.5 mm Wall - 200 µm UC	NO	YES	YES	*NA*	No bubbles from the porous head
8	1.5 mm Wall - 300 µm UC	1, 2, 3	YES	YES	NO	Slightly smaller bubbles than the MiniBio sparger
9	1.5 mm Wall - 400 µm UC	7	YES	YES	NO	
10	1.5 mm Wall - 500 µm UC	7	NO	YES	NO	
11	2 mm Wall - 200 µm UC	NO	YES	NO	*NA*	No bubbles from the porous head
12	2 mm Wall - 300 µm UC	2, 3	YES	YES	YES	Slightly smaller bubbles than the MiniBio sparger
13	2 mm Wall - 400 µm UC	4	YES	YES	YES	
14	2 mm Wall - 500 µm UC	8	NO	YES	*NO*	

Table 5.3. *Sparger analysis of bubbles output for the minibioreactor (250mL) at an air flowrate of 5mL/min*

5.7 Dynamic culture of UBMCs

Previous experiments with the bioreactor established improved culture processes. Next, the MiniBioreactor was prepared for a culture with UBMCs to analyse cell viability and culture stability. The reactor was setup with a sample tube (Figure 5.19). The vessel contained 40mL of HSC expansion medium (I-Medium) and calibrated pH and DO sensors were placed in the head plate. The pH was CO_2 and alkali controlled whereas the DO was only air controlled. A 'Medium Feed Vessel' contained 30mL of fresh I-medium and this medium was added gradually during the first week of culture. UBMC's were thawed, washed, and resuspended in 5mL of medium. Cell count was performed including viability analysis using Trypan Blue. Cell suspension was added to the MiniBioreactor and culture was initiated. The cell density used corresponded with that used in static cultures. Cell samples (200µL) were taken after 1 day, 4 days, 6 days, 8 days, and 20 days to analyse cell amplification and cell viability.

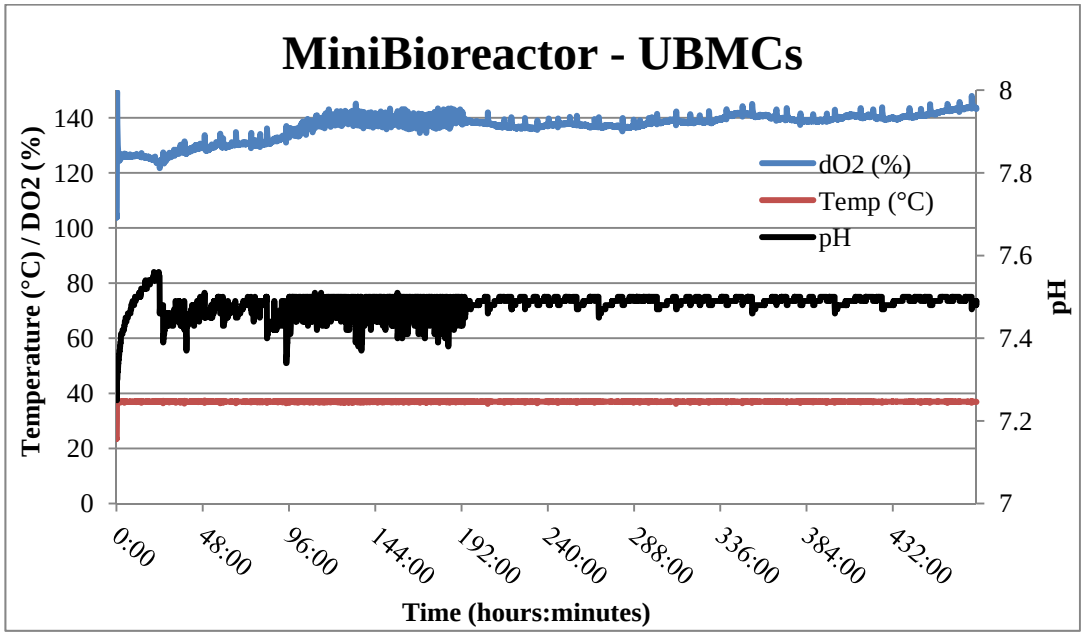

Fig. 5.28. *Sensor registration profile for the MiniBioreactor containing UBMC for HSC expansion*

During the dynamic culture the temperature (37ºC) remained stable whereas the pH fluctuated between 7.3 and 7.5 for the first 8 days of culture before stabilising at 7.45 for the

rest of the culture period. The recorded DO was ~120% for the first 24 hours of culture and rose to ~140% over the next 20 days (Figure 5.28).

The graph with the viability of UMBCs shows how the viability rate dropped rapidly during the first 4 days of culture with almost no viable cells after 6 days (Figure 5.29). The number of dead cells is high when compared to the number of viable cells and dropped in a similar fashion as the number of viable cells. On day 8, a slight rise in cell number was recorded. At day 20 no viable or dead cells were recorded.

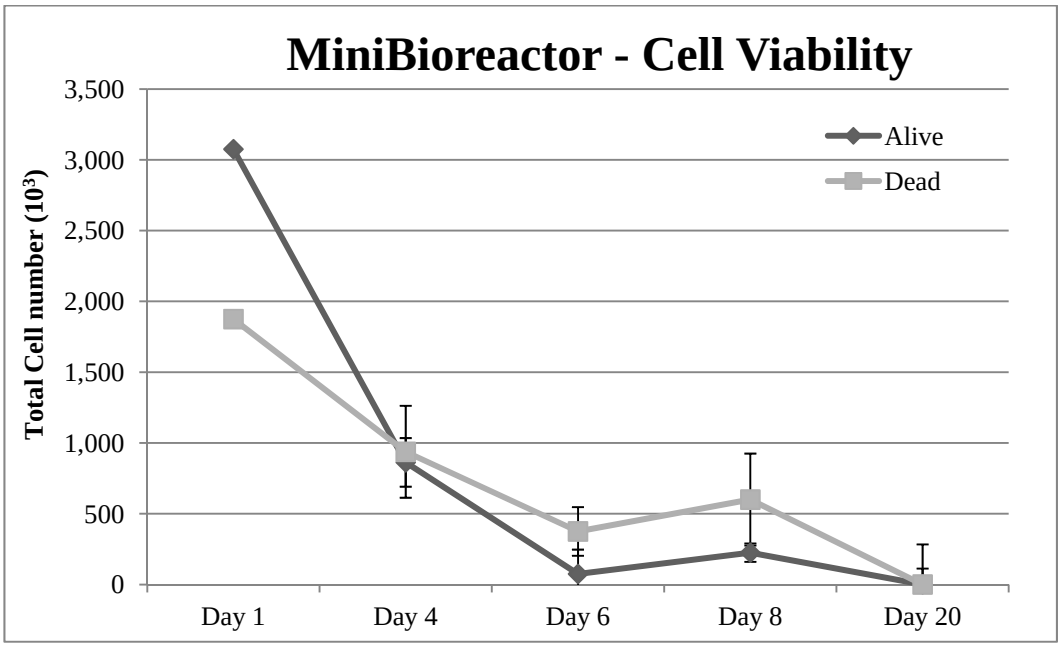

Fig. 5.29. *Cell Viability of UBMCs in a dynamic culture for 20 days (n=3, error bars represent StDev).*

5.8 Summary

A basic setup of a high volume (3L) bioreactor in a temperature controlled room (37ºC) was used to initiate dynamic cultures during this project and to determine requirements for successful cell expansion. Low cell viability indicated that improved control of the bioreactor was needed. Small scale cultures in a 250mL bioreactor were setup. Sensors were implemented and calibrated, a stirrer was added for distribution of nutrients to cells and data was logged over a prolonged period using specialised software (BioXpert Lite).

High cell viabilities were obtained using U937 cells. Stable temperature (37ºC) and pH (7.4) were achieved throughout the cultures. Control of DO was improved by not only using air, but also introducing nitrogen to the culture in order to lower the DO level.

The upscaling of cultures was tested by subsequently using a static culture, a MiniBioreactor (250mL), and a high volume Bioreactor (3L). Cell retention devices for the 3L Bioreactor were assessed as tools to refresh medium whilst maintaining cell retention in the bioreactor. Both the Spinfilter and the BioSep proved useful with the BioSep showing the most promising results and ease of handle. A range of porous spargers were tested to evaluate the means of introducing gases to the culture whilst minimising cell disturbance. A cell sample system was installed in the MiniBioreactor in order to analyse cell expansion and cell viability throughout the culture, under aseptic conditions.

The MiniBioreactor proved valuable to initiate and study small scale dynamic cultures. This will aid as a tool to develop and improve a range of bioreactor cultures. The developmental process described here requires further optimisation with the ongoing aim of producing erythrocytes.

5.9 Discussion

The main aim of this project was to translate 2D haematopoietic and erythroid cultures to 3D bioreactor cultures. The upscaling of the cultures should result in a higher cell output, increasing the manufacture of RBCs. The 3D upscaling of cultures is widely applied in the literature to produce higher cell numbers than possible in a 2D environment. A range of bioreactors and 3D materials (e.g scaffolds) have been used to study and increase cell expansion [69, 75, 77, 78]. As the research into the expansion and erythroid maturation of HSCs occurred in parallel, the human suspension cell line 'U937' was used to study and optimise the bioreactor processes. After successful optimisation, the bioreactor would be used to study and manufacture the erythroid maturation of HSCs.

During the early stages, adherent cells (*e.g.* MSCs) were used to analyse the parameters of the bioreactor under suboptimal conditions. During this initial phase it was established that all bioreactor models required pH and DO monitoring, a system for homogeneous distribution of cells and nutrients within the bioreactor, medium refreshment, temperature control, data recording, and cell sampling to allow for cell number and viability checks. As part of this analysis, feedback was provided to Applikon Biotechnology to provide required equipment, updates and advice in order to optimise 3D cell cultures and work together to improve culture conditions.

MiniBioreactors (small scale bioreactors), with a working volume up to 200mL, were used to establish the minimal requirements to cultivate cells. The analysis of cell viability and cell numbers were the main methods to determine these culture conditions. The bioreactors with a maintained pH of ~7.4 showed high cell viability (>95%) up to 9 days (Figure 5.4 and 5.6) whereas the bioreactor without maintained pH (fluctuating between 7.2 and 8) showed a low cell viability of ~68% (Figure 5.9). The importance of controlled medium addition (150mL) over a time period of 9 days, to support increasing cell viability, resulted in a higher cell density when compared to addition of medium over 3 days.

Another critical parameter was the fluctuation of DO during these and other bioreactor tests. As with the then current setup of the bioreactor control, only air and thus only upward DO control, was possible. To allow for downward DO control, an add-on was installed to allow for the addition of nitrogen, which should have resulted in lower controlled DO levels. It has been published that the human body represent low oxygen levels in the HSC niche, positioned in the BM, which stimulates erythropoiesis [116-118]. It was essential to precisely control the DO levels for creating culture conditions to stimulate HSC proliferation and subsequently the production of RBCs.

Published data stated that it is possible to create low oxygen conditions (hypoxia) in a bioreactor [119] and that hypoxia increases the proportion of long-term-reconstituting HSCs during *in vitro* culture [120]. A recent review analysed the influence of hypoxia and hyperoxia for HSCs and the importance for the balance between quiescence, self-renewal and cell commitment [121]. This advocates precise DO control in a bioreactor aimed for HSC expansion and erythroid maturation. The implementation and optimisation of the addition of air and nitrogen for DO control will be discussed after ‘cell retention’ and ‘upscaling and cell viability’ have been discussed.

5.9.1 Cell retention and viability

For cell retention two different devices were tested: an acoustic cell retention device (BioSep) and a spinfilter. Both the BioSep and the spinfilter were available for the 3L and 2L bioreactor, respectively. The aim of this project was to implement the highest performing device into a small scale bioreactor (working volume 200-400mL). The potential scalability of the device was also important. These two devices have been reviewed in depth [12-124] and an advantage of the acoustic cell retention device is the absence of a physical barrier and moving parts. Therefore, the acoustic device should be less prone to fouling , mechanical failure, and cell destruction [125].

It was found that both devices were suitable for cell retention with retention rates over 90% and cell viability of 90%. However initial experiments with the BioSep found low cell viability (~70%). This improved once the DO set point was decreased to a level similar to that used for the spinfilter (20-30%). This then resulted in the addition of air at a later stage of culture, after 96 hours instead of 24 hours, and in smaller volumes. Overall, the BioSep allowed for a higher cell density including improved cell retention, faster cell expansion, and higher cell viability rates (Figure 5.18) compared to the tested spinfilter (Figure 5.15).

5.9.2. Optimisation of DO control

The addition of air, to achieve and maintain a pre-set DO level, was applied via a sparger and when air was added, air bubbles entered the culture medium. The oxygenating bubbles can cause shear stress damage to the cells which has a negative effect on cell viability [126]. Cells adhere to the gas-liquid and are lifted to the surface where they may be damaged due to shear stress when bubbles penetrate the surface and burst 113-115, 127]. Another side effect of oxygenation via spargers is foam formation at the medium surface [126]. This is a high impact problem for small scale bioreactors (200-500mL) due to the smaller volume between medium surface and head plate to allow for foam formation. This volume available for foam production decreases with increasing medium volume (See appendix for foam production in bioreactor vessels).

After the installed add-on allowed for the addition of nitrogen, tests showed the precise control of the DO level in PBS (Figure 5.22). As oxygenation in culture medium produces unwanted foam, nitrogen and air were supplied to the bioreactor above the medium but tests showed the lack of accuracy in DO control and this method was rejected. The preferred method for addition of nitrogen and air to the bioreactor was via a sparger. Based on reports by collaborators of Applikon Biotechnology it was hypothesised that smaller bubbles would have a positive impact on cell viability and reducing foam formation [126]. A range of

spargers were tested and results showed that smaller air bubbles could be produced with a different design of porous sparger (Table 5.3). However, further analysis is needed to determine the effect on cell viability and, more importantly, foam production.

During these tests and other bioreactor cultures, it was observed that the My-Control (responsible for adjusting the set DO level) added nitrogen and air with a high pulse. Also, the lack of a dead band for the DO level (as is used by pH control) resulted in continuous correction of the DO level (*i.e.* continuous addition of nitrogen and air) contributing to continuous foam production. Feedback was supplied to Applikon Biotechnology and suggestions have been made to eventually update the control system. The scarce addition of CO_2 to influence and maintain pH did not seem to have a detrimental effect on cell viability and foam production was negligible during the maintenance of the pH level.

5.9.3 Dynamic culture of UBMCs

Analysis of UBMCs for culture in a 250mL MiniBioreactor was tested during bioreactor development process. UBMCs were used as insufficient UCB-$CD34^+$ cells were available to initiate a 30mL culture. Cell viability did drop during the first few days of culture, as expected and similar to static cultures, but cell numbers did not increase again during the additional days. The pH was maintained between 7.4 and 7.5. The monitored DO level was high throughout the culture (>100%) and no air (or nitrogen) was added. A possible cause for the decrease in cell viability was a low start cell density as it was similar to cell densities used during static cultures. The 3D environment with cells mixed throughout the medium volume by the impeller resulted in spreading the cells farther apart compared to static cultures. Optimal cell signalling was therefore obstructed and this problem might be overcome by applying higher cell densities (10^5-10^6 cells/mL). It was observed during the experiments in Chapter 3 and 4 that HSC expansion benefits from closely packed cells. Another issue affecting cell viability could be the shear stress induced by the marine impeller, however tests with U937 cells did not indicate this. Further tests with increased

UBMCs densities (~10^6 cells/mL) should be beneficial for cell expansion within the MiniBioreactor.

The process of translating the static HSC expansion and erythroid maturation into a 3D environment for upscaling cell culture should be continued. Major improvements have been made with the MiniBioreactor to successfully culture cells and optimise the cell culture environment. Ongoing work is needed and should lead to the achievement of HSCs culture in these MiniBioreactors in the near future.

Chapter 6

General Discussion and Conclusions

6.1 General discussion

The three research objectives were

1) to isolated PB-HSCs and optimise HSC culture conditions to allow for successful expansion beyond 7 days;

2) the production of RBCs from PB and UCB-isolated cells including the improvement of erythroid expansion rate, and

3) to use commercially-available bioreactors, by using cell line U937 as a model, for the development of an HSC culture system to expand and differentiate cells towards the erythroid lineage.

In short, this research focussed on creating an *in vitro* environment for increased production of tissue engineered RBCs.

Research groups have focussed on HSC expansion and the manufacture of artificial blood for many years. RBCs are responsible for oxygen transport throughout the human body and finding an alternative, cell-based, oxygen carrier could be tremendously beneficial for tackling shortages and improve safety in transfusion medicine. For this project we combined current progress made in this field to establish and improve the differentiation of HSCs into blood cell lineages to aim for clinically useful quantities.

Isolation of HSCs from PB proved difficult, predominately due to insufficient cell numbers available, and therefore UCB-$CD34^+$ cells were initially used as the main cell source for expansion studies. Data showed minimal expansion potential in long term cultures, but expansion cultures were maintained for a prolonged period by implementing an MSC-based stromal layer (*e.g.* BMSCs).

Literature demonstrated the benefit of using the $CD34^-$ cell population of PBMCs for erythroid maturation [41] and during this project, the principle of using MNCs was transferred to analyse their potential for HSC expansion. PBMCs and UBMCs, as an easy accessible cells source, showed prolonged cell culture potential when supported by cytokines (*i.e.* SCF, Flt3-L, TPO) and a BMSC stromal layer. Despite successful expansion rates of $CD34^+$ cells from UBMCs, the positive expression of CD34 did not exceed 35% for expanded PBMCs and UBMCs. With results showing the potential of using MNCs for CD34 expansion, more research is needed to compare the production rates of pre-CD34 cell isolation and HSC expansion with MNC expansion and subsequent isolation of CD34+ cells, and to further boost expansion of CD34+ cells within these MNC cultures.

The isolated cell populations (*e.g.* CD34+ cells, UBMCs) were used to produce RBCs following a widely cited protocol [8]. Successful erythroid maturation was proven by cell analysis and red cell pellets, but more research is needed to confirm the characteristic biconcave shape and cell function with RBC properties. The erythroid maturation of UCB-$CD34^+$ cells showed higher expansion potential compared to the differentiation of PB-$CD34^+$ cells but an essential limitation was the isolation of sufficient PB-CD34+ cells from available PB.

With the use of UBMCs, larger erythroid expansion rates were obtained, with cells showing positive for the RBC-associated markers CD235a and Hb-β [110]. However, analysis of maturated cells with hospital equipment, used for analysing patient's blood samples, could not be completed to confirm successful production of artificial

RBCs as higher cell quantities were required. Follow-up research should focus on full haematological analysis of cRBCs including Hb content, MCV, MCH, and enucleation and functionality tests. The expansion rates obtained for erythroid maturated UBMCs were lower than those published for differentiated UCB-$CD34^+$ cells [42, 45, 52], but when expansion was obtained for CD34+ cells within the heterogeneous MNC population and the erythroid maturated cells, expansions of over 400-fold were recorded. This demonstrates the potential of UBMCs in erythroid maturation and further optimisation of culture protocols for these cells should be beneficial for increasing production of cRBCs.

The development of a small scale bioreactor, aimed at expansion of HSCs and production of artificial RBCs, did not achieve these goals, but tremendous progress was made to optimise a dynamic culture environment. Cell retention devices were tested for their suitability in obtaining highly packed cell cultures within this bioreactor, with the aim of achieving RBC densities found in PB. Tests with the BioSep, utilising U937 cells, achieved high cell densities (*e.g.* ~$2x10^6$ cells/mL) with potential for continuous increase as can be obtained from Figure 5.18. Medium refreshment and precise pH and temperature control was paramount in achieving high cell densities and high cell viability rates. Upon implementing erythroid maturation of HSCs within this bioreactor, a further increase of cell density needs to be further investigated.

It was suggested that implementation of DO level control could aid in the achievement of hypoxia cultures, mimicking the HSC niche, for stimulation of erythropoiesis [116, 118]. Successful DO control was recorded, however, continuous

over-correction of the set DO level (*i.e.* continuous addition of air and nitrogen) contributed to excessive foam production. Feedback was therefore provided to Applikon Biotechnology with suggestions to update the control system (*e.g.* implementation of a dead band). In addition to this, a range of 3D printed porous spargers were assessed for their bubble size, to decrease the detrimental impact on cell viability and to reduce foam production [126]. It is paramount that follow-up research has access to improved DO level control before further hypoxia cell cultures are performed.

In this study, erythroid maturation has not yet been executed in the small scale bioreactors (250mL) but by analysing the 3D culture parameters progress to stimulate cell expansion has been made, while also studying the HSC expansion potential of MNCs and their ability to differentiate into RBCs in 2D cultures. Further research is required to optimise and combine these two areas for the increased production of artificial RBCs which could hypothetically be upscaled to larger volume bioreactors (2L).

Bioreactors which are currently used in this field for the use of HSCs and the production of blood lineage cells are largely experimentally focussed [69, 72-75] but reviews have been published mapping the route for production of artificial RBCs [54, 79, 128] and 3D tissue manufacturing [129-131] stating the complexity but also the potential for this field.

6.2 Conclusions

- During successful isolation, approximately $3x10^4$ $CD34^+$ cells can be isolated from 30mL of fresh PB (equals ~$4x10^7$ MNCs) but for sustainable expansion cell culture, higher quantities of PB (>200mL) are needed to isolate sufficient numbers of HSCs.
- The leukocyte cones used, provided insufficient PB-$CD34^+$ cells for sustainable expansion cultures.
- Cell densities used for expansion of UCB-$CD34^+$ cells (10^4 cells/mL) were not applicable to PB-$CD34^+$ cells, resulting in non-sustainable cultures.
- Cell cultures of pre-thawed isolated UCB-$CD34^+$ cells have better HSC expansion potential than post-thawed isolated UCB-$CD34^+$ cells.
- S-medium is a suitable basic medium for expansion of UCB-$CD34^+$ cells but requires an optimised cytokine cocktail for HSC expansion whereas serum-free I-medium is a suitable basic medium for expansion of UBMCs.
- UCB-$CD34^+$ cells in culture on a MSC stromal layer, using I-medium and a cytokine cocktail, demonstrated a prolonged round-like morphology for at least 14 days
- PB-$CD34^+$ cells cultured on a BMSC stromal layer demonstrated prolonged expansion potential but expression of CD34 decreased after 7 days of culture with I-medium proving more advantageous over a 21 day period when compared with P-medium and D-medium.
- Expansion of HSCs within a PBMC culture was the most successful on an MSC stromal layer using I-medium containing cytokines, resulting in almost 35% positive expression of CD34.

- Expansion of HSCs within a UBMC culture was the most successful on a BMSC stromal layer using I-medium containing cytokines, resulting in 85-fold expansion of $CD34^+$ cells over 7 days.
- The method published by Douay and Giarratana (2009) [86] proved successful for the manufacture of red cells from UCB isolated cells, with SCF, EPO, and IL-3 as suitable cytokines in erythroid maturation.
- Erythroid maturation of UCB-$CD34^+$ cells proved more successful when compared with PB-CD34+ cells in terms of cell expansion and the manufacture of red cells.
- UCB isolated cells in erythroid maturation showed a decrease in cell diameter, production of a red cell pellet, indication of enucleation, and presence of intracellular Hb-β.
- Erythroid maturation of UBMCs proved useful for the manufacture of RBCs, resulting in positive expression of RBC associated markers (*e.g.* CD235a and Hb-β), and a red cell pellet.
- Erythroid maturation of UBMCs requires further optimisation for creating a larger number of RBCs
- HSC expansion of UBMCs prior to erythroid maturation, in order to increase the number of $CD34^+$ cells within the MNC population, was not advantageous for the production of RBCs when compared to directly-maturated UBMCs.
- DO, pH and temperature sensors are required to monitor the cell environment and link obtained data to cell viability with maintenance of pH and temperature levels resulting in an improved cell viability and cell expansion rate.

- Active DO control can be obtained by using air and nitrogen but further research is needed to obtain stable DO levels in culture medium whilst avoiding excessive foam formation.
- Porous spargers are useful tools for obtaining smaller sized bubbles which should result in minimised detrimental effects on cell viability. From the examples tested, bubbles produced by the 3D printed 300μm UC spargers were the smallest in size.
- The BioSep is advantageous for cell retention when compared to a Spinfilter
- Precise management of medium volume expansion relating to optimal cell density and medium refreshment is needed to optimise cell expansion rates further.
- The MiniBioreactor is a useful tool to initiate small scale, dynamic, cultures.
- Dynamic expansion of UBMCs, and subsequent erythroid maturation, in a small scale bioreactor requires further optimisation in order to produce the required large cell quantities.

6.3 Suggestions for future work

HSC expansion for a prolonged period was only achieved with UBMCs but high $CD34^+$ expression was not achieved. Future research should focus on altering the cytokine cocktail, introducing hypoxia, and further optimisation of the culture medium to increase the output of HSCs [94, 118], [Tsiftsoglou *et al*, 2009]. These cells could then be evaluated for progenitor- and stem-cell frequencies within the haematopoietic spectrum by using a cobblestone area-forming cell (CAFC) assay assessing the quality of the optimised expansion method of UBMCs and PBMCs to

demonstrate their proliferative and differentiation capacities for blood cell lineages [132]. One of the main challenges remains to establish continuous self-renewal of HSCs whilst also being directed towards erythroid maturation.

Ongoing efforts should focus on the culture environment to enhance the production of RBCs in suspension, including feeder-free enhancement of enucleation rates. Recently, data has been published by Baek *et al* (2010) demonstrating positively charged culture plates to achieve efficient RBC production [133], rather than introducing the widely used stromal layer. This research contributes to establishing methods for the clinical manufacturing of RBCs whilst omitting the use of co-culture but attempting to mimic the stem cell niche.

Ultimately, large-scale automated production of RBCs *in vitro* will be paramount and bioreactors are essential tools for this process. Achieving high cell densities ($>10^8$ cells/mL) and expansion rates (10^7) have been reported [134]. Future studies should continue to boost these figures by focussing on creating the culture conditions that can be found in the stem cell niche. Also, current systems are merely focussed on either HSC expansion, or production of RBCs. A combined system, incorporating both cultures, should assist in creating a higher cell output than has currently been established.

Chapter 7

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7.1 References

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Appendix 1

Theun van Veen, John A. Hunt

Tissue engineering red blood cells: a therapeutic

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Appendix 2

Table and Figure Legends

Chapter 1 - Introduction

Chapter 2 – Materials and Methods

Chapter 3 – Results: Haematopoietic cell culture

Chapter 4 – Results: Manufacture of Red Blood Cells

Data is a collection of data as shown in Figures 4.10 and 4.13.

Chapter 5 – Results: Development of the bioreactor

Appendix 3

RBC analysis with SEM

RBC analysis with SEM

Native RBCs were processed and analysed for SEM as a method to visualise cell shape. It was hypothesised that native RBCs would show their characteristic biconcave shape. These data could then be compared to SEM images of cRBCs. Due to limited cell numbers no SEM data were collected for cRBCs. From Figures A3.1-4 it can be observed that the native RBCs show their characteristic morphology after being analysed with SEM and that this technique might have potential for analysing the cRBC morphology.

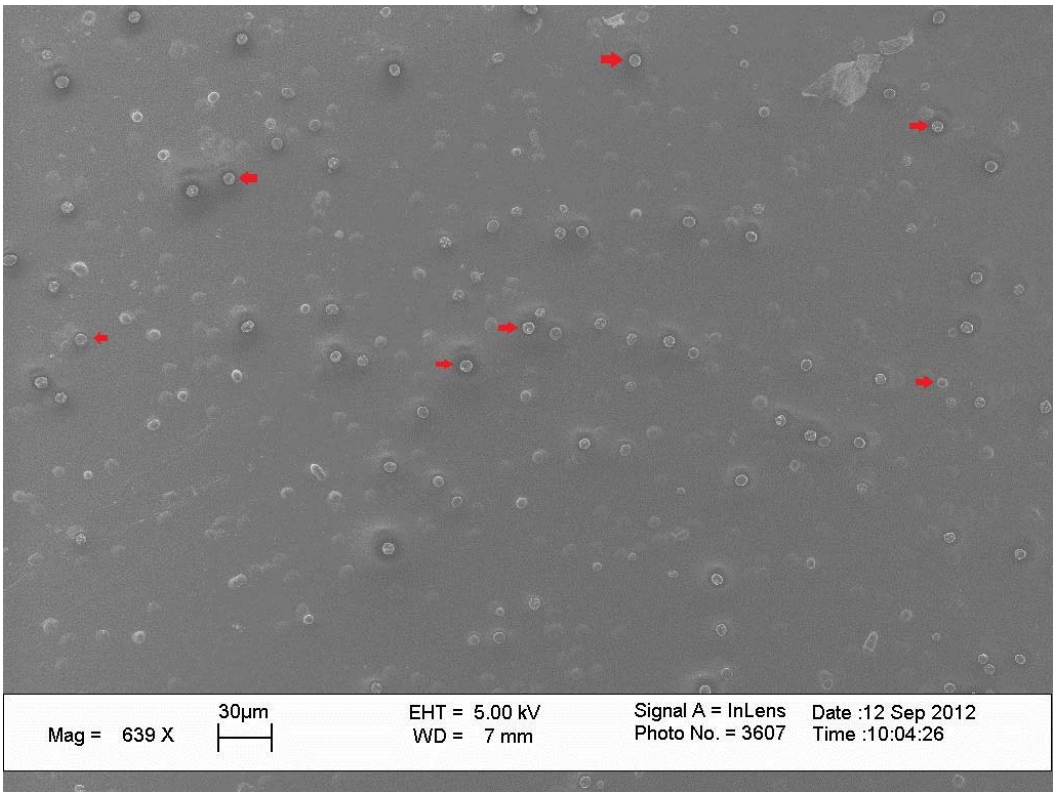

Fig. A3.1. *Overview image of PB isolated cells. RBCs are highlighted by red arrows. Scale bar represents 30µm*

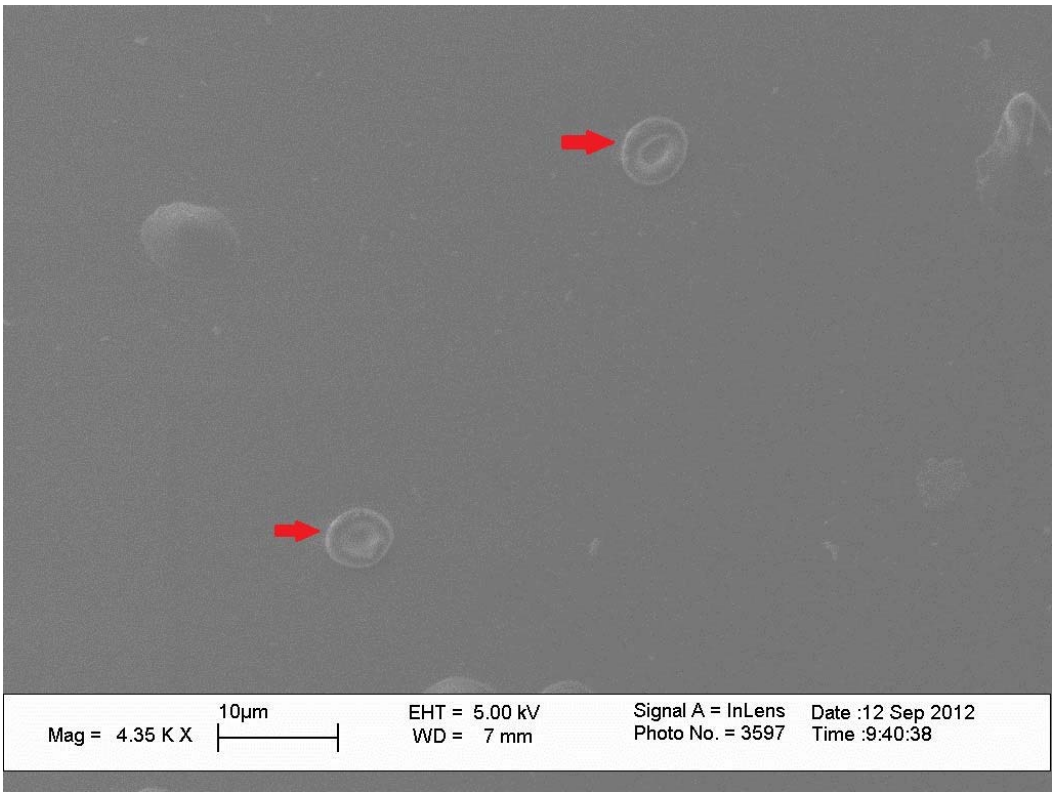

Fig. A3.2. *PB isolated RBCs are highlighted by red arrows and these cells show their distinctive biconcave shape. Scale bar represents 10µm*

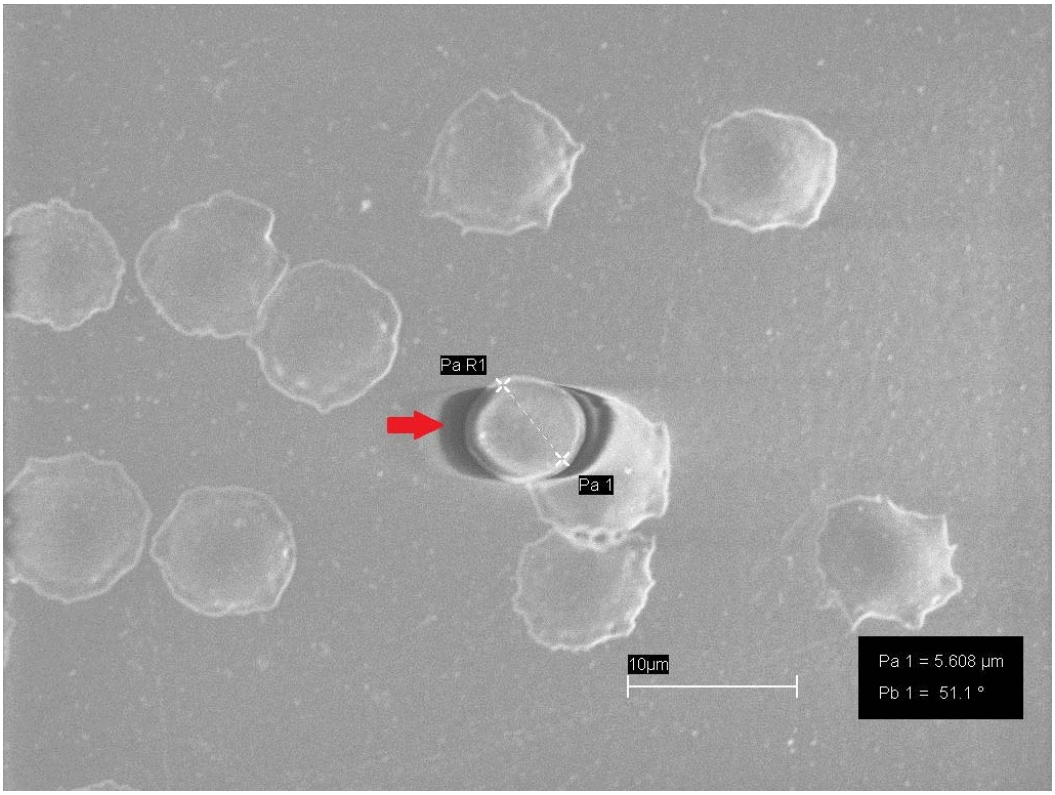

Fig. A3.3. *PB isolated RBC is highlighted by a red arrow and this cell has a diameter of ~5.6µm. Scale bar represents 10µm*

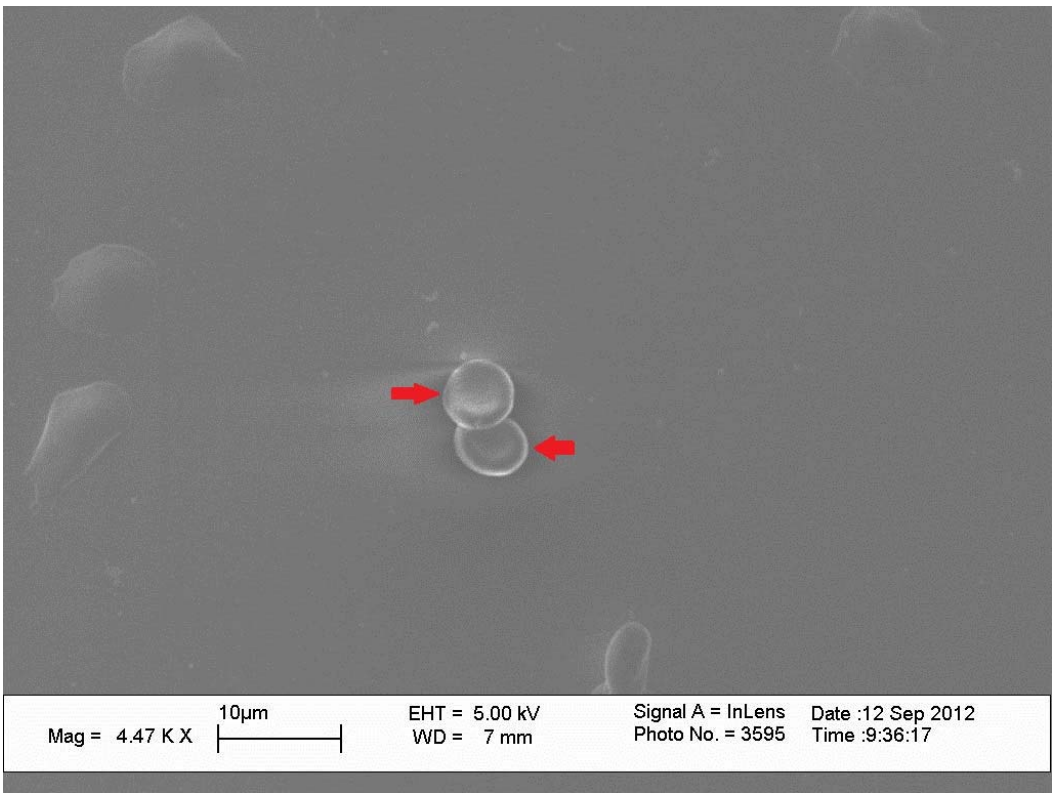

Fig. A3.4. *PB isolated RBCs are highlighted by red arrows and these cells show their distinctive biconcave shape. Scale bar represents 10µm*

Appendix 4

Surface marker profiles of stromal MSCs

Stromal MSC cell surface marker profile

MSCs were either isolated from BM or adipose, as described in section 2.2.3 and 2.2.4 respectively. Subsequently, these cells were analysed for a set of cell surface markers to establish their phenotype as MSCs, as described by (Dominici *et al.* 2006). All isolated MSCs did adhere to tissue culture treated plastic in standard culture conditions, but *in vitro* differentiation capacities for osteogenic, adipogenic, and chondrogenic behaviour were not analysed. The phenotype of these cells was established utilising the cell surface makers CD105, CD73, CD90, and CD29 (positive) and CD45 and CD34 (negative). Representative phenotypes of AMSCs and BMSCs can be found in Figures A4.1 and Figure A4.2. After successful confirmation of the cell properties, BMSCs and AMSCs were used as stromal cells in haematopoietic and erythroid cell cultures.

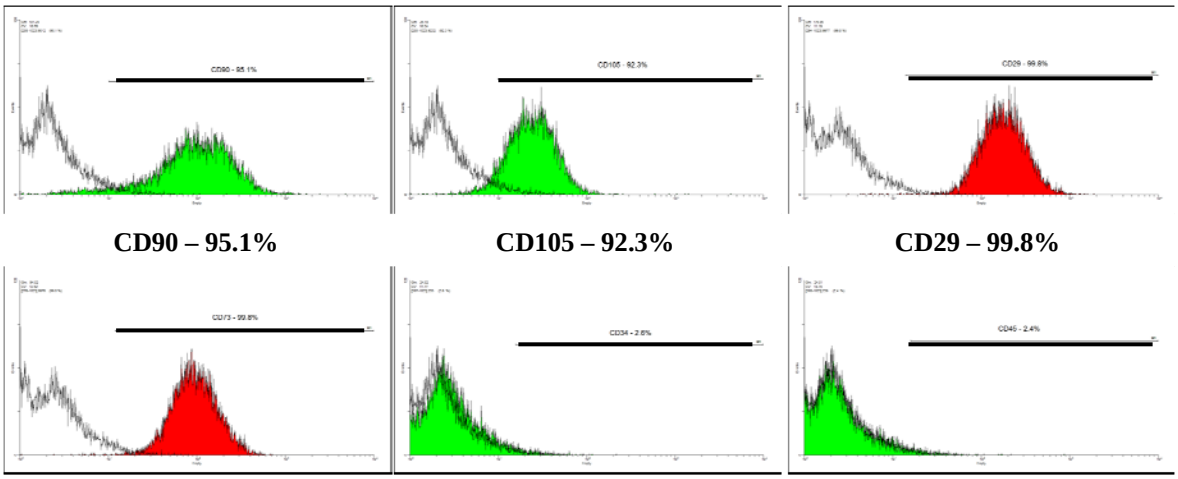

Fig. A4.1. *Representative phenotype of AMSCs used for haematopoietic and erythroid cell cultures*

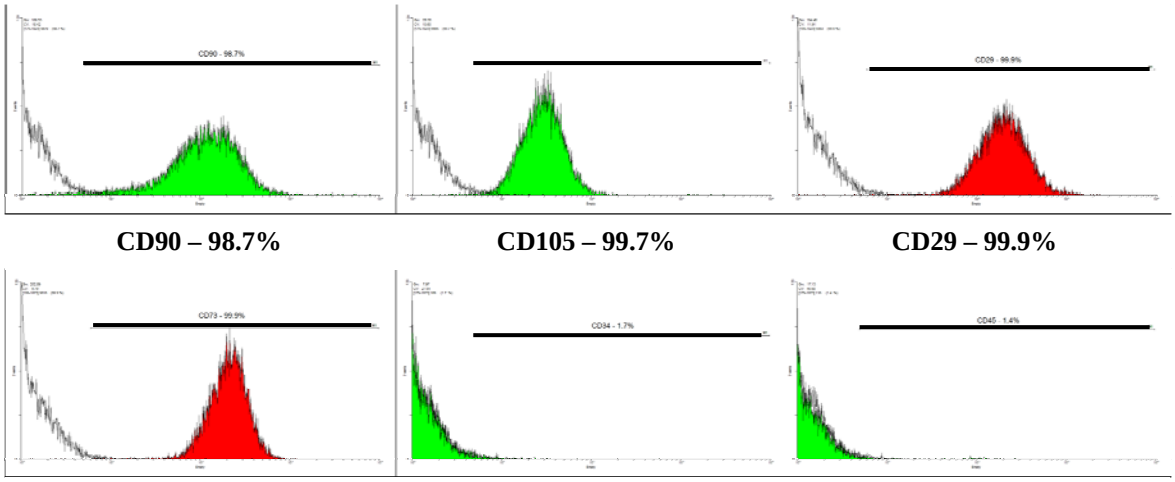

Fig. A4.2. *Representative phenotype of BMSCs used for haematopoietic and erythroid cell cultures*

Reference:

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, and Horwitz E. 2006, Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement, *Cytotherapy*, **8**: 315-317.