Producing large quantities of red blood cells from stem cells for transfusion purposes

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Juli 2012

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Bachelor scriptie

Ex vivo haematopoiesis

Abstract

Cultured human haematopoietic stem cells in the quantities required for blood transfusion could provide worldwide healthcare with a solid alternative to blood donation. Research into erythropoiesis in the embryo and foetus provides us with phase specific growth factors. *In vitro* generation of red blood cells from haematopoietic stem cells derived from hiPSCs now uses a minimal amount of factors (SCF, EPO, IL-3). The development of large-scale agitated bioreactors opens the doors to the industrial scale production of red blood cells for transfusion purposes. Nielsen et al. demonstrated that 560 units of RBCs can be derived per umbilical cord blood donation. These findings demonstrate the clinical feasibility of producing erythroid cells *ex vivo*. Human induced pluripotent stem cells (Yamanaka et al. 2010) provide us with unlimited amounts of $CD34^+$ stem cells. Current good manufacturing practice and optimized media resulted in the first clinical cultured red blood cell transfusion into humans (Douay L. et al 2011).

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Introduction

"So the heart is the beginning of life, the Sun of the Microcosm, as proportionably the Sun deserves to be call'd the heart of the world, by whose vertue, and pulsation, the blood is mov'd, perfected, made vegetable, and is defended from corruption and mattering; and this familiar household-god doth his duty to the whole body, by nourishing, cherishing, and vegetating, being the foundation of life, and author of all." - William Harvey (1649)

A Renaissance man, William Harvey was fascinated by the conversion of arterial blood into venous blood in a rapid, efficient and consistent manner within one system. Further on in time, in 1901, Karl Landsteiner discovered substances in the blood, antigens and antibodies, which made red blood cells clump when of different types, leading to the thus established concept of the AB0 blood groups. Several other blood groups were later identified and these findings had profound impact on blood transfusion principles: donors could now be matched to recipients in an orderly fashion and most complications could henceforth be avoided. Problems with incompatibility still manifested because antibodies were produced against previously unidentified antigens in the donor blood, resulting in an immune response.

Further studies on blood elucidated the anatomy of the circulatory system, the kinetics of blood flow and the constituency of blood, the latter of which laid the fundamentals for understanding hematopoietic morphogenesis: the process that generates all blood cells of the myeloid (*monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, dendritic cells, megakaryocytes and platelets*) and lymphoid (*T-cells, B-cells and NK-cells*) lineages and only until recently pluripotent stem cells, such as the embryo, were found to give rise to several multipotent stem cells including the hematopoietic stem cell, the basic unit of the hematopoietic differentiation process (See figure 1).

The understanding of these processes and understanding the way in which one cell differentiates into another cell and why not into another paves the way for hematological stem cell therapies and even the culturing of blood (Kaushansky K. 2006) or its components *ex vivo*, especially with the accumulating knowledge in the field of induced pluripotency (Suknuntha K. et al. 2012) which sets the requirements for obtaining hematopoietic stem cells to only a small sample of any patient-cell. This provides a way to culture entire batches of autologous hematopoietic cells for use in treatment of diseases or deficiencies by stem cell engraftment or cultured blood transfusion and simultaneously circumventing ethical concerns regarding experiments on embryonic stem cells. It is now of interest to develop a system that resembles the *in vivo* dynamics of haematopoietic stem cell self-renewal and differentiation as in the bone marrow in order to maintain a continuous production of functional adult red blood cells *ex vivo* in large quantities for the purpose of patient transfusions.

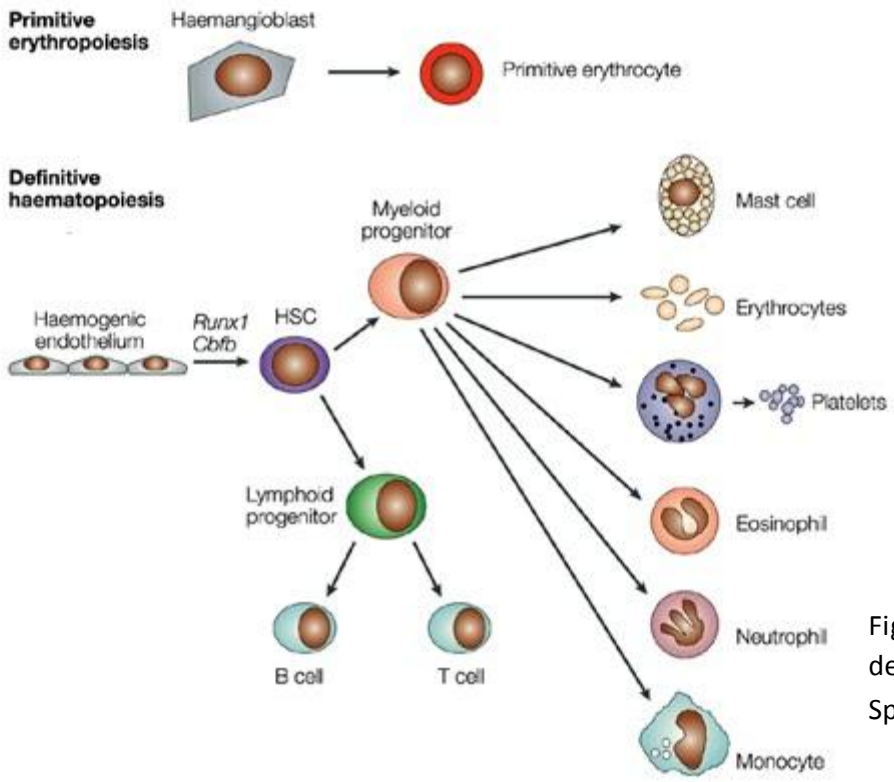

Figure 1: Haematopoietic development. (Adapted from Speck et al. 2002)

But here problems arise. The bodily response against any intruder is a tough barrier to overcome as the immune system recognizes the smallest error in consistency. When recognized, the system triggers a foreign-body response which can result in graft rejection (Claas F. H. J. et al. 2011), graft-versus-host-disease (Balkanov T. et al. 2011) and hemophagocytic syndrome (Ishida H. et al. 2007, Othman et al), among others, even when the stem cells were generated and cultured from the patient's own tissue. This challenges the treatment of hematological malignancies such as chronic and acute leukemia and it is thus necessary to overcome these physiological defense mechanisms to establish a stable and plentiful method with which to create any component of blood from the small scale for research purposes to the large scale manufacturing of autologous blood cells for transplantation and transfusion purposes (Douay L. et al. 2011, Nielsen L. K. et al. 2011), although the current methods are not yet fully standardized and validated (Haesook T. Kim et al. 2012, Rao M. S. et al. 2009, JACIE Standards for hematopoietic progenitor cell collection processing & transplantation. 2003). Red blood cell culturing is of the highest interest in the field of HSC culture since blood is a scarce resource and the implication of successfully creating safe and autologous blood is that transfusable, patient's-own blood can be created from any patient cell, which would dissolve the current issue of blood shortage due to population ageing and the difficulties in finding rare blood types (A. R. Migliaccio et al. 2012) and also would make blood donation partially obsolete (currently 92 million donations per year: www.who.int/worldblooddonorday/en/).

This thesis tries to underpin haematopoiesis and in this context optimal *ex vivo* haematopoietic stem cell differentiation protocols for producing significant quantities of stable transfusable adult red blood cells that survive *in vivo*.

Red blood cell development

The most abundant cells in the blood are the red blood cells (RBCs), constituting 45% of the whole blood, which carry the oxygen to the tissues by firstly binding the oxygen to a hemoglobin group. One microliter of blood contains approximately 4.2 million to 6.1 million erythrocytes.

Red blood cells are most primitively formed inside the yolk sac blood islands in 18 day old embryos (Takashina T. 1987) and are formed definitively inside the bone marrow in adults. Primitive and definitive haematopoietic stem cells arise from a common precursor stem cell. The development of red blood cells is favored by adhesion molecule interactions with a macrophage in the liver in the four-week old embryo and in the bone marrow in adults. Red blood cell development is called erythropoiesis (Figure 2). The formation of blood cells begins at the pluripotent haematopoietic stem cell, also called the hemocytoblast or the hemiangioblast that differentiates into the common myeloid progenitor. The common myeloid progenitor subsequently differentiates into a unipotent stem cell. The unipotent stem cell then develops into the pronormoblast, also called the pro-erythroblast. The earliest erythroid cell, the pro-erythroblast, undergoes several mitotic divisons which generate basophilic, polychromatophilic and orthochromatic erythroblasts which remain nucleated in embryonic development inside the yolk sac (Segel et al.). The orthochromatic erythroblast with decreased nuclear size and activity and an increase in hemoglobin concentration subsequently divides asymmetrically into the pyrenocyte and the reticulocyte. The reticulocyte has no nucleus and has a further increased concentration of hemoglobin and red blood cell specific proteins that are acquired during maturation within the vascular network.

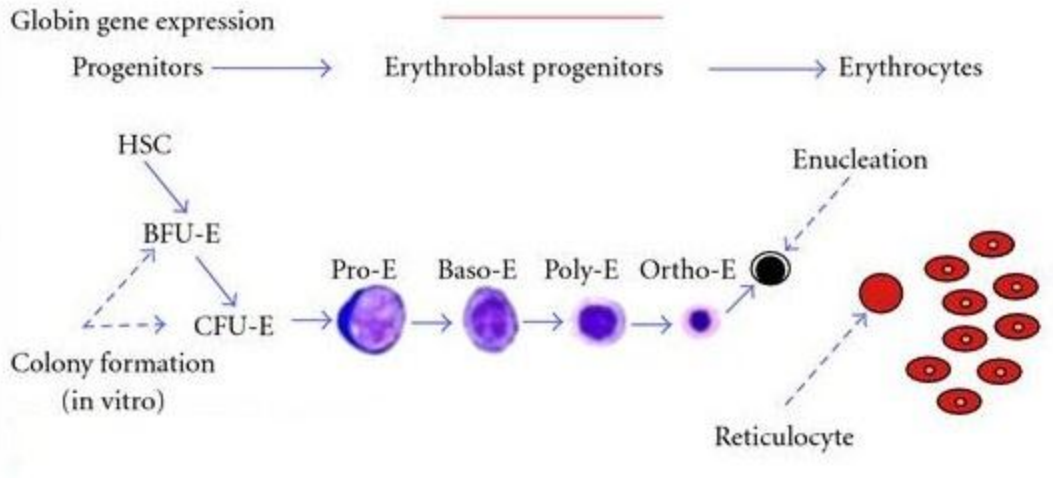

Figure 2: Erythropoiesis. (Adapted from Cianetti et al. 2009)

The spleen is considered to have an essential role in the remodeling process (Holroyde et al.). The reticulocyte is remodeled to a smaller biconclave disk that has lost residual organelles, several nucleic acids and CD71 erythrocyte marker expression (Giarratana et al.). Terminal maturation of the red blood cell is guided by chromatin condensation, actomyosin motors and vesicle trafficking (Keertshivan et al. 2011). After birth, beta-globin chains replace fetal gamma-globin chains which facilitate easier oxygen release into tissues. The process of erythropoietic maturation is tightly regulated by erythropoietin. Erythropoietin (EPO) plays a central role in definitive erythropoiesis and it is synthesized in the liver after birth. Erythropoietin ensures that the destruction of red blood cells is equal to the production of red blood cells. Compared to their definitive adult counterparts, yolk sac erythroblasts are more sensitive to erythropoietin, differentiate faster and contain more hemoglobin (Blau et al. 1966). Bone marrow transplant or erythropoietin administration which results in accelerated erythroid expansion leads to the production of erythroid cells that are similar to fetal erythroid cells (RJ et al. 1993). Plasma erythropoietin concentration decreases during neonatal development and correlates positively to hemoglobin concentration in reticulocytes. Newly formed erythrocytes circulate for about 110 days in the bloodstream before being recycled by macrophages. Individual differences in blood complexity named blood-type (or –group) arise due to differences in protein, glycoprotein, carbohydrate and glycolipid expression levels on the surface of RBCs and are inherited; defined by a specific chromosomal location (Table of blood group systems". International Society of Blood Transfusion. October 2008).

What is the optimal cell to be cultured into large RBC quantities ex vivo?

To begin making an optimal *ex vivo* culture of haematopoietic stem cells and committed erythroid cells an optimal base material must first be established (Table 1). There are currently three models under investigation, namely the human HSC which is derived from the umbilical cord blood, or the bone marrow or the peripheral blood of a patient, the human embryonic stem cell and the human induced pluripotent stem cell (hiPSCs), which can be derived from any patient cell that is subsequently de- and reprogrammed to the haematopoietic stem cell type. These methods c.q. techniques for deriving or obtaining haematopoietic progenitors are finally liable to the long-term *in vivo* engraftment and haematopoiesis-sustaining capabilities of the obtained haematopoietic stem cell. The haematopoietic end-product under scrutiny in this work is the enucleated red blood cell for transfusion purposes.

Cell type	Source	Special consent required	Advantages	Disadvantages
Peripheral blood mononuclear cells (PBMCs)	By product of leukoreduction	No	Donor with known phenotype	Limited expansion capacity
	Apheresis collection	Yes	≥3 products from single collection	Donors may become ineligible
	Mobilizing agent before apheresis	Yes (for mobilization and apheresis)	Expansion similar to cord blood with humanized media	Quality control requirement greater than blood
Cord blood	Low volume units unsuitable for transplantation	Yes	Hematopoietic progenitor cell number > PBMC 3–50 products phenotype must be determined	Multiple donations not possible Quality control moderately complex
iPSC	Fibroblast cultures from autologous or allogenic donors of known phenotype	Yes (for skin biopsy, fibroblast culture, and induction of pluripotency)	Unlimited expansion capacity	Quality control highly complex
hESC	Human embryos ≃1200 cell lines available	Yes	Unlimited expansion capacity	Ethical limitations on development and use Quality control highly complex

Table 1: Stem cell sources for the generation of red blood cells ex vivo (Adapted from Grazzini et al).

A complete profile of each potential candidate cell must be established to finally obtain a clinical-grade cell therapy product. Of significant importance are pluripotency marker expression levels, cell surface antigen expression kinetics, frequencies and levels, cell phenotype, karyotypic stability, stable differentiation patterns, homogeneity of the cell populations after multiple divisions, self-renewal capacity, viability and the safety of the end-product regarding adventitious agents and toxicity. In the final product, the red blood cell, phenotype, enucleation and adult haemoglobin content are of crucial importance. These characteristics are measured in the starting material and in the final cell product. Currently multiple stem cell sources are utilized for the *ex vivo* generation of red blood cells (Table 1) but an optimal model has not yet been fully demonstrated. Stem cells derived from cord blood, hESCs and hiPSCs are described below.

Stem cells derived from cord blood

Haematopoietic stem cells derived from the umbilical cord blood, peripheral blood or bone marrow are functionally relevant for studying ontogenic and molecular haematopoietic processes and give a total of one transplant possibility and thus the supply of these cells needs to be constantly secured. Compared to bone marrow derived stem cell transplants, cord blood derived haematopoietic stem cells engraft across the histocompatibility barrier with a lower incidence of graft-versus-host-disease (Kurtzberg et al. 1998) and that is why cord blood transplants have been used for over 24 years to treat malignant and non-malignant disorders in patients. Cord blood transplants are easily obtained, bring no risk to the donor and have a low immunogenicity and are best suited for HLA-mismatch recipient-donor transplantations (Toye et al.). Human erythroid progenitor cell lines have been created from cord blood stem cells and have shown to be able to form reticulocytes and have shown to be able to engraft *in vivo* (Douay et al. 2002, 2011). Also cord blood is a more abundant source of haematopoietic stem cells than adult blood (Tanavde et al). With the recent creation of a cord blood derived cell line capable of adult haemoglobinization as reported by Nakamura et al. and, beyond the scope of this topic, the technical advances in the enucleation of hiPSCs the large scale production of functional adult red blood cells is a realistic foresight as recently shown by Nielsen et al. (2011).

Human embryonic stem cells

Human embryonic stem cells (hESCs) were first clinically tested in 2010 by Colman et al. They have shown their potential for efficient differentiation towards the erythrocyte fate. HESCs are derived from the inner cell mass of the blastocyst and can be maintained indefinitely in culture and can also differentiate into all three germ layers: endodermal, ectodermal and mesodermal, the latter of which is differentiable into red blood cells. The problem with culturing hESCs towards the *in vivo* ontogenical equivalent of red blood cells is the generation of an adult beta-globin containing red blood cell. The definitive red blood is obtained *in vivo* by the erythroid maturation process via the migration of haematopoietic progenitors to the liver in the foetus and the bone marrow in the adult in which embryonic haematopoietic progenitors lose their fetal globin and nucleic acids. *In vitro* conditions must in this way resemble *in vivo* conditions. Also a problem in obtaining hESCs is that the procedure for obtaining these cells is ethically restriced, invasive and that only small quantities can be obtained at one time. These restrictions currently make hESCs a model of study for erythropoietic developmental processes more so than being the optimal starting point for producing large amounts of red cells *ex vivo.* Prior donor profiling for stem cell properties can provide laboratories with specific hESC cultures of known characteristics. In 2010 Colman A. et al. created six clinical grade hESC lines tightly following the available regulations, underlying the whole process from embryo

procurement to line characterization. These lines are karyotypically normal, maintain an undifferentiated state and are pluripotent as they formed embryoid bodies *in vitro* and teratomas in SCID immunodeficient mice in which cells of the endoderm, ectoderm and mesoderm were found. These cells correlate with the surface antigen expression profiles of each respective cell as previously established. These cells were differentiated into the cardomyogenic and pancreatic progenitor cells showing their potential for development into specialized somatic cell types (Colman A. et al. 2010). Also, they have been tested free for human and nonhuman pathogens, thus creating a solid framework and model for the creation of stable donor-specific human embryonic stem cell banks for use in transplantation, drug-screening settings and *ex vivo* haematopoiesis. In the right culturing conditions (serum-free erythroid induction medium), embryoid bodies are able to generate a large number of relatively pure erythroid cells (more than 90%) within 21 days of culture (Papayannopoulou T. et al. 2006). Many additional human embryonic stem cell lines are currently being created in trying to cover as many phenotypes as possible.

Human induced pluripotent stem cells

Currently the most promising haematopoietic stem cell source is by derivation of these cells from induced pluripotent stem cells (Yamanaka et al. 2010) since this technique circumvents ethical, obtainment, banking and preservation difficulties. The deprogramming, culturing and 'reprogramming' of somatic cells is different from in vivo embryonic development and this gives rise to the question whether iPS cells are finally different from ESCs. Research by Bock et al. (2011) showed that iPS cells and ES cells cannot be distinguished based on DNA methylation, or the epigenetic make-up, and gene expression characteristics. The following differentiation into a committed lineage does show differences in efficacy between iPS and ES cells, demonstrating that iPSC clones and ESC clones have overlapping degrees of variation, although the variations among different ESC clones are more completely known. Several iPS cell clones are *de facto* indistinguishable from their ES clone counterparts. Even so, all-iPSC mice do show minor genetic alterations compared to their parental cells (Quinlan et al., 2011), but with the constantly increasing stability of iPS derived stem cell lines (Yamanaka S. 2010), considering the development of non-integrating plasmids and even non-viral methods such as direct transduction of recombinant proteins, these cells seem to hold most promise for being the clinical-grade cell therapy product for producing red blood cells *ex vivo*. HSCs derived from cord blood are most likely a better option than skin-biopsy (Cheng et al. 2011) derived fibroblasts for deriving haematopoietic hiPSCs because they have a more favorable (epi-)genetic expression profile and proliferative capacities than adult $CD34^+$ cells or fibroblasts. Also they circumvent concerns regarding possible UV irradiation of the obtained dermal fibroblasts (Yamanaka S. 2010). Research by Cheng et al. (2011) tried to create functional hiPSC lines for the purpose of creating erythroblasts. In this study, per 2×10^6 mononuclear cells, which are obtained from 1 mL of cord blood,

more than 14 iPSC lines were derived which were pluripotent 'as confirmed by both *in vitro* and *in vivo* differentiation assays.' (Cheng et al. 2011). After expansion in erythroblast proliferation inducing culturing conditions nearly all the cells expressed erythroblast marker CD36 which suggests that these cells are highly differentiable towards the erythroblast fate. Additional evidence favoring the creation of hiPSCs from cord blood $CD34^+$ cells was brought forth by Sugiyama et al. in 2010 whose research compared six iPSC lines derived from mouse embryonic fibroblasts, tail-tip fibroblasts, hepatocytes and gastric endothelial cells for variance in haematopoietic potential. They found that embryonic mesodermal fibroblast cells, specifically the 20D17 (transfected with Oct3/4, Sox2, Klf4, T58A, c-Myc) and 178B5 (transfected with Oct3/4, Sox2, Klf4) (Nakagawa et al.) stem cell lines, cultured into embryoid bodies and subsequently cultured to form HSCs, had more haematopoietic potential than adult-derived iPS cells and that this potential was comparable to the haematopoietic potential of embryonic stem cells based on morphology, gene expression patterns, proliferation, pluripotency and epigenetic status. Douay L. et al recently (June 24, 2012) showed that hiPSCs can reach complete terminal maturation with the complete switch from fetal to adult hemoglobin. From a study in France it is estimated that 15 hiPSC clones could cover 100% of the needs of Caucasian patients with rare blood phenotypes/genotypes (T. Peyrard, L. Bardiaux, C. Krause et al. 2011).

Optimal cell

The optimal cell for culturing red blood cells *ex vivo* is a cell that is obtained most easily, is most cultivable towards the red blood cell fate and is most stable throughout its divisions. For each of these characteristics clinical observations have been made and it seems this way that the most promising cell, mostly because of the (epi-)genetic memory it retains of being a HSC, is a umbilical cord blood (CB) derived $CD34^+$ haematopoietic stem cell that is induced to a pluripotent fate by the least amount of factors possible for the least amount of incorporated noise (Yu et al.), that is karyotypically, phenotypically, morphogenetically, genetically and epigenotypically stable after differentiations from its committed to pluripotent to multipotent state and after maximal divisions *in vitro* and *in vivo*, that is able to wholly form enucleated red blood cells after several cytokine-induced lineage-commitment steps *in vitro* and that is finally a stereotypical cell that is equivalent in its overall efficiency compared to its counterpartal embryonic cell developmental efficiencies. Furthermore, the cell needs to be initially universal and be non-immunogenic. The optimal CB derived $CD34^+$ HSC cell is, leading up to the process of maximal expansion and qualifying for the above standing characteristics:

- Rhesus negative.
- O negative (absence of A- and B-transferase).

- Heterozygous for as many antigens as possible (almost 400 types in humans (Papayannopoulou et al.).
- Kell negative.
- Clean of neoantigens after final differentiation. (By polyethylene glycol (Garraty et al.) red blood cell surface cleaning.)
-

The prototypical candidate cell is from an hiPSC line derived from optimal cord blood $CD34^+$ HSCs preferably reprogrammed by a EBNA1/OriP plasmid containing four or less of the pluripotency inducing factors SOX2, Klf4, Oct3/4 and possibly LIN28 (Cheng et al. 2011). This emanates into an inexhaustive supply of $CD34^+$-like pluripotent stem cells that can be stored indefinitely (Miglaccio G. et al 2009) and can be grown very optimally for the purpose of producing large amounts (Nielsen et al. 2011) of HLA-compatible functional transfusable (Douay et al. 2011) adult red blood cells *ex vivo*.

Red blood cell production

Deriving high amounts of transfusable RBC units requires a red blood cell production process that is semi-analogous to the highly efficient generation of RBCs *in vivo* and is also capable of generating RBC amounts equal to those present in transfusions, namely 2.5×10^{12} cells/mL. Studies on the fetal and neonatal haematopoietic development and the patterns of definitive maturation of erythrocytes in the embryo elucidated the necessary components of the haematopoietic system, such as the fundamental role of EPO that ensures proliferation of progenitor cells and maturation of precursor cells (Papayannopoulou et al. 2009). Maturation of erythroid cells goes through several intermediate stages. The progenitor cell forms primitive colony forming unit erythroids, definitive burst forming unit erytrhoids and erythroid precursor cells. The differentiation into the myeloid (and lymphoid progenitors) and the subsequent differentiation into the committed precursor cells and the final differentiation into lineage committed cells (E. D. Thomas et al. 1999, Mohandas et al) is tightly regulated by several proteins (K. Kaushansky 2006). Leading up to the initial culture, CD34+ HSCs are isolated by density gradient centrifugation. When cultured *ex vivo*, CD34+ HSCs can be grown onto stromal feeder cells (Moore et al. 2006) and be directed to growing into the desired blood cell. For the production of large quantities of red blood cells, on the other hand, the ultimate derivation of red blood cells from these feeder cultures is not optimal (up to 5 RBC units per umbilical cord blood donation) as too much reacting surface area is necessary for the manufacturing of highly enucleated red blood cells in large amounts and in the density required for transfusion (Timmins et al.). New methods are being developed to increase RBC production efficiency. As Nielsen et al. mentioned in 2011, "Given the high capacity of the hematopoietic system in vivo, we believe that the comparatively low cell yields reported so far reflect limitations in the culture process, not an intrinsic biological limitation of the HPC source".

Human induced pluripotent stem cells differentiate with equal efficiency into the various blood cells compared to hESCs as shown by Lapillone and colleagues (2010). This means culturing techniques of driving hESCs towards the haematopoietic fate apply equally to hiPSCs. The most important first step in the creation of red blood cells from hiPSCs is the stable transformation of the cord blood derived $CD34^{+}$ hiPSCs into haematopoietic progenitors via progenitors with hemoangiogenic potential (Nishikawa et al. 1998). Douay et al. (2010) differentiate hiPSCs into human embryoid bodies using 5% human plasma supplemented with stem cell factor (SCF, 100 ng/mL), thrombopoietin (TPO, 100 ng/mL), FLT3 ligand (FL, 100 ng/mL), and recombinant human bone morphogenetic protein 4 (BMP4; 10 ng/mL), recombinant human vascular endothelial growth factor (VEGF-A165; 5 ng/mL), interleukin-3 (IL-3; 5 ng/mL), interleukin-6 (IL-6; 5 ng/mL) (Peprotech) and erythropoietin (Epo; 3 U/mL) (Eprex). (See figure 3). Day 20 hEBs in this system were considered to have the highest erythroid potential by the observation that the erythroid markers CD45, CD34 and CD71 were then most abundantly expressed. The embryoid bodies were differentiated into red blood cells in the presence of 10% human plasma, SCF, IL-3 and EPO. After 8 days a population of 99% erythroblasts was observed. Per 2 hiPSCs 3 embryoid bodies formed, from which $4.4x10^{8}$ cells were generated of terminal erythroid phenotype ($CD36^{neg}GlyA^{high}$). Carbon monoxide rebinding kinetics was nearly identical to fetal blood levels.

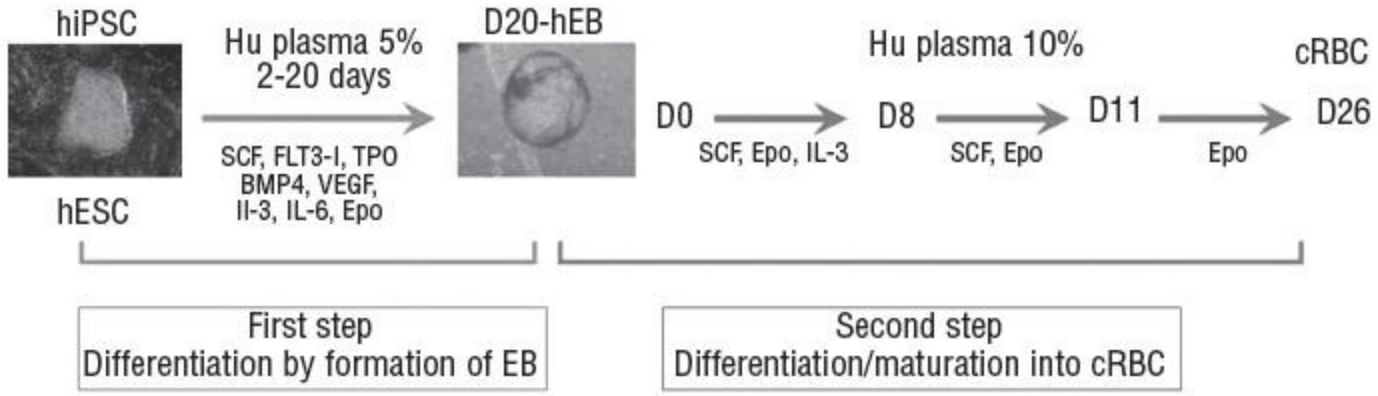

Figure 3: Induction of hiPSC towards human embryoid bodies and subsequent induction towards red blood cells. (Douay L. et al 2010).

VEGF is necessary for the differentiation and subsequent expansion of the erythroid precursor cells (EPCs). More recently however, Douay et al. (2011) showed that when the haematopoietic microenvironment was replicated using human serum VEGF and also IGF-II were not necessary for erythroid enucleation. This implies that the microenvironment gives enucleation cues. During the differentiation into EPCs constant epigenetic remodeling takes place that ensures activation of erythroid-specific genes (Wozniak et al.) and also this phase allows for the growth of the EPC population. Research by Nielsen et al. into the large-scale production of enucleated red blood cells has come up with a minimal

approach of adding the cytokines SCF, HC, EPO and IL-3 to the culture to drive proliferation and erythroid differentiation of cord blood derived $CD34^+$ HSCs. Removal of SCF, HC and EPO from the cell culture induced terminal differentiation and enucleation of the erythroid cells. Terminal maturation is characterized by loss of CD36 receptor expression and an increase in glycophorin-A expression (Miglaccio AR et al. 2009), also see Figure 4. Earlier methods by Miharada et al. used mifepristone and plasmanate to efficiently enucleate reticulocytes. Erythroid production efficiency can be increased by manipulating the culturing environment such as in agitated bioreactors, mimicked three-dimensional bone structures and in scaffolds that resemble the *in vivo* environment or manipulation of the transcriptional environment such as the ectopic expression of engineered Nup98-HoxA10 fusion protein (Hong S. et al.) and the down-regulation of miRNA-125 and miRNA-126 (Witte O. N. et al.). The addition of isogarcinol to the $CD34^+$ cell culture, a histone acetyltransferase inhibitor, increased the number of cord blood cells by a factor of 7.4 (Nishino et al.). Increasing the amount of generated red blood cells is attained by optimizing the differentiation towards the subsequent phase of erythropoiesis. Van den Akker et al. and Varricchio et al. add hydrocortisone and dexamethasone in the early stages of culture to maximize the number of pro-erythroblasts generated by the HSCs. A low oxygen concentration in early culture has also shown to be important for early erythroid development (Rogers et al.). Zimmerman et al. show that exposure of the cord blood culture to 0.2% hypotonic saline for five minutes lyses non-reticulocytes that results in an average 3.6-fold increase in reticulocyte numbers. For the purpose of reaching maximal red blood cell concentration Nielsen et al. use a feeder-free dilution strategy. Using this strategy they can currently produce 560 RBC units per umbilical cord blood donation by using an agitated bioreactor system based upon earlier work using stirred suspension bioreactors as by Zandstra et al. in 1994. Their feeder-free, macrophage-free protocol relies on diluting (10.000 cells/mL) the obtained EPCs in a minimal volume of 200mL to maximize the final density of enucleated red blood cell product. Their fully-defined animal component-free medium is comprised of IMDM (Invitrogen) supplemented with 100 ng/mL SCF (Amgen), 3U/mL EPO (Janssen-Cilag), 5 ng/mL IL-3 (ProSpec-Tany), and 10^{-3} M HC (Sigma-Aldrich), 1% fatty acid free bovine serum albumin (BSA; Sigma-Aldrich), 120 mg/mL holo-transferrin (Sigma-Aldrich), 10 mg/mL insulin (Sigma-Aldrich), 900 ng/mL ferrous sulfate (Sigma-Aldrich), and 90 ng/mL ferric nitrate (Sigma-Aldrich). After 21 days of low-density cell culture in the defined medium under static conditions the cell population showed a 1.73×10^6-fold increase in cell number. The cells exhibited a pure erythroid phenotype of mixed maturity. Subsequent removal of SCF and HC induced nuclear extrusion (>90%) and so induced the development of red blood cells. The EPCs derived from culture were expanded in 1L CultiBag® bioreactors and after 33 days the cell population was still expanding, showing a 2.25×10^8-fold increase, or 560 units of RBC per UCB donation (see figure 5), contrary to previous systems as by Giarratana et al. which use feeder-systems in which the cell populations stopped expanding significantly after 21 days of culturing. After maturation of the expanded erythrocytes, the CultiBag® cell culture was purified for enucleated red blood cells by using anti CD235a-APC (or glycophorin-A) and anti CD71-FITC (or transferrin receptor) antibody expressional characterization. The filtrate contained cell volumes equal to that of young donor reticulocytes (Gifford et al.) and was characterized by an average hemoglobin content of 30.3- 31.3 pg/ cell compared to 27–33 pg/cell for donated red blood cells.

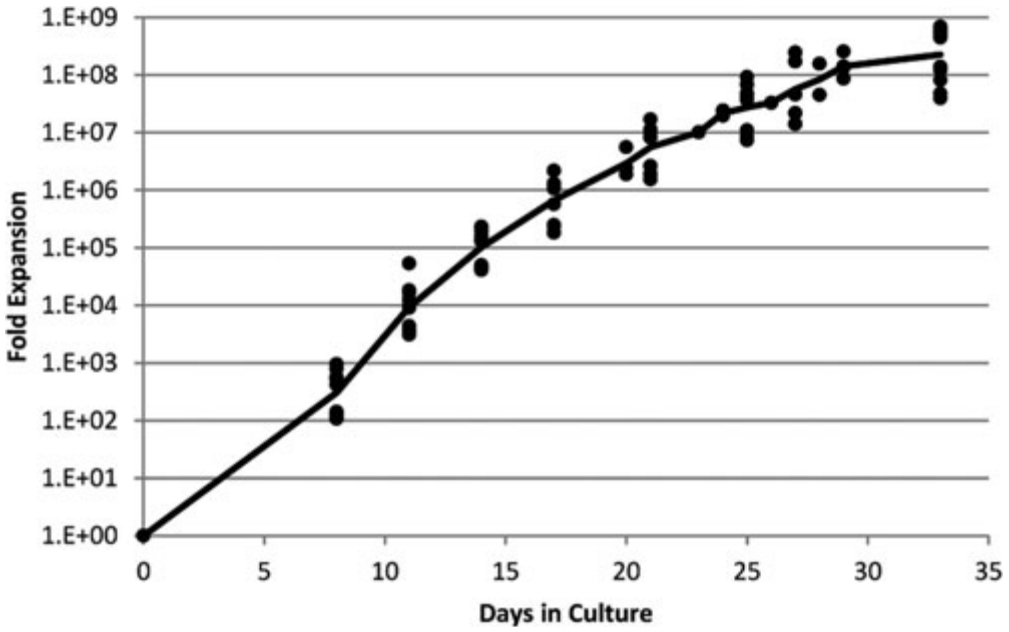

Figure 5: Ultra high yield expansion of EPCs as by Nielsen et al.

When cultured longer, the cell populations started showing signs of poor, inconsistent enucleation and lesser reddening of the red blood cells, henceforth continuing the expansion process is not clinically significant. Douay et al. in 2011 reported cells that are able to mature *in vivo*, to persist for several weeks *in vivo* in immunodeficient mice and in humans and were already shown in 2002 by this team to be able to amplify in *vivo* to about a hundred-fold, but only produced 3 transfusable units per cord blood donation at this time. Also this red blood cell population already showed decay after 17 days to 70% at day 21. In 2011 Douay et al. showed an increase in the cell culture stability to 40% decay by days 24-28 using a three-phase culturing system comprised of (1) HSC proliferation, (2) proliferation of EPCs and (3) terminal erythroid differentiation (Table 2). The enucleation rate in this protocol was 79-83%. This leads to believe that an optimal culturing strategy is a combination of the stable *in vivo* engraftable red blood cell culturing protocol by Douay et al. (2011) and the maximal expansion protocol by Nielsen et al. (2011) (Table 2).

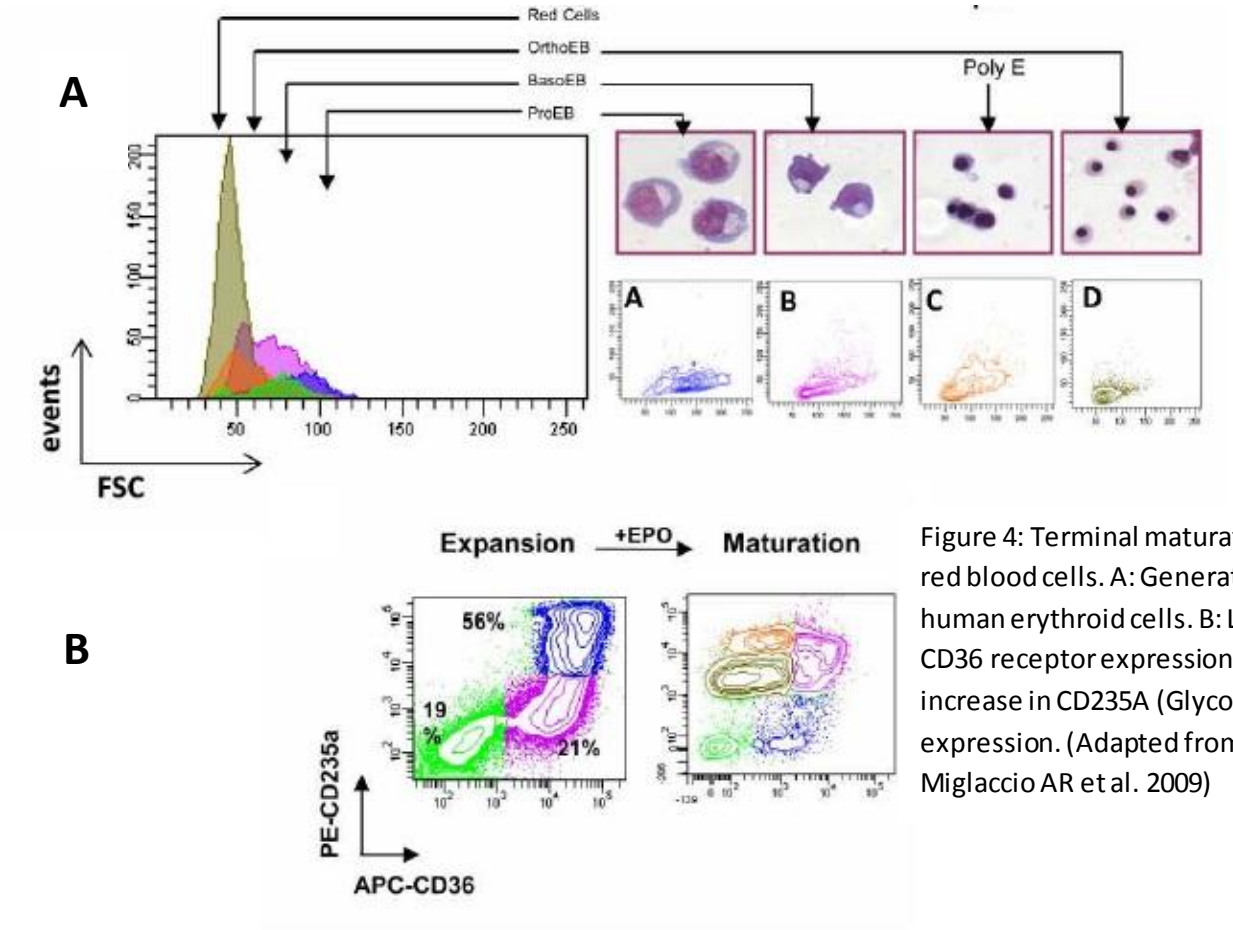

Figure 4: Terminal maturation of red blood cells. A: Generation of human erythroid cells. B: Loss of CD36 receptor expression and increase in CD235A (Glycophorin-A) expression. (Adapted from Miglaccio AR et al. 2009)

Increased purity of the red blood cell volume can be reached by filtering the population through a leukocyte filter, a technique also termed leukapheresis, followed by irradiation with 3000 cGy, a radiation strength that is functionally tolerated by erythrocytes (Papayannopoulou et al.). Obtained is a highly pure high-density enucleated red blood cell product of the lowest immunogenicity cultured in a fully defined medium.

Table 2			
	Douay et al. 2002	**Douay et al. 2011**	**Nielsen et al. 2011**
Starting cell amount	10.000 cells/mL	10.000 cells/mL	10.000 cells/mL
CD34+ cell fraction for expansion	1.000.000 cells	1.000.000 cells	5.000.000 cells
Final cell fold amplification towards RBCs	600.000-1.000.000-fold	37.000-fold	225.000.000-fold
Factors used in HSC proliferation	Flt3-L, SCF, Tpo	SCF, EPO, IL-3, M-HC	SCF, EPO, IL-3, M-HC
Factors used in EPC proliferation amplification	SCF, EPO, IGF-1	SCF, EPO	SCF, EPO, IL-3, M-HC
Factors used for terminal erythroid differentiation	EPO, IGF-1	EPO	EPO, IL-3
Enucleation efficiency	*99% (in vivo enucleation)*	81%	>90%
Number of derived RBC units per UCB donation	1 to 3	-	560
in vivo engraftment in NOD/SCID immunodeficient mice	yes	yes	-
in vivo engraftment in homo sapiens	-	yes	-

Table 2: Comparison between red blood cell culturing protocols as by Nielsen et al. (2011) and Douay et al. (2002, 2011). 1 UBC donation equals 5 x 10^6 CD34+ cells. 1 RBC unit equals 2.5 x 10^{12} red blood cells.

Transfusion

Now that there is a way to create a stable supply of red blood cells from cord blood derived $CD34^+$-like human induced pluripotent stem cells that are fully characterized the question remains whether the red blood cell product is suitable for transfusion. Significance for producing red blood cells *ex vivo* is given by the evidence that *ex vivo* generated red blood cells express the adhesion receptors Chemokine receptor-4, alpha-4 integrin and P-selectin more so than present in donor derived blood, which are necessary to propagate cell interactions once injected *in vivo* (Migliaccio et al.). Douay et al. already showed in 2002 that *ex vivo* generated erythroid cells generated from cord blood can differentiate into red blood cells *in vivo*. The team transplanted 30×10^6 late erythroid progenitors pre-labeled with CFSE into sub-lethally irradiated NOD/SCID immunodeficient mice. The erythroid cells were detectable in all organs and were found to have all become red blood cells after 3 days *in vivo* and expressed mainly adult haemoglobin, showing that there had been a transition *in vivo* from fetal to adult haemoglobin c.q. terminal maturation within the vascular network (Figure 6). More recently, in 2011, Douay et al. demonstrated that using good manufacturing practice functional reticulocytes could be transplanted into humans. These cells matured terminally *in vivo* as shown by the disappearance of the CD71 receptor, loss of nucleic acids and the formation of the reticulocytes into a biconclave disk. The definitive red blood cells persisted for several weeks inside the body. During the first 5 days post-transfusion 94%-100% of the cells were still alive. After 26 days 41% to 63% of the injected red blood cells had remained. The *in vivo* maturated red blood cells had similar half-life values (28 days) as observed by decay in fluorescence intensity (Figure 6), enzyme content (normal contents of glucose-6-P dehydrogenase and pyruvate kinase), deformability, antigen expression and hemoglobin content (88% adult hemoglobin) compared to native donor RBCs. After several weeks of cryopreservation at 4 degrees Celsius the cultured red blood cells showed no decay in function. As they put it: "Their survival in vivo testifies globally to their quality and functionality". Transplantation of stem cell products using good manufacturing practice (See Figure 7) requires that the product is safe for use in patients and contains no adventitious agents. Immune response against infused red blood cells is overcome partly by the use of iPSCs in a patient-specific setting, which naturally develop into autologous cells. Developing universal red blood cell banks from a single optimal donor does give rise to possible immune system rejections due to the expression of neoantigens on the surface of these blood cells that are inherent to the donor.

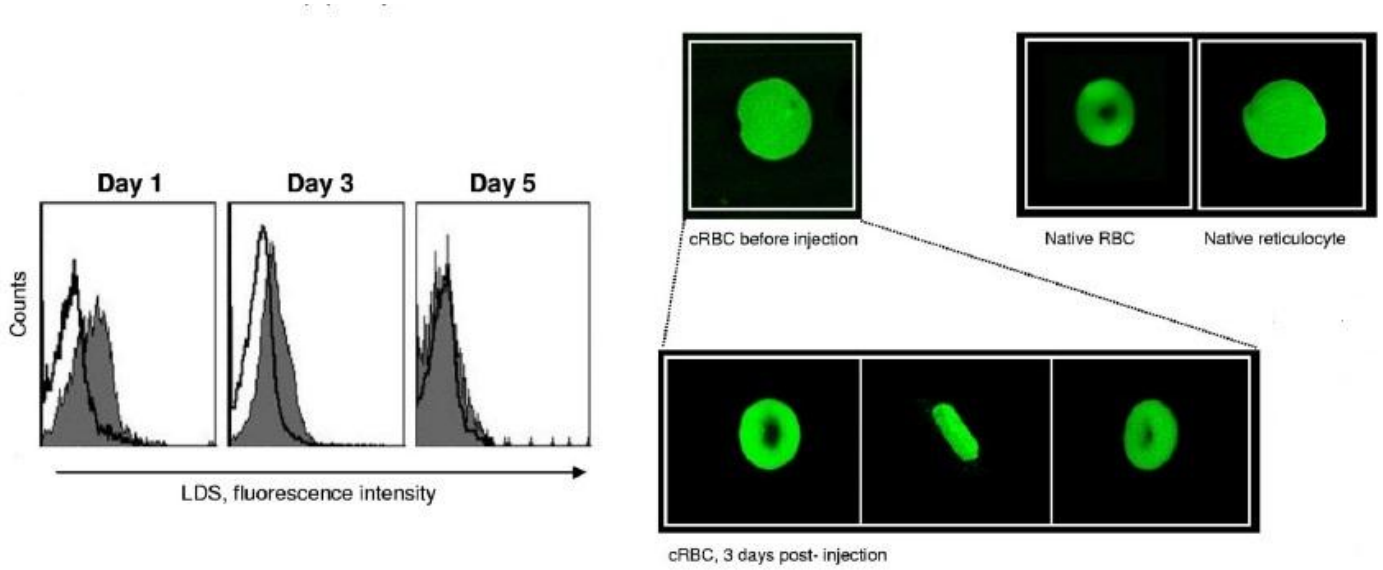

Figure 6: A: Lifespan of transfused cultured red blood cells as measured by fluorescence intensity. B: In vivo terminal maturation of reticulocytes into red blood cells (Douay L. et al. 2011).

One approach is to clear the red blood cells of their immunogenicity by engineering the final cells using polyethylene glycol which removes neoantigens on the surface of the red blood cells (Garraty et al., Rao et al.). The P-Capt filter that was developed by Judd et al. filters red blood cells for prions such as in Creutzfeld Jacob Disease transmission. At the same time it keeps red blood cells viable, free of neoantigen expression or immunogenic responses or safety issues after the infusion of a complete unit (Judd et al.). Summarizing, the possibility of the large-scale manufacturing of non-problematic storable and transfusable (to-be) adult red blood cells from a single UCB donation makes a central EPC/RBC production system a viable option.

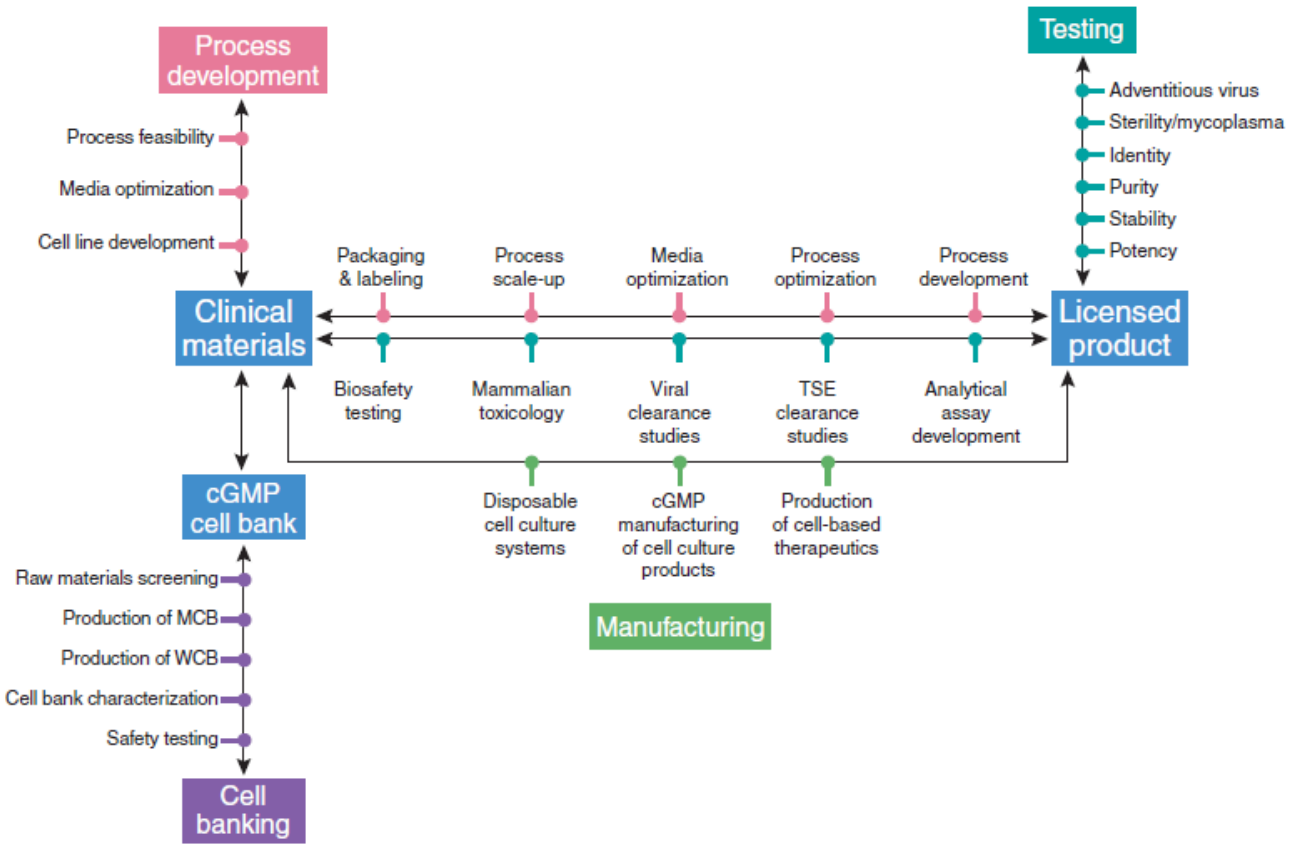

Figure 7: Development of a stem cell product (adapted from Rao et al. 2009)

Discussion

The development of new cell therapies and cellular programming processes has paved the way for a future in which red blood cell donation and RBC unit shortage is of the past. Especially hiPSCs (Yamanaka et al. 2010) show great promise for the creation of unlimited amounts of stable $CD34^+$ stem cells that can be banked and be used for drug-screening purposes. From a study in France it is estimated that 15 hiPSC clones could cover all blood phenotypes of white ancestry. Research into the process of erythropoiesis has elucidated definite phases in the development of haematopoietic stem cells towards the red blood cell fate. Proliferation and differentiation of stem cells using optimized animal-component-free media under fully characterized conditions has paved the way for the manufacturing of stable red blood cells capable of circulating in the human blood stream. $CD34^+$ umbilical cord blood derived stem cells can be cultured into large amounts (Nielsen et al. 2011) of red blood cell volumes that are of the proper density and that are similar to native red blood cells using current good manufacturing practice, but only until recently can cultured

red blood cells be transfused into patients as shown by Douay et al in 2011. *In vivo* transfused red blood cells terminally mature and acquire all characteristics of native definitive red blood cells. As Douay L. and colleagues conclude in their 'Proof of principle for transfusion of in vitro-generated red blood cells' (2011) article: "The ultimate and decisive challenge is […] to design a cost-effective automated industrial cell culture system capable of maintaining a self-renewing progenitor population, which provides an environment for efficient erythroid differentiation and allows sorting/purification and packaging of the end-product RBCs in a manner directly suitable for transfusion." The scaling up of red blood cell culturing has culminated into the development of an agitated bioreactor system that can produce up to 560 RBC units from a single umbilical cord blood donation (Nielsen et al. 2011) using a feeder layer-free protocol of defined media (Table 2). Optimizing the *in vivo* survival capability of the cultured red blood cells is now a matter of great importance.

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