Cell Therapy for Blood Substitutes

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Introduction

For decades, supplies of transfusable blood components have failed to keep up with increasing demand. This continual shortfall has prompted efforts to develop safe and effective blood substitutes which can be produced from non-immunoreactive sources and in limitless quantities. This chapter summarizes recent efforts to develop alternative sources for red blood cells (RBC) and platelets, two of the blood’s most critical life-savings elements.

RBCs, the oxygen-carrying component of the blood, are transfused in over half of all anemic patients admitted to intensive care units in the United States (Corwin et al., 1995; Littenberg et al., 1995; Corwin et al., 2004) and it is estimated that nearly 5 million patients receive approximately 14 million units of RBCs per year in the United States alone (Whitaker and Henry, 2005). Limitations in the supply of RBCs can have potentially life-threatening consequences for patients, specifically for those who have rare or unusual blood types with massive blood loss due to trauma or other emergency situations. Unfortunately, the supply of transfusable RBCs, especially “universal” donor type (O)Rh-negative, is often insufficient, particularly in the battlefield environment and/or major natural disasters due to the lack of blood type information and the limited time required for life-saving transfusion. Moreover, the low prevalence of (O)Rh-negative blood type in the general population (<8% in Western countries and <0.3% in Asia) further intensifies the consequences of blood shortages for emergency situations where blood typing may not be possible.

Platelets, anucleate discoid-shaped cell fragments released from megakaryocytes (MK) are essential to hemostasis, the biological process by which bleeding stops. Platelets adhere to damaged blood vessels and trigger a series of biochemical changes that stimulate clot formation and vascular repair. In cases of thrombocytopenia (platelet counts are less than $150 \times 10^{3}$/ul), the increased risk of bleeding can have life-threatening consequences (Patel et al., 2005). In the United States, approximately 1.5 million platelet
transfusions are performed annually to protect patients, including those treated with chemotherapy or stem cell transplantations, from the risk of thrombocytopenia and its related dangers (Kaushansky, 2008). Unfortunately, refractoriness occurs in approximately one out of every three patients who require repeated platelet transfusions (Slichter et al., 2005; Hod and Schwartz, 2008) and while both immunological and non-immunological complications may be to blame, HLA alloimmunization is the primary cause of refractoriness (Hod and Schwartz, 2008). Over the past few decades, a steady increase in demand for platelets in combination with their limited shelf-life has presented a constant challenge for blood centers and donor dependent programs. As with RBCs, there is a vast and continuous need for functional, transfusible platelets, especially in times of emergency. Pluripotent stem cells may be able to serve as an alternative source for producing transfusible RBCs and platelets. Here, we will review recent progress in developing these life-saving blood substitutes, including some of our own efforts in unlocking the potential use of human embryonic stem cells and induced pluripotent stem cells in these endeavors.
Red Blood Cells

Erythropoiesis:

Erythropoiesis is the highly regulated, multistep process by which the body generates mature red blood cells, or erythrocytes. During mammalian development, erythropoiesis consists of two major waves: (1) primitive erythropoiesis, which is initiated in the yolk sac with the generation of large nucleated erythroblasts, and (2) definitive erythropoiesis, which arises from the fetal liver with the development of smaller enucleated erythrocytes (Ney, 2006). Definitive erythropoiesis in fetal liver features the production of enucleated RBCs that quickly become dominant in embryonic circulation. The switch of hemoglobin to fetal types ($\alpha_2\gamma_2$) also occurs at the initiation of definitive erythropoiesis (Kovach et al., 1967; Brotherton et al., 1979; Stamatoyannopoulos, 2005). However, recent reports show that yolk sac-derived primitive erythroblasts can also enucleate in the circulation of a mouse embryo and persist throughout gestation (Kingsley et al., 2004; Fraser et al., 2007).

In adults, all blood cell types including lymphocytes, myeloid cells and RBCs are derived from hematopoietic stem cells (HSCs) residing in the bone marrow. The initial differentiation of a multipotential HSC into a common myeloid progenitor (CMP) determines its capacity to further differentiate into granulocytes, erythrocytes, megakaryocytes, and macrophages but not lymphoid cells. As the CMP continues to differentiate, it undergoes significant expansion and will eventually commit to one particular lineage. Erythroid unilineage commitment leads to the appearance of the pronormoblast (also called the proerythroblast or rubriblast). The pronormoblast will then pass through early, intermediate, and late normoblast (erythroblast) stages, prior to expelling its nucleus and becoming a reticulocyte. Upon exiting the bone marrow, reticulocytes enter the blood circulation and become fully mature RBCs. The various stages of erythropoiesis can be distinguished by characteristic morphological features in the cell cytoplasm and nucleus, which become evident after Wright-Giemsa staining. Additionally, using an in vitro colony-forming assay, CMP progenitors can be identified by their ability to form a characteristic colony forming unit, called the CFU-GEMM while early erythroid progenitors give rise to burst-forming units-erythroid (BFU-E), and late erythroid progenitors give rise to colony-forming units-erythroid (CFU-E) in this assay (Ney, 2006).

“Universal” blood generated by modifying RBC surface antigens:

While Karl Lansteiner’s nobel prize-winning discovery of ABO blood groups occurred over a century ago, there are now 30 known human blood group systems and the
complicated issues surrounding blood type incompatibility continue to frustrate clinicians and scientists. To circumvent these issues, researchers have been trying to develop universal blood for decades, primarily through chemical modifications on the surface of RBCs. Among these efforts, Goldstein and co-workers demonstrated that group B erythrocytes can be enzymatically converted to group O, and that the converted cells survived normally in A, B and O individuals (Goldstein et al., 1982). Since this groundbreaking discovery, there have been coordinated efforts to identify both clinically- and economically viable alternative enzymes. Recently, new classes of bacterial exoglycosidases have been discovered that can enzymatically perform group O conversions with faster kinetics (Olsson and Clausen, 2008; Liu et al., 2007), yet the likelihood of this technology playing a major role in blood transfusion practice is unclear. Currently, there are no reports showing the enzymatic conversion of other important blood typing groups such as the Rh factor. With the momentum of the rapidly advancing stem cell field in recent years, the concept of (O)Rh- negative RBCs derived from pluripotent stem cells clearly offers an attractive option for the future of blood transfusions.

**RBCs generated from adult stem cells in vitro:**

Erythrocytes have been derived from a variety of primary stem cell sources including umbilical cord blood (CB), peripheral blood (PB) and bone marrow (BM). CD34+ cells from CB, PB and BM have been isolated and differentiated into erythrocytes with 95% purity after a little over a week of culture using EPO, stem cell factor (SCF), and interleukin-3 (IL-3) (Leberbauer et al., 2005; Giarratana et al., 2005; Miharada et al., 2006). Co-culturing with a mouse MS-5 stromal cell line or human mesenchymal stem cells facilitates enucleation, the hallmark of mature RBCs. The stem cell-derived erythrocytes have similar properties to normal RBCs, including membrane deformation capacity, intrinsic enzymatic activity, and balanced adult/fetal forms of hemoglobin that can bind and release oxygen. Additionally, these erythrocytes have been found to survive in vivo in NOD/SCID mice, being detectable 3 days after transplantation. Stem cells from PB and BM have limited expansion capacity (29,000 and 16,500-fold, respectively) compared to those from CB (140,000-fold). Yet, cells from adult PB and BM are often easier to obtain and display mature forms of hemoglobin, whereas CB sources are more difficult to obtain and cells derived from them only express fetal globins. Despite their potential utility however, these primary cells still represent donor-limited sources of blood substitutes.

**RBCs generated from human embryonic stem cells:**

Human embryonic stem cells (hESC) represent an alternative stem cell source for generating blood components, one whose capacity for expansion far exceeds that of BM,
PB, or even CB. Hematopoietic precursors as well as more mature progeny representing erythroid, granulocyte, macrophage, megakaryocytic and lymphoid lineages have all been identified in differentiating hESC culture systems (Kaufman et al., 2001; Lu et al., 2004; Vodyanik et al., 2005; Wang et al., 2005; Chang et al., 2006; Qiu et al., 2005; Zhan et al., 2004). Therefore, many groups have focused their efforts on trying to steer the in vitro differentiation of entire hESC cultures into specific blood cell types, such as RBCs or megakaryocytes/platelets (Lu et al., 2008a; Gaur et al., 2006; Takayama et al., 2008).

The controlled differentiation of hESCs into erythrocytes has primarily been achieved by either embryoid body (EB) formation or by co-culturing with stromal cells followed by isolation of CD34+ cells and further expansion/differentiation. While somewhat different in approach, both systems have encountered the same obstacles in generating fully mature adult RBCs. For example, Chang et al. generated erythroid cells from hESCs by isolating and expanding non-adherent cells of day-14 EBs for an additional 15 to 56 days (Chang et al., 2006). The resulting cells coexpressed high levels of embryonic ε- and fetal γ-globins but little or no adult β-globin. In addition, the cells had not enucleated. Using a stroma co-culture method, erythroid cells could be generated by culturing hESCs with FHB-hTERT human fetal liver stromal cells for 14 to 35 days, isolating CD34+ cells and further differentiating them in a 4 step culture system. In steps 1 and 2, cocktails of cytokines were used to promote the proliferation and maturation of erythroid precursors. In steps 3 and 4, erythroid cells were transferred onto mouse BM stromal cells (MS5) to facilitate terminal maturation (Olivier et al., 2006). While these erythrocytes were generated on a relatively large scale (0.5 to 5 x 10^7 cells), the resulting cells had similar problems to those generated by the EB method; they mainly expressed embryonic ε and fetal γ globin isoforms, with only a trace amount of adult β-globin being detected. Despite these reports, other studies suggests that specific types of stroma can, in fact, facilitate the expression of adult β-globin in developing erythrocytes. Using immunostaining with globin chain specific monoclonal antibodies, Ma et al showed that almost 100% of hESC-derived erythrocytes expressed the adult β-globin chain after co-culture with murine fetal liver derived stromal cells (mFLC) (Ma et al., 2008). Yet, the majority of the cells still had not enucleated, a problem that does not seem to have an easily explainable mechanism or simple solution. For example, a study published in 2005 showed that co-culturing CD34+ CB, PB, and BM cells on MS5 stroma produced RBCs with up to 100% enucleation (Giarratana et al., 2005) while a year later, Miharada et al reported that up to 77% of cord-blood derived erythrocytes could achieve enucleation without the use of any stromal cells (Miharada et al., 2006).

The mechanism(s) by which stromal cells may facilitate erythrocyte enucleation are largely unknown and therefore, it remains to be determined whether or not stroma co-culture will be absolutely required for enucleation of hESC-derived erythrocytes. Stroma may secrete important soluble factors, provide critical cell-cell contact, and/or engulf of
nuclei and other organelles. Some studies suggest that the process of enucleation involves a critical asymmetric cell division (Chasis et al., 1989) and that Rac GTPases and their effector, mDia2 help extrude the pycnotic nucleus during this process (Ji et al., 2008). Other studies show that, in vivo, contact with macrophages is important and that the erythroblast-macrophage-protein, Emp plays a critical role in enucleation (Hanspal and Hanspal, 1994; Soni et al., 2006; Hanspal et al., 1998). Clearly, further studies will be required in order to determine how to improve the efficiency of both enucleation and globin switching in vitro. While co-culture with stroma may help circumvent problems with the processes, production of clinical grade RBC substitutes will demand stroma-free culture conditions.

Not only must in vitro generated RBC substitutes be fully matured and enucleated to use in the clinic, but they must also be capable of large scale production. Related to this issue, we recently developed a strategy that efficiently and reproducibly generates functional hemangioblasts (the common precursor cell to all hematopoietic and endothelial cell lineages) using a serum-free culture system and have been able to do so with high purity (>95%) and in a relatively large scale (Lu et al., 2007; Lu et al., 2008b). One of the characteristics of the primitive hemangioblast cell is its highly efficient generation of large BFU-E (Figure 1-i & 1-ii), CFU-GEMM (Figure 1-iii) and CFU-E (Figure 1-iv) colonies when cultured in methylcellulose-based medium. This prompted us to investigate whether or not hemangioblasts could be used as an intermediate cell source to generate large, clinically relevant quantities of blood components, such as erythrocytes and platelets. The overall strategy, as depicted in Figure 2, employs a basic three step approach to proceed from hESCs to the final blood cell products.

Using the hemangioblast system, we have generated functional RBCs (blood types A, B, O, and both RhD+ and RhD-) on a large scale from multiple hESC lines (Lu et al., 2008a). Three critical elements allowed us to do this; (1) the efficient generation of hemangioblasts without disruption of their colony forming environment; (2) expansion of hemangioblasts to erythroblasts in a high cell density (see Figure 3i and 3ii); and (3) culture in semi-solid methylcellulose-based media to provide optimal conditions for maximum expansion and high erythroid purity (Figure 3i). We generated approximately $10^{10}$ to $10^{11}$ erythroid cells per six-well plate of hESCs (Lu et al., 2008a), which is over a thousand-fold more efficient than previously reported methods (Olivier et al., 2006). Oxygen equilibrium curves of erythroid cells from day 19-21 of differentiation were comparable to normal transfusible RBCs and responded to changes in pH and 2,3-diphosphoglycerate. During the course of our studies, we found that extended in vitro culture facilitated further maturation of these erythroid cells, inducing a progressive decrease in size, increased expression of the erythrocyte cell surface marker, glycophorin A (CD235a), as well as chromatin and nuclear condensation. When the extended culture was performed on OP9 stromal cells, it resulted in the extrusion of the pycnotic nucleus.
in up to 65% of cells and the generation of enucleated erythrocytes with a diameter of approximately 6.8 µm (Figure 4). At this stage, the erythrocyte population is nearly 100% positive for glycophorin A (Figure 3iii), has a very high content of hemoglobin (Figure 3iv), and expresses ABO antigen (Figure 3v). Although the cells were found to express fetal and embryonic globin chains, globin chain specific-PCR and immunofluorescent analyses showed that after extended culture, expression of adult β-globin increased from 0% to 15% (Figure 3vi). Overall, these results show that it is feasible to differentiate and mature hESCs into functional oxygen-carrying erythrocytes on a large scale. The identification of a hESC line with a O(-) genotype would permit the production of ABO and RhD compatible (and pathogen-free) “universal donor” RBCs. While considerable effort is still needed to bring hESC-derived RBCs to clinical trials, these efforts certainly provide a promising lead.

Can RBCs be generated from human induced pluripotent stem cells?

The successful reprogramming of somatic cells into a pluripotent state has been achieved by ectopic expression of various combinations of transcription factors such as Oct4, Sox2, Klf4, c-Myc, LIN28 and Nanog cells (Yu et al., 2007; Takahashi et al., 2007). The derivation of these induced pluripotent stem cells (iPSCs) is less controversial than that of hESCs and thus, they open up an exciting new route to obtain pluripotent stem cells. Moreover, the fact that iPSCs can be produced in a patient-specific manner will eliminate the issue of immuno-rejection in cell, tissue, or organ replacement therapies in the future. We and others have demonstrated that human iPSCs can successfully differentiate into erythrocytes which possess classic morphology, express glycophorin A, and have abundant hemoglobin content (Choi et al., 2009; Ye et al., 2009; Lengerke et al., 2009; Feng et al., 2010). However, our recent study has revealed some intrinsic molecular and cellular abnormalities in the iPSC derivatives such as increased apoptosis, limited CFU capability and limited expansion (Feng et al., 2010). The exact cause(s) of these abnormalities is unclear at this time but may be due to alterations caused by the modified genome of virally reprogrammed cells. More research will be needed to determine whether iPSCs generated with viral-free methods have the same abnormalities as the virally reprogrammed ones or if they offer a better alternative for the generation of patient-specific blood replacement cells.

Where do we go from here?

The manufacture of safe and effective red blood cell substitutes will help alleviate many of the risks, complications, and hardships associated with donor-dependent RBC sources. As summarized here, significant progress has been made towards this end by manipulating the differentiation potential of hESCs and iPSCs and driving them towards erythrocyte development. In vitro differentiation systems that can be scaled up for mass
production of RBC substitutes have already been developed and the hemangioblast methodology described in this chapter represents one such possibility. Despite many exciting advances with in vitro culture systems, problems associated with the final stages of erythrocyte maturation, namely enucleation and globin switching will still need to be fully resolved before hESC/iPSC-derived RBCs can be produced in a stroma-free manner, scaled up for mass production, and brought to the clinic.
Megakaryocytes and Platelets

Megakaryopoiesis

Due to their role in clot formation and blood vessel repair, platelets are essential for cessation of bleeding and maintaining an abundant supply of them is vitally important. Megakaryocytes, the large multinucleate precursors to platelets provide a constant, renewable source of platelets to the blood system and are themselves produced through a process called megakaryopoiesis. As previously mentioned, hematopoietic stem cells differentiate into CMPs, which then undergo significant proliferation and expansion as they further mature and differentiate. Once exclusively committed to the megakaryocyte lineage, however, they only retain limited expansion capacity. On a cellular level, megakaryocyte maturation involves many changes such as an increase in expression of the cell surface markers, GPIIb/IIIa (also known as CD41 or αIIb/βIII integrin receptor) and GPIb/GPIX/GPV receptors and an increase in cytoplasmic and nuclear mass, with cells expanding to 50-100 microns in diameter. The increase in nuclear mass encompasses several rounds of endomitosis, i.e. chromosome replication without cytokinesis (Lordier et al., 2008), which results nuclear polyploidization and cells with up to 128N (Tomer, 2004).

In vivo, the drastic increase in cytoplasmic mass that occurs during maturation facilitates production of several thousand platelets per mature megakaryocyte (Long, 1998) as it allows the accumulation of several components crucial for platelet biogenesis, such as α granules, dense bodies, and platelet associated proteins (eg., vWF, PF4) (Schmitt et al., 2001; Deutsch and Tomer, 2006). In addition, the intracellular membrane system (demarcation membrane system or DMS) undergoes various structural and functional changes that are necessary for platelet formation. Recently, it has been reported that SDF/CXCR4 signaling encourages maturing megakaryocytes to migrate close to blood vessels (Lane et al., 2000; Majka et al., 2000; Kowalska et al., 1999; Avecilla et al., 2004) and this potentially helps explain why morphologically distinct mature megakaryocytes are often observed in close proximity to the bone marrow sinusoidal vascular cavity surrounded by endothelial cells.

On a molecular level, thrombopoietin (TPO) is the primary physiological regulator of megakaryocyte and platelet generation (Kaushansky, 2008), however numerous other cytokines, growth factors, and small molecules have been found to be important as well. Cytokines including IL3, IL6, IL9, IL11, BMP4, FL, SCF have been reported to synergistically stimulate the proliferation of megakaryocyte progenitors (Gordon and Hoffman, 1992; Deutsch and Tomer, 2006) while TGF-β1 and PF4 have been found to inhibit their development and maturation (Eslin et al., 2004). Downstream
of cytokine signaling pathways, various transcription factors help orchestrate the progressive lineage commitment and maturation of developing megakaryocytes. For example, the GATA1/FOG1 complex synergistically controls the expression of megakaryocyte-specific genes including integrin αIIb (CD41a) (Wang et al., 2002; Gaines et al., 2000). Targeted disruption of either GATA1 or FOG1 can lead to severe anemia and embryonic lethality (Fujiwara et al., 1996) whereas disruption of GATA1 in a megakaryocyte lineage-restricted manner results in significant thrombocytopenia and differentiation defects in megakaryocytes (Shivdasani et al., 1997). Evidence from in vitro studies and mouse genetic models has identified other transcription factors such as GATA2, Fli1, NFE2 and RUNX1 as being critical regulators of megakaryopoiesis as well (reviewed by Pang et al., 2005).

**Biogenesis of Platelets**

While not mutually exclusive, two models have been proposed to describe the assembly and release of platelets from megakaryocytes into the blood stream, a process known as thrombopoiesis (reviewed by Kosaki, 2005 and Patel et al., 2005). In the global fragmentation model, cellular processes termed proplatelets undergo mutual detachment to produce small functional platelets within the megakaryocyte cytoplasm, which then explodes and releases the cell’s entire content of platelets simultaneously. This theory, conceived in the 1970s, is supported by electron microscopy of mature megakaryocytes, as well as in vivo and ex vivo observations of platelet release. More recent experimental evidence lends support to the proplatelet model of platelet biogenesis. In this model, proplatelets actually extend from the mature megakaryocyte cell body, traverse the vascular endothelium and enter the bone marrow sinusoids, where the shear force of the blood stream facilitates release of nascent platelets from proplatelet ends. On a molecular level, thrombopoiesis is thought to be a highly coordinate process, with sophisticated reorganization of membrane and microtubules and precise distributions of granules and organelles (Junt et al., 2007). It also appears as though localized apoptosis may play important roles in proplatelet formation and platelet release (Clarke et al., 2003). Despite these recent advances in our understanding of platelet biogenesis, mechanistic details underlying membrane reorganization, initiation of proplatelets, transportation of platelet organelles and secretary granules, and control of platelet size remain to be elucidated.

**Production of Megakaryocytes and Platelets from adult stem cells**

Various sources of adult HSCs have been used for studying megakaryocyte/platelet differentiation. Choi and colleagues were the first to report that functional platelets could be produced from CD34+ HSC isolated from peripheral blood (Choi et al., 1995). During 10 to 12 day of culture, aplastic canine serum was used to promote in vitro
vitro megakaryocyte lineage commitment/maturation and was then replaced by human serum to enhance platelet production from the megakaryocytes. Platelets generated from this system demonstrated aggregation capacity when stimulated with either ADP or thrombin, the physiological agonists for normal blood platelets. Subsequent studies have shown that HSCs from PB, BM, and CB are also all capable of producing megakaryocytes and functional platelets (Norol et al., 1998; Bruno et al., 2003; Ungerer et al., 2004).

Matsunaga et al. used an in vitro culture system to demonstrate the feasibility of producing functional platelets for clinical use (Matsunaga et al., 2006). Using this protocol, the calculated yield of platelets from $5 \times 10^6$ CD34+ cells was $1.26-1.68 \times 10^{11}$, which is equivalent to approximately 3 units of donor-derived platelets. However, these calculations were based on extrapolating data from experiments using just 500 starting CD34+ HSCs. A 3-D culture system described by Sullenbarger B. et al provides additional evidence that it is possible to produce platelets in scalable quantities (Sullenbarger et al., 2009). In this system, about 20 platelet-like particles were produced from each input CD34+ cell. Both of the above protocols consisted of 3 distinct culture steps, the first of which amplifies hematopoietic progenitors from cord blood CD34+ HSCs over the course of approximately 14 days. The second step, lasting ~5 to 7 days, encourages megakaryocyte differentiation while the third step alters culture conditions to enhance platelet biogenesis. Other protocols have described two step culture methods, with the first step consisting of hematopoietic progenitor amplification and the second step lumping megakaryocyte/platelet production together, however these systems appear to be less efficient than the 3 step methods of Matsunaga and Sullenbarger.

**Megakaryocytes and platelets generated from human embryonic stem cells:**

Following initial successes with murine ES cells (Eto et al., 2002; Fujimoto et al., 2003; Nishikii et al., 2008), megakaryocyte differentiation from hESCs has been achieved in co-culture systems with animal stromal cells (Gaur et al., 2006; Takayama et al., 2008). Using hESCs co-cultured with either OP9 or C3H10T1/2 stroma, Takayama et al. reported production of $4.8 \times 10^6$ functional platelets from $10^5$ hESCs (Takayama et al., 2008). After initial induction of hematopoietic differentiation, single cells from hESC-derived structures called ES sacs were replated onto stroma to allow megakaryocyte differentiation and platelet production during 24 days of culture.

As mentioned in the first part of this chapter, our group has produced an in vitro hemangioblast differentiation system that enables production of functional erythrocytes without stromal cells (Lu et al., 2008a). We have adopted this system for in vitro production of megakaryocytes and have observed significantly improved efficiency as compared to other methods; obtaining approximately 100 CD41+ megakaryocytes from
one input hESC (Li et al, submitted for publication). In vitro derived megakaryocytes from this system are shown in Figure 5. Upon further maturation, the hESC derived megakaryocytes gave rise to platelet-like particules which, when treated with thrombin, demonstrated comparable functionalities (eg., spreading and clot formation/retraction) to normal human blood platelets (Li et al, submitted for publication). Yet again, as observed with RBCs, stromal co-culture enhances megakaryocyte maturation and efficiency of platelet production. Collectively, these results indicate that it is feasible to induce the in vitro differentiation and maturation of hESCs into functional platelets and this is an important step in generating large-scale and donorless supplies of platelets for clinical use.

**Improving the efficiency for in vitro platelet production**

The studies described above provide an important proof of principle for the in vitro manufacturing of functional platelets from HSCs or hESCs, yet the efficiency of platelet production will need to be significantly improved in order to achieve clinically relevant yields. A potential work flow diagram for mass in vitro platelet production is depicted in Figure 6. Considering the optimal in vivo capacity of megakaryocyte development from HSCs and platelet production from megakaryocytes, both the initial hematopoietic amplification stage and downstream platelet biogenesis could stand to be optimized. Strategies to increase the efficiency of megakaryocyte/platelet production include the development of novel culture systems that mimic the in vivo bone marrow microenvironment as well as the optimization of media formulations and concentrations of cytokines, small molecule mimetics, and nutrients. For instance, TPO receptor agonists such as YM477, AMG531 have been found to enhance megakaryopoiesis and thrombopoiesis (Fukushima-Shintani et al., 2008; Broudy and Lin, 2004). In addition, physiological parameters such as pH, media viscosity, and oxygen levels may all be optimized for increased platelet biogenesis. Lastly, in vivo observations which helped formulate the proplatelet model of platelet biogenesis suggest that shear force could play an important role in platelet release (Junt et al., 2007; Patel et al., 2005). Adaptation of such mechanical force in culture systems may also significantly promote proplatelet growth and platelet release.

**Perspectives**

Limitations in the supply of RBCs and platelets can have potentially life-threatening consequences for transfusion-dependent patients with unusual/rare blood types, particularly those who are alloimmunized and patients with cancer or leukemia who, often develop platelet alloimmunity. Although cord blood, bone marrow, and peripheral blood have been investigated as sources of progenitors for the generation of large-scale transfusable RBCs and platelets, (Leberbauer et al., 2005; Giarratana et al.,
2005; Miharada et al., 2006; Sullenbarger et al., 2009; Matsunaga et al., 2006), it is clear that even after expansion and differentiation, these progenitors represent donor-limited sources of RBCs and platelets. hESCs and iPSCs, especially virus-free human iPSCs (Woltjen et al., 2009; Kaji et al., 2009; Yu et al., 2009; Kim et al., 2009), represent a new source of stem cells that can be propagated and expanded in vitro indefinitely, providing a potentially inexhaustible and donorless source of RBCs and platelets for human therapy. For hESCs, the ability to create banks of cell lines with matched or reduced incompatibility could potentially decrease or eliminate the need for immunosuppressive drugs and/or immunomodulatory protocols (e.g., O negative lines for the generation of universal RBCs). Inasmuch as iPSCs could potentially be produced from a patient’s own cells, they carry enormous potential as an alternative source of stem cells for treating human diseases related to many different organ systems, and could eliminate tissue incompatibility issues altogether.
References


piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 458, 766-770.


Figures

**Figure 1**: Human ESC-derived hemangioblasts showed tremendous CFU capability when cultured in CFC-medium. (i and ii): early BFU-E (x4, x10), (iii): large CFU-GEMM (x10); and (iv): late CFU-E (x10).
Figure 2: Graphic illustration of hemangioblastic derivatives such as RBCs and platelets from pluripotent stem cells *in vitro*. A dramatic expansion in cell number between steps 1 and 2 makes this system amenable to large scale production. In addition to RBCs and platelets, derivatives such as white blood cells (WBCs), smooth muscle cells (SMCs), and endothelium may also be generated using this *in vitro* differentiation system.
Figure 3: (i-ii): Morphology of a typical high density expansion/differentiation of immature hESC-derived erythrocytes cultured with medium containing methylcellulose (x10); (iii): Maturing hESC-derived erythrocytes stain positive for erythrocyte marker CD235a, with small fraction of cell showing enucleation (arrows); (iv): Giemsa/benzidine double staining of maturing hESC-derived erythrocytes showing high content of intracellular hemoglobin (brown). Arrow indicates a fully enucleated red blood cells (x100); (v): Maturing hESC-derived erythrocytes express A type surface antigen (Red, x100 magnification); (vi): Some hESC-derived erythrocytes express beta chain hemoglobin (green, x100) after elongated culture in vitro. Cellular nuclei in all fluorescence images were stained with DAPI (blue).
**Figure 4**: Enucleation of *in vitro* cultured erythrocytes derived from hESCs. Top: low magnification image of Giemsa-stained enucleated erythrocytes (x20); bottom: high magnification (x100) image of enucleated erythrocytes (black arrows).
Figure 5: Megakaryocytes generated from hESCs in vitro. Polyploid nuclei are evident in Giemsa staining (left panel) and represent a key morphological feature of mature megakaryocytes. Immunofluorescent staining (right panel) shows CD41 cell surface marker expression (green), dapi (blue)-positive nuclei, and granular von Willebrand factor (vWF) in the cytoplasm (red) of mature megakaryocytes (merged image).
Figure 6: *in vitro* biogenesis of platelets from different stem cells sources. The expansion at the hematopoietic progenitor stage and the efficiency of platelet biogenesis are the key stages for large scale *in vitro* production of platelets.