CELL & GENE THERAPY INSIGHTS

ADVANCED CELL THERAPIES – PROGRESS TOWARDS THE CLINIC

SPOTLIGHT

EXPERT INSIGHT

In vitro red cell manufacture for clinical application

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Red cell transfusion is an important part of current medical practice with around 92 million units transfused per annum worldwide; however global provision is limited by factors including sufficiency of supply, immunological incompatibility and the risk of transfusion-transmitted infections. The production of red cells *in vitro* is therefore an attractive goal; however such endeavors face many challenges including those arising from the complex biology of erythropoiesis, the scale of production required – a single red cell unit contains approximately $2x10^{12}$ cells – and the new developments needed in both upstream and downstream processing. Many of these challenges are common to other next-generation cellular therapeutics and so development of *in vitro* red cell production may also inform more general approaches to large-scale cell manufacturing where the cell, rather than a protein or factor it produces, is the desired product. In this article we discuss the alternative strategies for production of red cells *in vitro* and the progress on the scientific and engineering challenges that need to be addressed to make manufactured red cells a clinical reality.

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In 1816, John Henry Leacock, a medical student in Edinburgh from Barbados, carried out the first systematic experiments on intra-species blood transfusion. He demonstrated that exsanguinated dogs could be resuscitated with a transfusion from another dog but not from a cat, thus establishing the requirement for a proper understanding of the nature of the product, a clear medical rationale and compatibility between donor and recipient. Leacock did not further develop his research, but it was picked up by a fellow medical student, James Blundell, who went on to carry out the first human–human transfusions in Guys Hospital in London [1].

Today, around 92 million red cell transfusions are administered per annum worldwide for a wide variety of clinical conditions. However, significant limitations persist, particularly when looked at from a global perspective, including sufficiency of supply, immunological incompatibility and the risk of the transfusion of transmitted infection.

For many decades, researchers have sought pharmaceutical alternatives to red cell transfusion, including the development of synthetic oxygen-carrying molecules and hemoglobin-containing solutions, but with limited success. This is perhaps unsurprising in light of the complex way in which red cells interact with the microcirculation in the regulation of tissue oxygenation and acid–base balance. The hematological system was the field within which the existence of stem cells was first postulated by Papenheim [2], demonstrated experimentally by McCulloch and Till [3], and clinically applied as bone marrow transplantation by E Donnall Thomas *et al.* [4]. Hence the generation of red cells *in vitro* is an attractive proposition in light of our scientific understanding of hematopoiesis, experience of this type of product and well-established clinical indications. However, the quest to achieve scalable *in vitro* red cell production is anything but simple and exemplifies the challenges in stem cell and developmental biology, analytic and process development and bioengineering characteristic of the next generation of cellular therapeutics.

HEMATOPOIETIC ONTOGENY & THE IMPORTANCE OF MICROENVIRONMENT

In humans, primitive hematopoiesis arises in the extra-embryonic mesoderm in the yolk sac at around day 21 of gestation, resulting in the production of megaloblastic nucleated red cells expressing embryonic hemoglobins (Gower 1, Gower 2 and Portland), which have higher oxygen affinity than fetal or adult hemoglobins and display less co-operativity and pH sensitivity. Specialized embryonic hematopoietic progenitor cells (HPC) arise in the yolk sac shortly thereafter [5]. Definitive hematopoietic stem cells (HSCs) arise independently from the intra-embryonic mesoderm, specifically from the hemogenic endothelium in the ventral floor of the dorsal aorta (and other arterial vessels) at around day 27 and differentiate into normoblastic enucleated erythrocytes expressing fetal hemoglobin [6,7]. From there they migrate to the embryonic liver at approximately day 32 where they massively expand and become the main source of fetal definitive hematopoiesis from around 6 weeks onwards. From around 20 weeks, hematopoietic stem and progenitor cells (HSPCs) migrate to the bone marrow, which is the source of adult definitive hematopoiesis following birth. Thus hematopoietic ontogeny involves a complex temporal and spatial interplay between HSPCs of differing origin and potentiality, and a variety of different microenvironments. This complexity needs to be properly understood and managed in order to achieve successful *in vitro* differentiation of erythrocytes from stem cell sources **(Figure 1)**.

ERYTHROID DIFFERENTIATION FROM HEMATOPOIETIC STEM & PROGENITOR CELLS

In the adult human, HSCs are thought to comprise around 1 out of 10^5 of the nucleated cells in the bone marrow and are capable of self-renewal and differentiation into the multiple different mature cell

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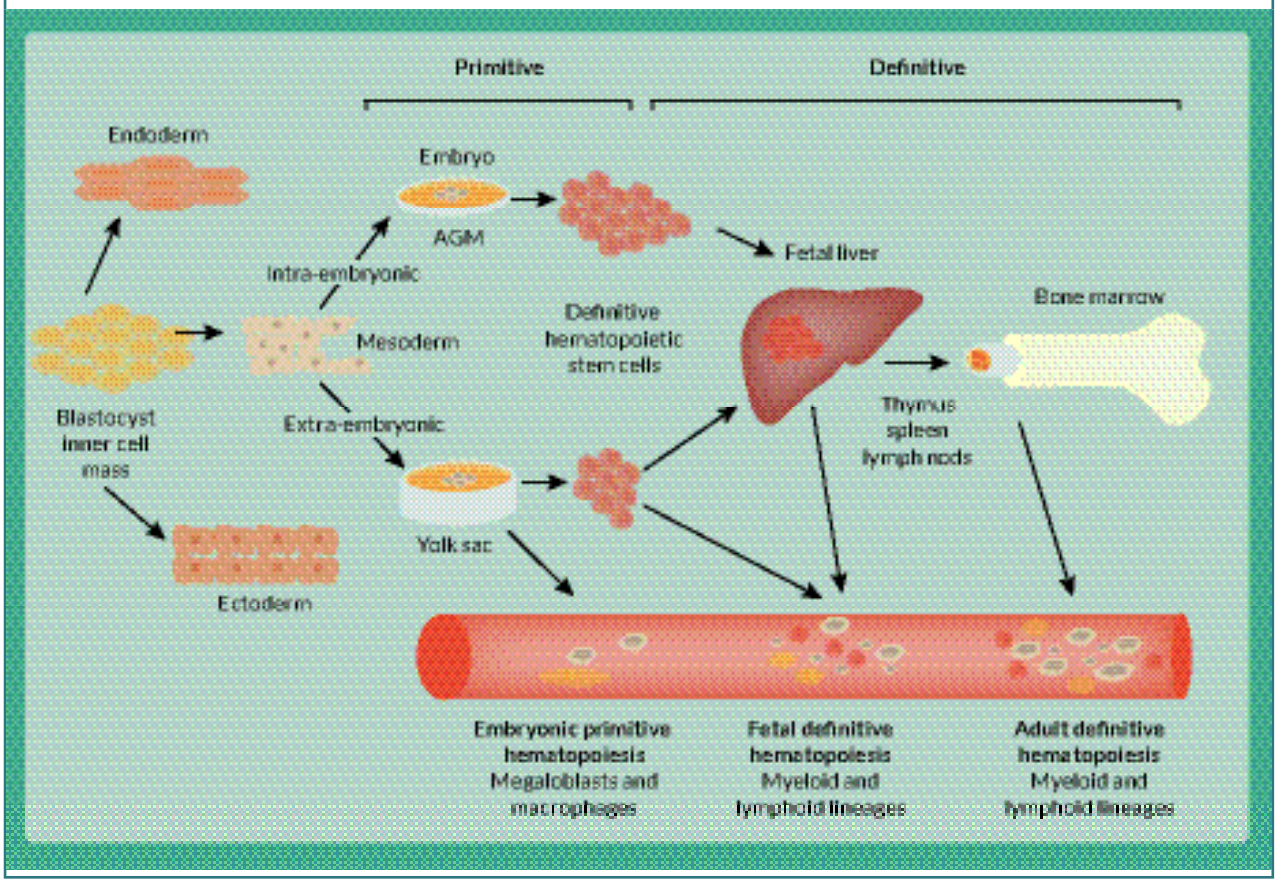
FIGURE 1

Ontogeny of hematopoiesis.



types of the adult hematopoietic and immune systems. They reside adjacent to the endosteal surface of the bone and their progeny migrate centripetally during differentiation. Erythoid precursors from the pro-erythroblast onwards are found in association with macrophages in erythroblastic islands close to the central venules. During enucleation the macrophages phagocytose the extruded nuclei and organelles, whilst the resulting reticulocyte undergoes substantive membrane remodeling as it is released into the circulation.

In clinical practice, CD34 is used as a surrogate marker of HSC potential, though in reality more than 99% of this phenotypically defined population are mature HPCs and endothelial cells. HPCs can be cultured short term *in vitro* in semi-solid matrices within which each will develop into a discrete colony as a result of a limited number of cell divisions followed by terminal differentiation. This system is, however, a one-stage continuous culture with limited flexibility and scope for expansion.

These limitations can be circumvented in suspension culture systems, which can be adjusted to allow sequential support of proliferation and differentiation. Over the past 20 years, a number of groups have developed a variety of suspension culture systems that use $CD34^+$ enriched populations derived from bone marrow, granulocyte colony-stimulating factor mobilized

peripheral blood or umbilical cord blood as a starting material.

The most simple of these used single-phase liquid cultures and a variety of cytokines in various combinations including interleukin-3 (IL-3), granulocyte-monocyte colony stimulating factor (GM-CSF) and erythopoietin (EPO) [8]. After 18–21 days in culture, erythroblastic islands developed followed by enucleation of 10–40% of cells. The enucleated cells had the characteristics of reticulocytes and expressed gamma globins (cord blood) or beta globin (adult bone marrow and peripheral blood). A higher level of expansion was reported by Panzenbock *et al.* who cultured $CD34^+$ cells in EPO, stem cell factor (SCF), insulin-like growth factor 1 (IGF-1), dexamethasone and estradiol, resulting in a 10^5-fold expansion in erythroid progenitors by days 15–18 [9]. Erythroid progenitors were recovered and cultured further in EPO and insulin in order to induce terminal differentiation to erythrocytes. Similarly, Freyssinier *et al.* cultured $CD34^+$ cells for 7 days in serum-free conditions with SCF, IL-3 and IL-6, resulting in a 30-fold expansion in cell numbers and the appearance of high numbers of $CD36^+$ cells [10]. The $CD36^+$ cells were purified by immunomagnetic separation and further cultured in the same medium and cytokines with the addition of EPO for an additional 3 days, leading to a 100-fold cell expansion and maturation to glycophorin A-positive erythroblasts and a small proportion of enucleated red cells. Unlike in Malik *et al*'s [8] study, enucleation did not seem to be dependent on the presence of macrophages in this system. Similarly, Miharada *et al.* developed a method to produce enucleated erythrocytes using vascular endothelial growth factor (VEGF), IGF-II and mifepristone (a glucocorticoid antagonist) but did not make use of feeder cells or macrophages [11]. Maggakis-Kelemen *et al.* [12] also used a similar culture system to that of Malik *et al.* [8], but with the addition of dimethylsulphoxide (DMSO), ferrous citrate and transferrin. They found that the latter dramatically enhanced hemoglobin synthesis but that whilst precursor cells showed a high degree of deformability, the cultured reticulocytes and erythrocytes showed reduced shear modulus compared to controls. Fujimi *et al.* [13] showed that co-culture with macrophages significantly enhanced the production of red cells from $CD34^+$ HSPCs.

The most extensive functional analysis of *in vitro* generated erythroid cells has been carried out by Douay and colleagues who developed a protocol for differentiation of cord blood $CD34^+$ cells in defined media, using a 3-step protocol involving stimulation of $CD34^+$ HSPCs using SCF, Flt3-ligand and thrombopoietin (TPO), followed by expansion of erythroid progenitors using SCF, EPO and IGF-1; and terminal erythroid differentiation using EPO and IGF-1 [14,15]. The system produced 2 x 10^5-fold amplification with dominant erythroid differentiation. This group have subsequently modified their protocol for application to adult blood and bone marrow [16,17] substituting IL-3 for Flt3-ligand in the first phase, culture with EPO in the presence of a murine stromal cell line (MS5) or human mesenchymal cells for 3 days; followed by the stromal cells alone without growth factors for up to a further 10 days [18,19]. Commitment towards

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the erythroid lineage was morphologically apparent from day 8, with terminal differentiation to 65–80% reticulocytes by day 15 and maturation to erythrocytes from days 15 to 18 evidenced by loss of transferrin receptor (CD71) expression, 90–100% enucleation and typical morphological characteristics. These cells also showed normal glucose 6 phosphate dehydrogenase (G6PD) and pyruvate kinase (PK) enzyme levels, membrane deformability and oxygen dissociation characteristics and similar survival to native red cells following carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling and intraperitoneal infusion into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. Douay and colleagues have used a Good Manufacturing Process (GMP) version of this protocol, omitting the co-culture step, to manufacture 10^{10} cultured erythroblasts from autologous peripheral blood $CD34^+$ cells that were ^{51}Cr labeled and reinfused into the human volunteer. The level of the cells in the circulation after 26 days was comparable to that seen with peripheral blood red cells, supporting their overall quality, safety and compatibility, and amounting to clinical proof of principle [20].

Anstee and his colleagues have developed a simpler, feeder-free two-stage culture system using enriched $CD34^+$ cells cultured for 5 days in serum-free medium supplemented with SCF, IL-3 and EPO, lipoprotein and tacrolimus, and then transferred to medium supplemented with human AB serum, transferrin, EPO, insulin and T3 [21,22]. This system produces a 10^4-fold expansion in cell numbers with 55–95% enucleation. Leucocyte filtration was used to remove normoblasts, free nuclei and R1 reticulocytes leaving approximately 3 x 10^{10} R2 reticulocytes and erythrocytes, which displayed normal morphology, protein expression and glycosylation, membrane deformability and oxygen dissociation and superior recovery and survival in immunodeficient mice. A larger scale clinical recovery and survival study using a GMP version of this protocol is due to commence in 2017.

Overall, although it is clear that bone marrow, cord blood and peripheral blood can be used as sources of HSPC to generate sufficient functional red cells for a small number of units, large-scale manufacture is not likely to be possible because of the limited replication capacity of HSCs and the remaining need for donation of this starting material.

ERYTHROID DIFFERENTIATION FROM PLURIPOTENT STEM CELLS

Even though long-term repopulating HSCs have not yet been successfully generated from human pluripotent stem cells (PSCs), several groups have demonstrated differentiation of downstram hematopoietic cells [23,24] using two different approaches.

The first approach entails co-culturing PSCs with murine stromal lines, such as S17 or OP9 [25], or the yolk sac endothelial cell line C166 [26] to promote or support the commitment of pluripotent PSCs specifically to the hematopoietic lineage. Vodyanik *et al.* [25] showed that OP9 stroma supports the production of up to 10 times more HSPCs compared with other murine bone marrow stromal cell

lines. This group demonstrated production of up to 20% $CD34^+$ cells and isolated up to 107 $CD34^+$ cells from a similar number of hPSCs. In an attempt to recapitulate the microenvironment of developing HSCs in the embryo, Ledran *et al.* showed that co-culture of hPSC with monolayers of stromal cells derived from murine AGM, fetal liver or other developmental niches enhanced early differentiation of $CD34^+$ HSPCs [27]. These HSPCs were capable of primary and secondary engraftment into immunodeficient mice at low levels and also generated both erythroid and myeloid cells in colony-forming assays. Interestingly, the AGM-derived AM201B4 line resulted in the best reconstituting capacity suggesting, perhaps logically, that PSCs may be better supported by stroma from the sites where definitive hematopoiesis first occurs, rather than sites such as the bone marrow, which is more active in the adult.

The Bouhassira group have shown that hESC culture on hTERT-transformed human fetal liver cell lines can generate 2–10% $CD34^+$ cells, which can then be differentiated into an erythroid population using a 4-stage liquid culture protocol [28,29]. Using this system, they further reported that increasing the duration of culture on the FH-hTERT cell line from 14 to 21 or 35 days lead to a 5000-fold increase in cell number of erythroid cells with differentiation of the predominant population of megaloblastic nucleated erythroblasts to a small population of normoblastic enucleated red cells with an increase in alpha/zeta and beta/epsilon chain ratios recapitulating *in vitro* the shift from primitive to definitive hematopoiesis [29–31].

The second approach entails the removal of PSCs from feeder cells and culture in suspension, which leads to the formation of embryoid bodies (EBs). Chadwick *et al.* demonstrated that bone morphogenetic protein 4 (BMP4), a ventral mesoderm inducer, strongly promotes hematopoietic differentiation in the context of a combination of hematopoietic cytokines (SCF, Flt-3 ligand, IL-3, IL-6 and G-CSF) [32]. It has also been demonstrated that addition of VEGF-A165, which is normally produced in response to hypoxia, further promotes erythropoiesis, particularly when augmented by EPO [33]. Chang *et al.* [34] have described the generation of erythroid cells from PSCs by subsequent processing of cells present at either early or late stages of EB formation, whereas Zambidis *et al.* [35] have more closely defined the progress of differentiation in EB through primitive and definitive stages of hematopoiesis. Much of the seminal work characterizing hematopoiesis in EB has come from the Keller group who have demonstrated that at 3–4 days, EB differentiation give rise to cell colonies expressing both endothelial and hematopoietic markers (KDR [FLK1] and CD117) [36,37]. Other groups have also shown the appearance of $CD34^+$ cells from 5 days of culture, peaking at day 12–15, with evidence of primitive erythropoiesis between 7 and 12 days and definitive erythropoiesis emerging from days 12– 20 [32,33,35].

One of the more clinically relevant published studies is that of Lu *et al.*, who have demonstrated the differentiation of hESCs into functional erythrocytes on a large scale using a 4-step procedure [38]. The first step involved the formation

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of EBs by culturing PSCs in serum-free media, BMP4, VEGF and bFGF with the addition of SCF, TPO and FLT-3 ligand after 48 h. The second involved dissociation of the EBs with trypsin and culture of the single-cell suspension in blast colony growth medium, bFGF and recombinant tPTD-HOXB4 fusion protein for 10 d. The third step consisted of further culture with EPO for 5 days and thereafter with SCF, EPO and methylcellulose. Finally, the erythroid cells were enriched through a plastic adherence step. Starting with one 6-well plate of hESC, they generated 10^{11}–10^{12} nucleated erythroid cells with comparable oxygen dissociation curves, response to pH (Bohr effect) and 2,3-diphosphoglycerate (DPG) activity to normal adult red cells, despite expressing mainly embryonic and fetal globins. However, after 28 days of culture only 16% of the cells expressed beta globin and 10–30% of the cells became enucleated. Also, in order to achieve this impressive expansion in cell numbers during differentiation, it was necessary to treat the cells with a recombinant HOXB4 protein.

These two methods of inducing hematopoietic differentiation have also been combined; Wang *et al.* demonstrated that the treatment of PSCs during EB formation with a combination of low-dose cytokines and human bone marrow stroma significantly increased hematopoietic differentiation [39]. In a very promising study, Ma *et al.* demonstrated that PSC co-cultured with murine fetal liver-derived stromal cells after an initial EB stage, produced large numbers of erythroid progenitors that underwent definitive differentiation with a shift from epsilon-globin expression to beta-globin expression and production of enucleated erythrocytes. Comparative analysis against cord blood-derived progenitors showed similar expression of glycophorin A, CD71 and CD81, G6PD activity and oxygen-carrying capacity [40]. However, they were only able to achieve a 100-fold expansion from PSCs to mature erythrocytes.

Most recently, Jaichandran and colleagues [41] have reported a serum-free chemically defined microcarrier-based suspension culture based on the differentiation protocol developed by Lu *et al.* resulting in a 6-fold improvement in HPC expansion and an 80-fold improvement in erythroid cell generation compared with their standard EB method. They report efficient terminal maturation and production of up to 50% mature enucleated erythrocytes using co-culture with mesenchymal stromal cells.

We feel that the key to improving the process and making it possible to efficiently achieve hematopoiesis and erythroid maturation at scale and in a manner that is current GMP-compliant, is to identify and replace the array of signals delivered by stroma with a defined animal- and feeder-free culture system. We have developed such a system using combinations of cytokines and small molecules [41]. To induce differentiation, hPSCs are cultured in BMP4, VEGF, Activin A, Wnt3A, and GSKIIIbeta inhibitor VIII or A-A014418 (Mix A). On day 2 the existing set of cytokines and small molecules is augmented and FGF, SCF and beta-estradiol are added. On day 3 the EBs are dissociated and re-suspended in medium containing BMP4, VEGF, FGFa, SCF, IGF, TPO, heparin, IBMX and beta-estradiol (Mix B). On day

5 this medium was refreshed and StemRegenin added. On day 10 the cells are replated in erythroid liquid culture conditions with hydrocortisone, SCF, Flt3-ligand, BMP4, IL3, IL11, IBMX and EPO (Mix C). On day 17 the cells are cultured in medium containing BSA, insulin, transferrin, lipid mixture, hydrocortisone, SCF, IGF, IL3, IL11 and EPO (Mix D). From day 24 the cells are suspended in medium containing EPO and from day 26 in medium alone [42]. This protocol supports 50,000–200,000-fold expansion in cell numbers by day 31 of which 99% are CD235a-positive with 90–95% fetal hemoglobin. The approach has been demonstrated to be reproducible across a number of embryonic and induced PSC lines. However, enucleation of these cells appears to be unstable giving rise to abnormal R1 reticulocytes, which fragment easily leaving only around 10% stable reticulocytes. The cause of this is unclear, but may represent the PSCs adopting a fate similar to that of the transient but definitive erythroid/myeloid precursor population reported by the Palis laboratory [5].

ERYTHROID CELL LINES

In light of the challenges in achieving definitive adult erythropoiesis and stable reticulocytes through PSC differentiation, several groups have explored the possibility of generating immortalized erythroid cell lines (ECL). Hirose *et al.* described the immortalization of erythroblasts from hiPSC, using doxycycline (DOX)-inducible overexpression of BCL-XL and c-MYC to establish a highly proliferative population of immortalized erythrocyte progenitor cells that underwent differentiation on removal of DOX [43]. Neither gene alone was sufficient to cause the immortalization, and survival and proliferation of the clonal imERYPC lines in the presence of DOX was dependent only on EPO. After removal of DOX, the maturing normoblasts were shown to have gene expression profiles broadly similar to those derived from CB CD34$^+$ cells including predominant expression of HbF (~80%) and some HbA and HbE. Enucleation of the differentiated immortalized erythroid progenitors was low in this report – seemingly only occurring in 1–2% of cells, but injection of erythroblasts into clodronate-treated NOD-SCID mice resulted in extensive enucleation *in vivo*. This line offers a very good system for studying erythroid development but the potential to use such a line to generate therapeutic red blood cells is limited by the lack of *in vitro* enucleation and the expression of HbF rather than HbA. These important properties might be improved by the use of a similar immortalization strategy using adult CD34$^+$ as the starting material rather than iPSC.

Kurita *et al.* transfected both human PSC and HSPC with lentiviral vectors containing human papilloma virus (HPV)-16 E6/E7 under a tetracycline–inducible promoter. CD34$^+$ cells were cultured in serum-free media with SCF, TPO and Flt3-ligand for 1 day and the HPV-16 E6/E7 expression system was then introduced by lentiviral transduction [44]. They were further cultured for 4 days in SCF, EPO and dexamethasone. Non-adherent cells were collected and cultured in the presence of SCF, EPO, dexamethasone and doxycycline for a prolonged period with regular changes of medium.

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The cells proliferated continuously for more than 6 months (80 population doublings). Erythroid cell lines have an erythroblastic morphology and express Glycophorin A, CD71, CD36 and c-kit. In our hands, terminal differentiation can be induced through culture in EPO alone to produce 15–30% stable reticulocytes expressing fetal or adult hemoglobin dependent on the source of the $CD34^+$ starting material.

PROSPECTS & CHALLENGES FOR MANUFACTURE & CLINICAL APPLICATION

The manufacture of red cells for clinical application remains very challenging. Whilst the culture of $CD34^+$ cells derived from bone marrow, peripheral blood or umbilical cord blood gives rise to essentially normal reticulocytes with high levels of efficiency, overall expansion of cell numbers is low and given that a unit of clinical red cell concentrate contains around 2.5×10^{12} erythrocytes, the prospect of manufacturing more than a handful of such units from each donation of starting material appears to be low. PSCs offer the possibility of indefinite expansion of the starting material and considerable expansion during differentiation; however, the differentiation process remains incompletely understood and may require additional signaling to achieve stable enucleation. ECLs offer the prospect of simplifying the complexity of the differentiation process and produce stable reticulocytes, but the majority of the cell expansion would need to be provided by the cell line itself. Moreover, both PSCs and ECLs engender the risk of infection during long-term culture and of neoplasia due to the use of oncogenes during derivation, insertional mutagenesis or genetic instability within the cell line itself. Whilst it can be argued that the depletion of nucleated cells from the final product and/or the use of pathogen reduction technologies may reduce these risks, they remain of concern.

The scale and complexity of the bioprocess required to produce a unit of red cells is challenging. It is not simply a matter of scale up (or scale out), but also of intensifying the cell culture, achieving control over a multifactorial process during which the cells undergo substantial phenotypic and metabolic changes, and controlling the costs of manufacture. The intensification challenge can be evaluated with respect to the known limits on bioprocess in more established suspension cell cultures such as CHO lines for protein manufacture. In such systems, there is a mechanical stress tolerance of the cells that determines the mixing and gas delivery operation of the bioreactor, an oxygen demand of the cells that (in conjunction with dissolved oxygen input rate to the bioreactor) determines the absolute density limit at which cells can be cultured, and a tolerance to soluble factors such as nutrients and cytokines or detrimental metabolites that determines medium provision and exchange strategy. Such limitations have tended to restrict culture systems for common cell lines to absolute maximum densities in the order of 1×10^7 cells/ml after substantial optimization and, in some cases, cell line engineering. These densities need to be surpassed without requiring uneconomic medium perfusion or exchange rates to produce 2×10^{12} red cell doses in economically reasonable volumes.

It has been shown that erythroid progenitors through to enucleated reticulocytes can be cultured in various scalable platforms including rocked bags and mechanically stirred tanks. There is therefore no compatibility barrier between stresses imposed by standard manufacturing equipment and reticulocyte production. Further, oxygen demand of cells during erythroid commitment is low relative to cell lines for protein manufacture. Similarly, studies of common nutrient and metabolite demand have not identified primary limits of culture intensification. This is both a challenge and an opportunity; it suggests very high intensity production is plausible with respect to common mass transfer and metabolic constraints, but achieving it may require identifying and addressing novel inhibitory mechanisms.

Given that manufacturing platforms are not incompatible with the process, manufacturing challenges (beyond autocrine growth inhibition) fall into four key areas. A particular challenge of bioprocess for red cell culture, similar to other stem cell products, is that the engineering considerations are different for each phenotypic stage of development. Further, metabolic factors governed by nutrient supply, or autocrine exposure determined by media exchange strategy, can themselves alter the phenotypic trajectory of cells, either changing the nature of the end product or altering the total proliferative capacity of the culture. This creates a complex control challenge where the control window is tracking a moving target, and any deviations can have compounding effects that necessitate control changes throughout the rest of the process. Secondly, production is not only limited by the intensification of the system at any phenotypic point (cells produced per volume), but also by the total proliferative capacity of the system that determines end product yield (cells produced per input cell). Emerging cell-line-based approaches to expansion may overcome absolute proliferative limits, but the metabolic state and sensitivities of the cells responsible for the bulk of production will be key to controlling cost of goods; proliferation needs to be weighted in the part of the process with lower bioprocess demand and therefore greater intensification potential. Thirdly, phenotypic heterogeneity in culture will dictate the yield curve with time of final enucleated product; greater homogeneity will allow harvest of more homogenously aged product and reduce the requirement for bioprocess conditions that support both the latter stages of culture and enucleated cell stability. The homogeneity of maturation and stability of enucleated cells in culture will determine whether continual or multiple harvest strategies are required to deliver required efficiency and product quality. Finally, downstream processing of enucleated cells at scale will require new technologies; current leukofiltration is not necessarily optimized to deal with the balance of cell material from a bioprocess. Although specification of downstream process requirements won't be fully definable until the harvest material is defined, it is likely that tangential flow filtration or similar strategies may be necessary to deliver success at manufacturing scale.

Some of these challenges require mathematical process models to address them. In particular,

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the intensification of the system at a given point of phenotypic development, and the impact of that intensification on the trajectory and proliferative capacity of the culture, require models that can describe cell culture dynamics and evolving consequences of autocrine exposure or nutrient depletion. The limitation to the application of such approaches are the skills barrier to entry and a poor understanding of the level of model complexity required to provide manufacturing utility in the field. The mechanistic understanding associated with such models is necessary to define current system potential and the requirement for new or modified bioprocess engineering solutions.

Realizing the long-term objective of clinical supply of manufactured red cells requires further work on understanding and abbreviating erythroid differentiation protocols, achieving more consistent and stable enucleation, and intensification of both upstream and downstream bioprocessing. Most important though is clear demonstration that the red cell products of the manufacturing process are fit for purpose both *in vitro* and *in vivo*. The task remains challenging, but is helping to inform generic approaches to the large-scale manufacture of cellular therapeutics.

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