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REVIEW ARTICLE

Towards manufactured red blood cells for the treatment of inherited anemia

Ferrata Storti Foundation

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ABSTRACT

Patients with inherited anemia and hemoglobinopathies (such as sickle cell disease and β-thalassemia) are treated with red blood cell (RBC) transfusions to alleviate their symptoms. Some of these patients may have rare blood group types or go on to develop alloimmune reactions, which can make it difficult to source compatible blood in the donor population. Laboratory-grown RBC represent a particularly attractive alternative which could satisfy an unmet clinical need. The challenge, however, is to produce - from a limited number of stem cells - the $2x10^{12}$ RBC required for a standard adult therapeutic dose. Encouraging progress has been made in RBC production from adult stem cells under good manufacturing practice. In 2011, the Douay group conducted a successful proof-of-principle mini-transfusion of autologous manufactured RBC in a single volunteer. In the UK, a trial is planned to assess whether manufactured RBC are equivalent to RBC produced naturally in donors, by testing an allogeneic mini-dose of laboratory-grown manufactured RBC in multiple volunteers. This review discusses recent progress in the erythroid culture field as well as opportunities for further scaling up of manufactured RBC production for transfusion practice.

Introduction

Red blood cell (RBC) transfusions are the mainstay treatment for anemic patients and are given routinely in hospitals around the world. Depending on the clinical presentation, blood transfusions are likely to be administered to improve the patients' quality of life and are administered either intermittently when needed, or for extended periods, as in the case of 50-90% of patients suffering from myelodysplastic syndromes and for transfusion-dependent patients with inherited anemia and hemoglobinopathies (such as sickle cell disease and β-thalassemia). Chronic RBC transfusions introduce secondary complications which contribute to morbidity, due mainly to transfusion-induced iron overload and erythrocyte alloimmunization.[1,2] It is particularly challenging for blood services to source RBC compatible for multiply alloimmunized patients with chronic transfusion-dependent anemia or rare blood types.[3,4]

Breakthroughs in the field of erythropoiesis research have led to the development of reproducible protocols that can yield large numbers of cultured human reticulocytes, often referred to as laboratory-grown or cultured red blood cells (cRBC). In addition to being an excellent model system for exploring human erythropoiesis in health and disease, this work has laid the foundations for the interest in producing human cRBC for transfusion purposes or as a vehicle for red cell-based therapeutics. The production of cRBC from stem cells or other cellular sources (see below) may one day fill the unmet clinical need for transfusion-dependent patients, but only if the challenge of growing enough clinical grade RBC can be met.

The term "manufactured RBC" (mRBC) refers to clinical grade cRBC grown under good manufacturing practises (GMP). Both cRBC and mRBC are in fact nascent RBC, known as reticulocytes[5-7] and they are referred to only as mRBC within this manuscript from now on to avoid confusion. One added benefit of mRBC, compared to standard donor-derived RBC, is that they are a homogeneous population of immature RBC that should last the normal 120-day lifetime in the circulation. This regenerative medicine product is anticipated to reduce transfusion fre-

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quency and the associated iron overload.[6] Support for this idea comes from studies showing that transfusion of young RBC (also called neocytes) is beneficial to patients with inherited anemias, reducing iron overload and increasing the interval between transfusions.[8-12]

mRBC have been tested in immunocompromised mice models[5,6,13] and non-human primates.[13] Importantly, a proof-of-principle mini-transfusion of autologous mRBC has been conducted in a single volunteer, illustrating that mRBC can survive in the circulation and are safe for use in humans.[5] The benefit of mRBC in allogeneic transfusions across multiple recipients still needs to be demonstrated before moving to patients. The commercial company Rubius Therapeutics has a business model built around producing novel mRBC for therapeutics. To date, Rubius has conducted one injection of mRBC engineered for treatment of phenylketonuria in a single patient (Rubius press release 12th March 2020[14]), but no further specific information was released. In the UK, the National Health Service Blood and Transplant (England's Blood Service) is intending to conduct a single-center, randomized, allogeneic, controlled, phase I, cross-over trial denominated RESTORE (Recovery and Survival of Stem Cell Originated Red Cells (ISRCTN:42886452 and EudraCT: 2017-002178-38). This healthy volunteer trial has faced significant delays, most recently due to the COVID-19 pandemic but will, it is hoped, be carried out in the near future to assess the recovery and survival of a mini-dose of mRBC derived from $CD34^+$ cells isolated from adult blood donors *versus* the standard RBC from the same donor.

Laboratory-grown RBC offer the greatest potential in terms of sourcing rare blood groups for sickle cell and thalassemia patients with alloimmunity. It must be acknowledged however, that these patients also present the greatest challenge in terms of requirements for blood. Adult patients require multiple units of blood per month.[15] Realistically, the first therapeutic use of mRBC is likely to take place in a pediatric setting or for red cell-based therapeutics, such as enzyme replacement therapies, as both these applications require smaller numbers of mRBC. There is also a need to determine the number of mRBC that represents a therapeutic dose. For adult patients, one unit of standard RBC is estimated to consist of approximately $2x10^{12}$ RBC which raises the hemoglobin of an average adult by 1 g/dL. For pediatric patients, doses are more variable as they depend on the weight of the patient but are lower than an adult dose. It should be noted that a proportion (5-10%) of standard RBC are lost within the first 24 hours after transfusion[16] and this increases to 25% or more with blood storage time. Therefore, the actual number of RBC required to treat anemia is likely to be lower if the majority of the cells are nascent.

Many excellent reviews have been written on mRBC and the prospect of using mRBC for transfusion.[17-25] We therefore offer below a concise overview of the progress to date, highlighting the relevant issues and opportunities for optimizing and increasing the mRBC yield to an adult therapeutic dose.

Overview of the erythroid culture process

The recapitulation of erythropoiesis using primary hematopoietic stem and progenitor cells (HSPC) *ex vivo* requires specific combinations of cytokines and growth factors in order to first expand the HSPC, and then to direct lineage specification to ensure full differentiation to the reticulocyte stage (see Figure 1). Over the last 20 years, multiple laboratories have developed two-dimensional liquid culture systems that reproduce the process and stages of human erythropoiesis to generate reticulocytes. These include two to four stages, each characterized by the inclusion or omission of specific growth factors (Table 1). The general consensus is for the inclusion of a primary stage favoring HSPC expansion with interleukin-3 and stem cell factor, a secondary erythroblast expansion stage including stem cell factor and erythropoietin, followed by a terminal differentiation stage with erythropoietin. Notably, holotransferrin is included throughout the culture period. Some laboratories further modify the initial-stage culture media by including, for example, thrombopoietin and fms-like tyrosine kinase 3 (Flt-3) to enhance stem cell proliferation[13] and may also include glucocorticoids to increase expansion prior to differentiation.[26] The more recent culture protocols listed in Table 1 have been undertaken at considerably larger scale (i.e., at least 1 L), with some reports of successful generation of large numbers of reticulocytes. The challenge for the field is to increase the production even further to generate the equivalent of a therapeutically useful adult dose.

Starting material

The studies reporting the highest yields all use HSPC specifically isolated from cord blood,[13,27] mobilized[5] or standard peripheral blood[6,28] (Table 1). Another option is to use the whole peripheral blood mononuclear cell (PBMNC) component for production of mRBC, thereby omitting the expensive step of $CD34^+$ isolation.[7,29] As well as reducing costs, the use of PBMNC as the starting material under the correct culture conditions can contribute towards increasing the yield of mRBC. Indeed, PBMNC include all cells with erythroid lineage potential, some of which are $CD34^-$ that can enhance culture yield.[29] PBMNC also include $CD14^+$ cells that might act as helper/feeder cells that can limit the cell death of erythroid progenitors during the first few days of culture when volumes are still small and cells are kept in static tissue culture flasks or dishes.[30,31]

We highlight that there are other sources that are gaining traction, including immortalized pluripotent stem cells and immortalized cell lines,[32,33] which can be used to differentiate to reticulocytes, but we will not discuss these here because these have not yet been grown at scale. These sustainable cellular sources have great potential for continual blood production once the technical challenges of growing them have been circumvented and are likely to comprise a second wave of blood products after stem cell-derived mRBC.

Natural donor variation and yields

The genetic makeup of the donor-derived cellular starting material has long been recognized to have an impact on yield, which is problematic when trying to consistently produce a high number of mRBC using random donors.[34] This variation could be due to the number of HSPC present per volume of blood, which is highly variable between

donors, or down to subtle variations in genetic factors that influence how HSPC and erythroid progenitors proliferate, enucleate and/or respond to culture media composition. More work is therefore needed to explain the differences in yields observed between donors under standardized and reproducible culture conditions. The payoff from this painstaking work would be the identification of potential genetic variations that could be pre-screened for or utilized by genetically engineering alterations the starting material to benefit production.

Genome-wide association studies as well as identification of rare phenotypes linked to specific RBC traits might help to identify genetic variants suitable for reliably producing large numbers of mRBC. An example of such an approach was carried out by Sankaran's group.[35] LNK/SH2B3 is an adaptor protein that negatively regulates hematopoietic cytokine signaling. Rare lost-of-function *SH2B3* alleles have been associated with *JAK2*-mutation-negative erythrocytosis[36,37] and a hypomorphic allele of *SH2B3* (single nucleotide polymorphism rs3184504) was found to be significantly associated with high hemoglobin levels, packed cell volume and RBC count *in vivo*.[38] Using shRNA knockdown in adult, mobilized, peripheral blood and cord blood CD34$^+$ cells, Giani *et al.*[35] suppressed the expression of the LNK/SH2B3 protein and reported a 2- to 7-fold increase in yield of enucleated RBC in shRNA-treated cells compared to cells transduced with a control shRNA. More recently, a study of rare MAM-negative individuals by Thornton and colleagues[39] showed that peripheral blood CD34$^+$ cells from two MAM-negative individuals had a proliferation advantage in *ex-vivo* erythroid cultures, resulting in an average 5-fold increase in cell number compared to four age- and gender-matched MAM-positive controls. Whether the same observations concerning loss-of-function *SH2B3* and MAM-negative cells hold true for large-scale cultures and across multiple donors still needs to be determined.

Beyond yield, the choice of donor can also affect the quality of the final mRBC product due to the donor's own RBC intrinsic characteristics – not just in terms of blood group which can be selected for, but also in terms of storage characteristics or even longevity in circulation once transfused. The planned RESTORE clinical trial may provide data on this as the survival time of transfused stem cell-derived mRBC in the circulation of the recipient will be directly compared to the survival time in circulation of the same donor's standard RBC.

Genetic manipulation and small molecules

A key challenge for the field is to prevent the attrition of the self-renewal capacity of HSPC and to maintain the expansion capacity of erythroid progenitors (burst-forming and colony-forming units-erythroid) for a longer period before terminal erythroid differentiation occurs. One way to do this is through the use of glucocorticoids (see below) which can potentially improve the asynchronicity of enucleation in cultures, and may then improve reticulocyte stability and filtration efficiency. Another way is to

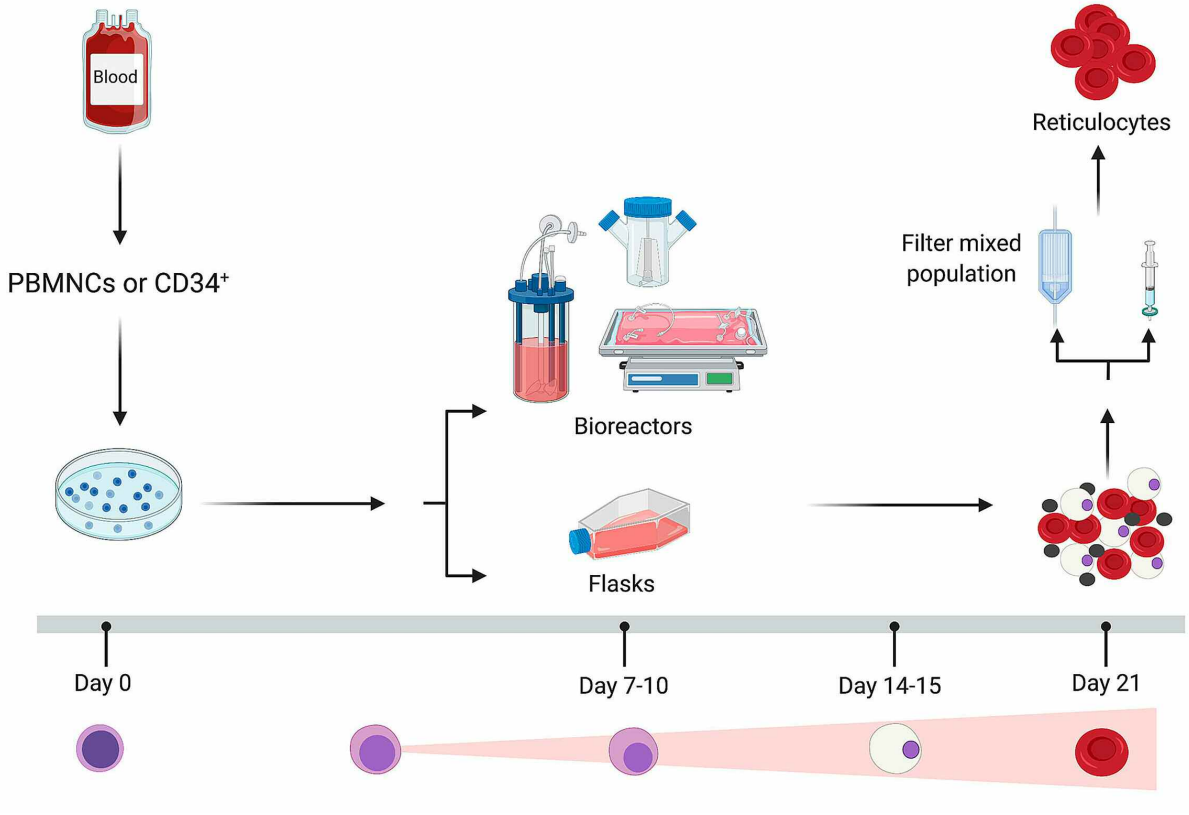

Figure 1. Overview of the erythroid culture process. *Ex vivo* culture systems require the isolation of peripheral blood mononuclear cells (PBMNC) or magnetic sorting of the CD34$^+$ cells as starting material. Culture systems then employ either flasks, spinner flasks or a bioreactor system depending on scale. The volume of the culture will increase dramatically as the cells expand and differentiate through days 7-14. Upon generation of a mixed population of reticulocytes, nucleated cells and pyrenocytes at day 21, cells then require filtration using either a syringe (small scale) or multiple leukocyte filters (large scale) depending on volume to give a pure reticulocyte population. Diagram made using biorender.com.

use culture manipulation to try and maintain stemness and proliferation potential. For example, there are small molecule inhibitors that are reported to enhance cord blood HSPC self-renewal, such as UM171[40] and the aryl hydrocarbon receptor agonist SR-1.[41] There are also factors such as angiopoietin-like 5 and IGBFP2[42] and notch ligand.[43] Although there is evidence that these small molecules or factors enhance HSPC expansion, there are no data yet to suggest that they can enhance the yield of reticulocytes when cultured on a large scale.

Perhaps the most exciting advancement in erythroid progenitor manipulation is the recently reported overexpression of BMI1 in human HSPC, which gave a 10^{12} fold increase of erythroblasts.[44] Not only does the extensive expansion give the potential for higher yields (assuming the cells do not differentiate spontaneously when grown in large volumes), it also confers the opportunity for further genetic manipulation due to the extended time frame. Genetic manipulation in the form of *YTHDF2* knockdown also generated a reported 14.3-fold increase in CD34$^+$ frequency in the culture conditions used by a separate group of researchers.[45] Alternatively, better biomimicry of the stem cell niche to recapitulate conditions *ex vivo* has the potential to maintain HSPC stemness for longer periods and therefore increase yield; however, these technologies still require further development.[46-52] It will be very interesting to see if applications of these innovations can translate into higher yields for large-scale mRBC production.

Media composition and optimization

As well as exploiting cell-intrinsic properties, the base medium composition could be further developed and supplemented. Erythroid progenitors are generally cultured in Iscove modified Dulbecco medium (IMDM) and laboratories maintain consistency with suppliers whenever possible. There are many different IMDM commercially available, some better than others in terms of supporting the proliferation and enucleation of erythroid progenitors. Studies are needed to determine exactly what nutrients are required to support the highest proliferation rates of HSPC and erythroid progenitors in culture, particularly important when culturing at high cell densities. Interestingly Heshusius *et al.* supplemented their IMDM with nucleosides and a range of trace elements to make a more defined GMP-compliant medium.[7] Zhang and colleagues[13] added folic acid and selenium to their large-scale cultures of human cord blood CD34$^+$ cells. An experimental approach, using parallel stirred tank micro-bioreactors, is needed to identify the definitive media and supplements to use for erythroid culture.

The lipid sources added to base media by different laboratories also vary significantly, with some groups favoring different amounts of plasma, serum (human or bovine) or serum-free conditions supplemented with animal, human or plant-derived lipid-rich reagents. For compliance with GMP, animal sources must eventually be substituted, which can have an impact on yields. In their recent report Heshuvius *et al.* also highlighted the importance of albumin purity for proliferation.[7] Interestingly, Wilkinson *et al.*[53] showed that 0.1% human serum albumin can be replaced by 0.1% polyvinyl alcohol for cultures of human umbilical cord blood-derived CD34$^+$ cells but as yet this observation has not been tested on a large scale.

Glucocorticoids

The importance of glucocorticoids in promoting stress erythropoiesis was originally discovered in avian and mice studies[54,55] and glucocorticoids have been used to

Table 1. Summary of recently published, large-scale erythroid culture systems, with expansion and enucleation rates where provided as well as bioreactor and GMP/non-GMP media constituents where applicable. Exhaustive reviews of small-scale erythroid cultures can be found elsewhere.[20,22,25]

Source and culture period	General protocol	Expansion	Enucleation rate	Key points	Reference
Cord blood, 33 days	Two-stage	$2.3x10^8$ by extrapolation	>90%	First demonstration of bioreactor use; 1 L cultures in wave-type bioreactor; non-GMP (use of BSA)	Timmins *et al.*, 2011
Peripheral blood CD34$^+$ cells	Three-stage	$6.15x10^4$ fold Large cultures (actual yield)	68%	1 culture of 2.5 mL packed filtered mRBC under GMP conditions autologous human transfusion	Giarratana *et al.*, 2011
Peripheral blood CD34$^+$ cells, 20 days	Three-stage	$>10^4$ fold Large cultures (actual yield)	55-95%	5 mL packed filtered mRBC, constant batch feeding in spinner flasks (no medium changes); non GMP	Griffiths *et al.*, 2012
Peripheral blood and cord blood CD34$^+$ cells 20 days	Three-stage	$>10^5$ fold Large cultures (actual yield)	63%	Large scale cultures (~25 L) 10 mL packed filtered mRBC using constant batch feeding in spinner flasks under GMP conditions	Kupzig *et al.*, 2017
Cord blood CD34$^+$ cells	Four-stage	$2.9x10^5$ fold Large cultures (actual yield)		Large scale culture in rotating wall vessels; non-GMP (use of 15% FBS in steps 2 & 3)	Zhang *et al.*, 2017
Peripheral blood MNC (no CD34$^+$ cell isolation) 21 to 37 days (due to expansion stage)	Three-stage	10^7 fold by extrapolation	> 90%	G-Rex bioreactor GMP compliant: serum-free and plant derived lipids for expansion; 5% human plasma for differentiation	Heshusius *et al.*, 2019

GMP: Good Manufacturing Practice; BSA: bovine serum albumin, mRBC: manufactured red blood cells, FBS: fetal bovine serum; MNC: mononuclear cells.

increase the yield of human mRBC.[26,29,56,57] Three of the larger-scale erythroid culture protocols reported to date incorporate glucocorticoids, using either dexamethasone in serum-free conditions[7] or hydrocortisone in the presence of 5% plasma[5] or in serum-free conditions.[27]

Recent studies have highlighted both the importance of lipid metabolism during terminal erythroid differentiation[58] and the fact that exposure to glucocorticoids can affect the lipid metabolism in cultured erythroid cells.[59,60] For erythroid cells cultured in the presence of hydrocortisone, the defect in lipid metabolism and resulting fragility of mRBC can be counteracted by supplementing the medium with cholesterol-rich lipids.[60] Interestingly though, addition of cholesterol-rich lipids slightly accelerated differentiation[60] and Heshuvius *et al.* reported that the addition of plasma caused premature differentiation in the presence of dexamethasone.[7] The interplay between glucocorticoid exposure time and lipid metabo-

Table 2. Highlighted strategies to improve current culture systems for manufactured red blood cells.

Improving yield & quality of mRBC	
Starting material	• selection of donors with genetic markers linked to specific RBC traits (high hemoglobin, packed cell volume or RBC count) • selection of donors with consistently high HSPC counts • use of whole mononuclear cell population (PBMNC) not just $CD34^+$ cells • genetic manipulation of starting material to enhance proliferation and compatibility
Media composition and supplements	• slow down attrition of HSPC self-renewal capacity • maximize expansion of erythroid progenitors (CFU-E) • maximize enucleation and maintain nascent reticulocyte viability
Filtration	• novel filtration technology to minimize loss of mRBC • maturation of mRBC to erythrocytes will enhance filtration efficiency
Storage	• optimal reticulocyte storage conditions to minimize loss of filtered mRBC until transfusion • maturation of mRBC to erythrocytes to enhance storage times
GMP-compliance and reproducibility	
Starting material	• minimize stem cell donor variability and cell loss during isolation
Media composition and supplements	• use defined reagents of known reliability and controlled provenance • multiple suppliers identified for key reagents • supplier surveys and site visits
Bioreactors and filtration	• closed systems • scalable GMP-compliant bioreactors with ease of use • close monitoring of the culture growth • GMP-compliant filtration process
Other considerations	• define release criteria of product and storage times • identification of optimum therapeutic dose of mRBC
Cost reduction	
Starting material	• use of PBMNC to circumvent expensive $CD34^+$ cell isolation
Media composition and supplements	• defined in-house media constituents • in-house growth factors • replace or reduce the most expensive constituents (e.g. holotransferrin) • develop protocols/bioreactors that use less medium overall without affecting the yield and quality of mRBC obtained • enhance media to enable increases in cell density
Bioreactors and filtration	• fully automated culture processes • automated filtration • minimal footprint and labor requirement

RBC: red blood cells; mRBC: manufactured RBC; HSPC: hematopoietic stem and progenitor cells; PBMNC: peripheral blood mononuclear cells; CFU-E: colony-forming unit – erythroid; GMP: Good Manufacturing Practice.

lism needs further investigation to maximize yield without compromising mRBC quality.

Holotransferrin

The majority of current protocols use between 0.33 and 0.7 mg/mL holotransferrin isolated from human plasma,[5-7] which represents the highest costing individual culture reagent. Holotransferrin is the natural carrier used to deliver iron to the developing erythroid cell by binding to CD71 and being internalized. It is then recycled by the cell and released back into the medium in its apo-form. A cheaper, plant-derived recombinant holotransferrin, optiferrin, is available but this is still expensive. Theoretically, the transferrin concentration could be reduced in culture media as long as iron is also supplemented to bind apotransferrin, without causing cell toxicity or increasing the likelihood of bacterial growth. For example, Timmins and colleagues used 0.12 mg/mL holotransferrin in combination with 900 ng/mL ferrous sulfate and 90 ng/mL ferric nitrate.[27] Olivier *et al.* used holotransferrin at a concentration of 0.05 mg/mL and 3 mM FeIII-EDTA in small scale immortalized pluripotent stem cell cultures, reportedly without affecting the yield of reticulocytes, but the potential impact on mean cell hemoglobin concentrations and viability during storage of the mRBC produced was not measured.[61] Other iron supplements that could be tested include reagents that would deliver iron to erythroid cells in a CD71-independent manner. These include the small lipophilic molecule hinokitiol that can carry iron across the cell membrane into erythroid cells[62,63] or alternatively, ferric carboxymaltose and iron sucrose, both already prescribed to patients suffering from iron deficiency.[64]

Bioreactors and growing erythroid cells at larger scale

The majority of erythroid cultures described in the literature are small and rely on the use of static tissue plastic flasks. For the reported larger scale cultures, a variety of culture vessels have been utilized. The original 2.5 mL packed mRBC produced under GMP conditions and tested in a single volunteer were cultured in static plastic flasks.[5] Spinner flasks (of 1.5 L and 3 L volumes) have since been used successfully from day 7 onwards for constantly batch-fed cultures reaching a volume of ~28 L to produce 10 mL of packed filtered reticulocytes.[6] Zhang *et al.* used rotating wall vessels to grow $2x10^8$ cells from cord blood $CD34^+$.[13] Most recently, Heshusius and colleagues used a 1 L gas-permeable, rapid expansion bioreactor (G-Rex; Wilsonwolf) which, as well as facilitating partial media replenishment, allowed 90% of the expansion medium to be removed and replaced by differentiation medium. Although the expansion was 10-fold lower in the G-Rex compared to static dishes, the enucleation rate was similar and by extrapolation the authors predicted that ~4.5 mL mRBC could be produced using this bioreactor.[7] The cell numbers from these studies are encouraging but are still a long way from the prediction by Timmins *et al.*,[27] who suggested it may be possible to produce 500 units from a single cord blood donation.

There are still many types of bioreactors to choose from and explore further for erythroid culture including: (i) continuous stirred tank bioreactors or spinner flasks which contain internal impellers; (ii) fluidized bed bioreactors in which cells are kept in suspension by the culture medium moving upwards; (iii) rocking heated platforms (wave-like bioreactors) onto which large disposable bags are attached; (iv) rotating wall vessel bioreactors also known as roller bottles and finally, (v) multi-layered static flasks. Whatever becomes the bioreactor of choice for erythroid cell culture, it will need to facilitate higher density culture, be scalable and incorporate automation. In the long term, this will make erythroid culture more cost-effective by: (i) reducing labor costs - cells would be cultured in a single container making the cultures easier to feed and less labor-intensive, with the possibility of automated, remote feeding of media and/or specific depleted nutrients; (ii) reducing the footprint and space required for each batch production; (iii) easing scale up; and (iv) minimizing human error and batch-to-batch variation by carefully controlling different parameters (such as pH, agitation and oxygenation) for optimal culture conditions. One can eventually then imagine rooms filled with bioreactors manufacturing mRBC continuously at scale for clinical use.

The challenge for producing mRBC in any of the above types of bioreactors type lies in the variety of culture conditions required during this 3-week process. All cultures are initiated from a small number of HSPC or approximately $100x10^6$ PBMNC, seeded in a small volume of medium. The erythroid cells then proliferate reaching a fold expansion of $>10^5$ and requiring low cell densities for optimum growth ($2\text{-}8x10^5$ cells/mL) or medium replenishment (constant *versus* repeated batch feeding). Orthochromatic erythroblasts then enucleate and are relatively fragile during this process. Moreover, erythroid cultures are inherently asynchronous, a proportion of cells start enucleating while others are still proliferating; this is noticeable in the last week of culture. To maximize yield, culture conditions have to support cells at different stages of terminal differentiation and nascent reticulocytes must remain viable until the highest percentage enucleation is reached. A manufacturing process that uses a bioreactor efficiently, minimizing the transitions between different types of culture vessels during the 21 days of culture, producing a high yield of reticulocytes and automating feeding, still needs to be identified.

Once the final product is made (i.e., mRBC have been filtered and stored), new cultures have to be reinitiated using new donor-derived HSPC. This is where immortalized pluripotent stem cells or an immortalized cell line that enucleates efficiently would be a game-changer as stocks of the same cell phenotype could be maintained.

Reticulocyte filtration and storage

At the end of the procedure, large-scale erythroid cultures need to be volume reduced and filtered to separate the mRBC from the nucleated cells and expelled nuclei (also known as pyrenocytes). Currently, the filters used are dead-end leukoreduction filters routinely used by blood banks. These have been designed to filter whole blood, which typically consists of ~ $5x10^9$ RBC and only ~$5x10^6$ nucleated cells per milliliter of blood. In comparison, the percentage enucleation for mRBC cultures is approximately 80% and the medium contains pyrenocytes as well as free DNA released by disintegrated nuclei.

It is notable that very few studies on mRBC yield test the filterability of their final product or report the yield after filtration. This is a key parameter as the mRBC must be purified before clinical use and this process currently alters the yield by 30-50%. Strategies and more research are needed to improve reticulocyte recovery after filtration, which will lead to a dramatic increase in yield. There are open-ended filter technologies, such as tangential flow filtration systems or acoustic resonance cell filtration,[65] which could potentially be used but are as yet untested. More experimental approaches include lab-on-a-chip microfluidic label-free reticulocyte-sorting methods.[66] All these possibilities need testing and optimizing on a large culture scale which is expensive, therefore, highlighting filtration as a key area of research calling for innovation as well as commercial investment and collaboration.

Once filtered, the mRBC need to be stored whilst quality control tests are carried out and until they can be transfused into a patient. As reticulocytes, mRBC are more fragile than RBC and more optimal storage conditions need to be developed. An alternative approach is to promote maturation of cultured reticulocytes into *bona fide* erythrocytes, which is another area of active research.

Compliance with Good Manufacturing Practises and quality control

Finally, it should be remembered that the challenge for mRBC production is not only to deliver a manufacturing process that can produce enough mRBC at scale but to develop a process that is GMP-compliant. Often GMP compliance includes the use of clean rooms, highly trained staff specialized in GMP, using closed processes to minimize any risk of infection, robust batch manufacturing protocols, as well as specific manufacturing processing and quality release criteria which are not required in standard R&D laboratories. The challenges of GMP compliance on large-scale erythroid cultures for a transfusion product may have an impact on yield, add pressure to culture times and increase costs, so will need to be considered and planned for from the outset.

Summary

Technologies to revolutionize transfusion options for patients with anemia, in particular for those receiving regular life-long transfusions, are much needed. There is great potential for laboratory-grown mRBC to be used in transfusion practice once production is mastered at scale. In the meantime, these culture systems at smaller scale have proven to be brilliant tools for understanding human erythropoiesis and optimizing culture methodology. The efficient production of mRBC at scale is now essentially a biotechnological challenge that requires multidisciplinary efforts. We have highlighted some of the key areas, breakthroughs and challenges (summarized in Table 2), in which investment together with intensive research into further optimization of culture systems and use of bioreactors at scale are needed to make the clinical use of adult therapeutic doses of mRBC become a reality. Focused research and collaboration between academics, blood banks, commercial entities and new spinouts, especially around the use of RBC-based therapeutics, will no doubt help to drive the development and efficiency of mRBC production under GMP conditions into the clinic.

Disclosures

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Contributions

SP, CES and AMT wrote the review together and all authors approved the final submitted version.

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