Cell Transplantation
Volume 29: 1–5

DOI: 10.1177/0963689720946658
journals.sagepub.com/home/cll
SAGE

In Vitro Generation of Red Blood Cells from Stem Cell and Targeted Therapy

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Abstract

Red blood cell (RBC) transfusion is a common therapeutic intervention, which is necessary for patients with emergency or hematological disorders to reduce morbidity and mortality. However, to date, blood available for transfusion is a limited resource, and the transfusion coverage system still depends on the volunteer-based collection system. The scarcity of blood supplies commonly develops because of local conditions that transiently affect collection. Moreover, donor-derived infectious disease transmission events also remain a risk. Thus, there is a huge demand for artificial blood. The production of cultured RBCs from stem cells is slowly emerging as a potential alternative to donor-derived red cell transfusion products. In this concise review, we summarize the recent in vitro expansion of RBCs from various stem cell sources, targeted therapy, prospects, and remaining challenges.

Keywords

stem cell, red blood cell, targeted therapy, diversity

Introduction

Red blood cells (RBCs) are anucleate blood components indispensable for oxygen delivery. RBC transfusion is a life-saving treatment in numerous therapies. The current system is based on voluntary blood donations with several shortcomings, such as chronic shortages for rare blood groups, sporadic restrictions in association with natural or man-made disasters, insufficient development of blood collection systems, and so on.[1]

The increased usage by aging population and the detrimental effects of storage on RBCs will eventually lead to insufficient blood supply[1]. In addition, the clinical demand for RBC transfusion remains high in surgical interventions and hematologic malignancies. Rather, the most threatening scenarios involve long-term disruption of the supply chain because of a major pandemic that would decrease the ability of the population to donate blood for an extended period of time[2]. In order to alleviate the intensified imbalance and shortfalls in blood supply and demand, therapeutic in vitro generation of RBCs via biotechnologies became an urgent need in global demand for transfusion applications. Many attempts had been made worldwide for in vitro generation of blood cells from different stem cell sources[3] because their immediate cell sources and precursors can be cryopreserved and stored long-term for repeated study.

Formation of RBCs from Hematopoietic Stem Cells (HSC)

HSCs are rare cells present in the blood and bone marrow that are capable of generating an entire hematopoietic system with their pluripotency and self-renewal properties. HSCs are also the stem cells that give rise to other mature blood cells, such as RBCs, platelets, and white blood cells. The formation process is regulated by signaling through both

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Submitted: June 8, 2020. Revised: July 7, 2020. Accepted: July 13, 2020.

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external factors, such as cytokines and fibronectin, and intracellular factors, such as transcription factors and miRNAs[4].

CD34+ also plays an important role in RBC production from HSCs[5]. The CD34 is a glycoprotein found in the bone marrow and expressed in early HSCs and are also found in cord blood (CB) and in small amounts as granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood stem cell concentrates in the peripheral blood[6]. Several groups have reported that CD34+ cells from CB and peripheral blood can be used to reproduce the hematopoietic process[7,8]. Nowadays, the differentiation process of HSCs into RBCs has already been thoroughly elucidated. HSCs differentiate into common myeloid progenitors and megakaryocyte-erythroid progenitors. Then, HSCs sequentially differentiate into unipotent progenitors restricted to the erythroid lineage. These unipotent erythroid progenitors are composed of burst-forming unit erythroid, colony-forming unit erythroid, and the morphologically recognizable erythroblast series that terminally differentiate into orthochromatic erythroblasts. Ultimately, orthochromatic erythroblasts enucleate into reticulocytes and mature into RBCs[4].

The late-stage maturity RBCs have been successfully generated by promoting erythroid differentiation of primary HSCs derived from CB units[9–11], mobilized apheresis products[7], or cell fractions discarded during the leukoreduction process of adult blood donations[12]. The generation of RBCs from HSCs takes about 21 days in vitro[10,13], making it unaffordable for clinical applications. According to Lalita and colleagues, using transforming growth factor β1 can significantly accelerate the process of *in vitro* RBC formation up by 3 days from HSCs by stimulating mitophagy and thereby making the large-scale production possible[14].

Nevertheless, the low number of HSCs is achieved even by donation and is hard to scale up. This is also the reason why human pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), currently represent the alternative approach for blood cells and components' derivation. To improve the scalable industrial production of RBCs, as a consequence, ESCs and iPSCs are investigated as an alternative stem cell source as their indefinite expansion capacity in vitro[15–17].

Formation of RBCs from ESCs

Human ESCs (hESCs) are capable of unlimited proliferation while maintaining the ability to form all the cells of the body, including blood cells[18,19]. It provides a potentially inexhaustible and donorless source of cells for human therapy.

Hematopoietic differentiation of hESCs has been widely investigated in vitro, and hematopoietic precursors have been identified in differentiating hESC cultures[18,20,21]. It has been reported that primitive erythroid cells can be produced from hESCs by embryoid body (EB) formation and coculturing with stromal cells[21–23]. However, the efficiency of differentiation of hESCs into homogenous RBCs still needs to be improved. Enforced expression of HOXB4 has been found to enhance the production of hematopoietic progenitors but has no effect on the maturation of RBCs[24]. Thus, another critical issue is whether hESCs can generate terminally mature progenies with normal function and be utilized in the clinic. Ma and colleagues recently developed a method for the efficient production of hematopoietic progenitors from hESCs by coculture with murine fetal liver-derived stromal cells[25].

Lu and colleagues were able to grow blood types A, B, O, and both Rhesus D positive and Rhesus D negative but unable to produce the O Rhesus D negative blood type, the so-called "universal" donor[15]. The differentiation of hESCs into functional oxygen-carrying RBCs on a large scale (1010–1011 cells/six-well plate), with up to 60% enucleation rate[15]. Elcheva and colleagues subsequently reported that GATA2 and TAL1 transcription factors are capable to directly convert hESC to endothelium having the potential to transform into blood cells. This study accelerates and enhances the generation of 33 million $CD43^+$ cells from 1 million transduced H1 hESCs after 7 days of expansion[26]. However, the clinical relevance of ESC is limited due to ethical and immunological concerns[27], and so the attention has turned to iPSCs.

Formation of RBCs from iPSCs

iPSCs are embryonic-like cells reprogrammed from adult somatic cells through retroviral transduction of defined factors and possess various properties of embryonic stem cells[28]. iPSCs can be created from dermal skin fibroblasts, and patient-specific iPSCs could avoid the immune rejection problems that might occur if heterologous sources of ESCs were used[29]. As a consequence, iPSCs are also investigated as an alternative stem cell source[17].

The sequential addition of cytokines at defined concentrations led to the in vitro differentiation of iPSCs into mature blood cell types, which is also the most difficult step in the manufacture[30]. The generation of RBCs from iPSCs takes about 26 days in vitro[17]. The differentiation of iPSCs by the formation of human EBs (hEBs) in EB medium takes about 20 days. Then, hEBs differentiate into the stage of mature cultured RBCs in the presence of supporting cytokines, including stem cell factor (SCF), erythropoietin (EPO), and interleukin-3 (IL-3) for 6 days[17]. However, in vitro erythropoiesis from iPSCs is currently limited due to low efficiency and unphysiological conditions of common culture systems. Especially, the absence of a physiological niche may impair cell growth and lineage-specific differentiation.

In 2019, Bernecker and colleagues reported a simplified but robust, xeno-free and feeder-free, culture system for prolonged RBC generation using a low concentration of supporting cytokines, such as SCF, EPO, and IL-3[31]. Colonies of undifferentiated human iPSCs were transferred into low-binding plates to induce EB formation for 5 days. Then, the spherical EBs were cultured in adherent plates in albumin polyvinyl alcohol essential lipid medium containing

SCF, EPO, and IL-3, which was changed weekly. Within 2 weeks, a hematopoietic cell-forming complex was established, from which hematopoietic cells were continuously released into the supernatant and harvested. Ultimately, cells released into the supernatant were harvested and differentiated into RBCs in a three-phase erythropoiesis system for 18 days[31]. This model is more cost affordable and less artificial when compared with conventional systems.

Formation of RBCs from Mesenchymal Stem Cells (MSCs)

Although RBCs have been derived from human PSCs, the risk of potential tumorigenicity cannot be ignored, and a majority of these cells produced from PSCs express embryonic ε-globins and fetal γ-globins with little or no adult β-globin and remain nucleated[32]. Lu and colleagues reported a method to generate RBCs from human hair follicle MSCs (hHFMSCs) by enforcing OCT4 gene expression and cytokine stimulation[32]. The adult β-globin chain with a minimum level of the fetal γ-globin chain was found in the cells generated from hHFMSCs. Moreover, these cells formed enucleated RBCs with a biconcave disc shape via multiple maturation events. In this study, the authors also revealed that OCT4 regulated the expression of genes associated with both pluripotency and erythroid development during hHFMSC transdifferentiation toward RBCs[32]. Other than that, it has also been found that MSCs can promote CD34+ HSC proliferation with preserved RBC differentiation capacity[33]. These findings indicate that mature RBCs can be derived from adult somatic cells, which may also serve as an alternative source of RBCs for potential autologous transfusion.

Targeted Therapy

To date, the use of in vitro stem cell-derived RBCs has not proved practical for routine transfusion. Despite the major worldwide research efforts to achieve the goal of RBC production have received great attention, the problems with large-scale production and cost-effectiveness have yet to prove practical usefulness. Therefore, although vast advances have been made in stem cell-derived RBC research, it is still in the beginning stages for clinical transfusion use by making RBCs available, in both quantity and quality.

RBCs, however, have additional clinical applications that do not require a large number of cells. Thus, there are some realistic intermediate therapeutic goals that could be achieved with the current technology, such as drug delivery, drug discovery, and reagent RBCs for antibody identification[34,35].

RBC with CD47 expressed on the cell surface is signaling to the immune system to avoid RBC uptake[36]. The recent progress of in vitro differentiation of stem cells into mature RBCs has boosted the possibility of drug discovery. In 2006, Chang and colleagues reported that the use of RBCs for systemic drug delivery was obtained in mouse models for Hemophilia, an X-linked recessive congenital disorder of coagulation due to factor VIII or IX deficiency[37]. In 2017, Doulatov and colleagues have reported the drug discovery for Diamond-Blackfan anemia using reprogrammed hematopoietic progenitors[38].

In order to predict the suitability of donors with rare blood types for alloimmunized patients, in vitro tests via RBCs are used. The RBCs from rare donors are usually with limited numbers. Thus, the generation of RBCs in vitro from mononuclear cells that are usually discarded during the leukoreduction process (or from iPSCs derived from these cells) may represent valuable substitutes for in vivo-generated reagent RBCs in these assays. Recently, the identification of drugs for personalized therapy of diverse disorders, such as inducers of hemoglobin F production for thalassemia and sickle cell anemia, inhibitors of 11-kDa nonstructural protein-mediated caspase-10 activation to prevent B19 parvovirus infection, and cellular-based antimalarial therapies, was considered to use in vitro assays by *ex vivo* expanded erythroid progenitor cells[39]. Substantial progress in these intermediate clinical applications is likely to ensure that the in vitro artificial RBCs will become a reality in the future.

Prospects and Challenges

These studies have provided the foundation for rational monitoring of in vitro differentiation into the mature RBCs to establish a reliable, efficient RBC generation protocol. Especially, the differentiation of iPSCs into RBCs provides opportunities for the development of novel technologies for manufacturing patient-customized blood products. Equally importantly, these studies also make it possible to proceed through the whole protocol from the stem cell generation to RBC maturation under good manufacturing practice conditions, which will be an essential requirement to use in vitro-generated biomaterials in the clinical field.

One of the next key steps toward clinical usage for blood shortage is bioreactor based-technology for further scaling up of cell production. It is a significant technical challenge to produce a sufficient number of RBCs to contribute to the existing transfusion system, even a few percent of the total RBCs transfused. The maximal concentration of cells that can be achieved in a bioreactor is the major issue. Since a unit of blood contains approximately 1–2 $\times$ 1012 cells, more than 1000 l of medium would be necessary to produce that many cells in static flask culture, which allows a maximal density of approximately 2 $\times$ 106 cells/ml[2,40].

Summary

All of the cell sources discussed above have the potential to eventually reach the clinical needs of RBCs. These studies would also surely promote the development of RBC clinical treatment. The race to develop the winning technology of

RBC generation will be one of the great scientific and technological interests. Overall, these continuous efforts to establish advanced strategies for a cost-effective, highly potent RBC culture system combined with engineering techniques would ultimately contribute to the practical utilization of in vitro-generated RBCs in the near future.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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