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 supply¹. 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 time². 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 sources³ 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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external factors, such as cytokines and fibronectin, and intra-
cellular factors, such as transcription factors and miRNAs\textsuperscript{4}.

CD34\textsuperscript{+} also plays an important role in RBC production from HSCs\textsuperscript{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
colonystimulating factor (G-CSF) mobilized peripheral
blood stem cell concentrates in the peripheral blood\textsuperscript{6}. Several
groups have reported that CD34\textsuperscript{+} cells from CB and
peripheral blood can be used to reproduce the hematopoietic
process\textsuperscript{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 sequen-
tially differentiate into unipotent progenitors restricted to the
erthroid lineage. These unipotent erythroid progenitors are
composed of burst-forming unit erythroid, colony-forming
unit erythroid, and the morphologically recognizable ery-
throblast series that terminally differentiate into orthochro-
matic erythroblasts. Ultimately, orthochromatic
erythroblasts enucleate into reticulocytes and mature into
RBCs\textsuperscript{9–11}.

The late-stage maturity RBCs have been successfully
generated by promoting erythroid differentiation of primary
HSCs derived from CB units\textsuperscript{9–11}, mobilized apheresis produ-
cts\textsuperscript{7}, or cell fractions discarded during the leukoreduction
process of adult blood donations\textsuperscript{12}. The generation of RBCs
from HSCs takes about 21 days in vitro\textsuperscript{10,13}, making it unaffor-
dable for clinical applications. According to Lalita and
colleagues, using transforming growth factor \(\beta\)1 can signif-
ically accelerate the process of \textsuperscript{in vitro} RBC formation up
by 3 days from HSCs by stimulating mitophagy and thereby
making the large-scale production possible\textsuperscript{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
core as their indefinite expansion capacity in vitro\textsuperscript{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\textsuperscript{18,19}. It provides a potentially inexhaus-
tible 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\textsuperscript{1,8,20,21}. It has
been reported that primitive erythroid cells can be produced
from hESCs by embryoid body (EB) formation and coculturing
with stromal cells\textsuperscript{21–23}. However, the efficiency of dif-
ferentiation of hESCs into homogenous RBCs still needs to
be improved. Enforced expression of HOXB4 has been
found to enhance the production of hematopoietic progeni-
tors but has no effect on the maturation of RBCs\textsuperscript{24}. Thus,
another critical issue is whether hESCs can generate termi-
nally 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\textsuperscript{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\textsuperscript{15}. The differentiation of hESCs into func-
tional oxygen-carrying RBCs on a large scale (1010–1011
cells/six-well plate), with up to 60\% enucleation rate\textsuperscript{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 genera-
tion of 33 million CD43\textsuperscript{+} cells from 1 million transduced H1
hESCs after 7 days of expansion\textsuperscript{26}. However, the clinical
relevance of ESC is limited due to ethical and immunological
concerns\textsuperscript{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 fac-
tors and possess various properties of embryonic stem
cells\textsuperscript{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\textsuperscript{29}. As a consequence, iPSCs are also investigated
as an alternative stem cell source\textsuperscript{17}.

The sequential addition of cytokines at defined concentra-
tions led to the in vitro differentiation of iPSCs into mature
blood cell types, which is also the most difficult step in the
manufacture\textsuperscript{30}. The generation of RBCs from iPSCs takes
about 26 days in vitro\textsuperscript{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\textsuperscript{17}. However, in vitro erythro-
poiesis 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\textsuperscript{31}. Colon-
ies 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 albu-
min 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. 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. Lu and colleagues reported a method to generate RBCs from human hair follicle MSCs (hHFMSCs) by enforcing OCT4 gene expression and cytokine stimulation. 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. Other than that, it has also been found that MSCs can promote CD34+ HSC proliferation with preserved RBC differentiation capacity. 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.

RBC with CD47 expressed on the cell surface is signaling to the immune system to avoid RBC uptake. 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. In 2017, Doulatov and colleagues have reported the drug discovery for Diamond-Blackfan anemia using reprogrammed hematopoietic progenitors.

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. 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 × 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 × 106 cells/ml.

**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.

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