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Review

Research advances in erythrocyte regeneration sources and methods *in vitro*

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ABSTRACT

Erythrocytes (red blood cells, RBCs) facilitate gas exchange in the lungs and transport oxygen to the tissues. The human body must maintain erythrocyte regeneration to support metabolically active cells and tissues. In many hematological diseases, erythrocyte regeneration is impaired. Researchers have studied erythrocyte regeneration for many years both *in vivo* and *in vitro*. In this review, we summarize the sources and main culture methods for generating mature and functional red blood cells *in vitro*. Hematopoietic stem cells (HSCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are classic sources for erythrocyte regeneration. In addition, alternative sources such as immortalized adult human erythroid cell lines and transformed fibroblasts have also been generated and have produced functional red blood cells. The culture systems for erythrocytes differ among laboratories. Researchers hope that improvements in culture techniques may contribute to improved RBC outcomes for blood transfusions, drug delivery and the treatment of hematological diseases.

1. Introduction

Oxygen delivery to cells and tissues is very important in developmental, physiological and regenerative processes. Erythrocytes perform this important task. Erythrocytes are produced by the differentiation of hematopoietic stem cells (HSCs) in their microenvironment (niche) *in vivo*. Erythropoiesis is divided into three major stages. Stage I (early erythroid differentiation) involves the staged differentiation of HSCs into erythroid progenitor cells (burst forming units-erythroid, BFU-E, and colony forming units-erythroid, CFU-E). Stage II (terminal erythroid differentiation) involves the differentiation of erythroblast and the enucleation of late erythroblast, including proerythroblast (Pro-E), basophilic erythroblast (Baso-E), polychromatic erythroblast (Poly-E) and orthochromatic erythroblast (Ortho-E). Stage III (maturation) involves the development of reticulocytes (Retics) into mature red blood cells (RBCs).[1]

For many years, researchers have been very interested in studying erythrocyte regeneration, not only to elucidate the developmental mechanism,[2–5] but also to improve hematological disease treatments.[6,7] The worldwide shortage of blood for transfusions has become increasingly evident, especially for rare blood types. There are three stem cell sources from which erythrocytes are artificially induced *in vitro*: HSCs, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs).[8] ESCs and iPSCs differ from HSCs in their *in vitro* proliferative capacity, and they provide unlimited cellular resources for inducing erythrocyte production. Recently, studies have found alternative ways to generate erythrocytes from immortalized cell lines or transformed fibroblasts. For erythrocyte regeneration *in vitro*, three major bottlenecks need to be overcome: limited cell proliferation, difficulty in transforming embryonic globin into adult globin, and low enucleation rates. We summarize the research advances in erythrocyte regeneration sources and methods in this review (Fig. 1, Table 1).

2. From native HS/PCs to erythrocytes

Studies on native hematopoietic stem and progenitor cells (HS/PCs) have provided essential information about human erythropoiesis. In the last decade, researchers have refined methods of

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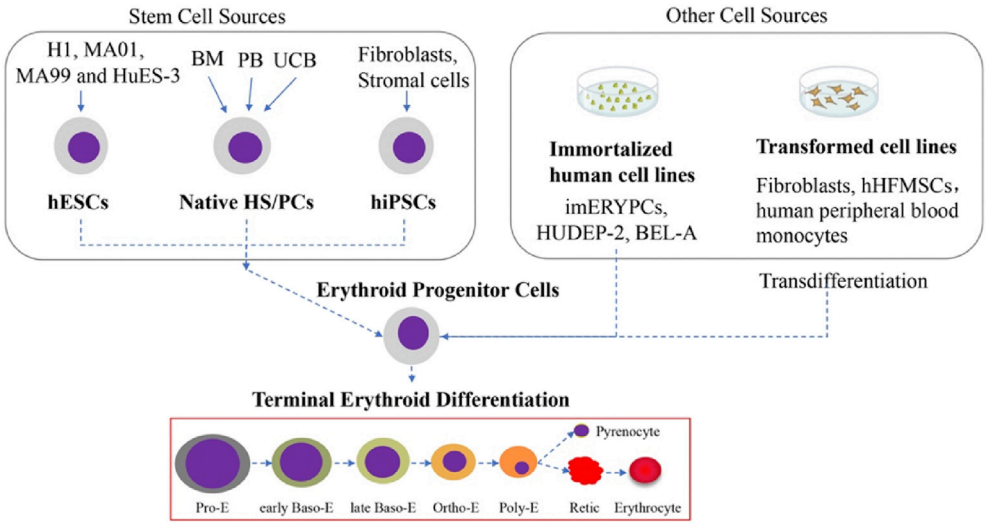

Fig. 1. Multiple Sources for Erythrocytes Regeneration. Native HS/PCs, hESCs, hiPSCs, immortalized human cell lines (imERYPCs, HUDEP-2, BEL-A), and transformed cell lines are sources for erythrocytes regeneration *in vitro* researches. Dotted line stands for the indirect cell type transformation.

Table 1
The mainly erythrocyte regeneration sources and methods *in vitro*.

Cell types	Origin and sources	Feeder cells	Hemoglobin expression	Enucleation ratio	References
Native HS/PCs	BM- or PB-derived CD34 + cells	human mesenchymal cells or MS-5 cell line	β-globin	> 95%	Douay, L. group[19,20]
	UCB-derived CD34 + cells	feeder cell-free	γ- and β-globin	77.5% ± 2.8%	Miharada, K. et al[21]
hESCs	H1, MA01, MA99 or HuES-3 cell lines	mFLSCs or mitomycin C treated MEFs	γ- and β-globin	> 60%	Ma, F. et al[31] and Lu, S.J. et al[32]
hiPSCs	IMR90 or FD-136 cell lines	mitomycin C treated MEFs	ε- and γ-globin	~ 4%	Lapillonne, H. et al[8]
	PB04 cell line from sickle cell disease (SCD)	mitomycin C treated MEFs and MS-5 cell line	ε-, γ- and β-globin	20–26%	Kobari, L. et al[36]
	CTRL-2 c1 or CTRL-1 c3 cell lines	C3H10T1/2 and OP9 cell lines	β- and γ-globin	very low	Fujita, A. et al[38]
	BMSCs and PBEPs from SCD	C3H10T1/2 and OP9 cell lines	β-, ε- and γ-globin	very low	Uchida, N. et al[39]
Transformed cell lines	fibroblasts with OCT4 overexpression	Matrigel-coated plate	β- and ε-globin	very low	Szabo, E. et al[41]
	hHFMSCs	Matrigel-coated dishe	β- with a little γ-globin	very low	Liu, Z. et al[43]
Immortalized cell lines	imERYPCs	feeder cell-free	γ-globin with a little ε- and β-globin	very low	Hirose, S. et al[45]
	HUDEP-2	feeder cell-free	β- without γ-globin	very low	Kurita, R. et al[46]
	BEL-A	feeder cell-free	β- without γ-globin	~ 30%	Trakarnsanga, K. et al[47]

primary human cell culture to obtain larger numbers of erythroid cells from relatively few HS/PCs obtained from bone marrow (BM), bone marrow mobilized cells in peripheral blood (PB), or umbilical cord blood (UCB).

Erythropoiesis was first studied *in vitro* mainly through semi-solid culture in which erythroid progenitors develop into discrete colonies.[9,10] Subsequently, researchers succeeded in the expansion of erythroid progenitors using a liquid culture system.[11–13] Terminal erythroid differentiation has also been reported in a two-step culture system.[14,15] Briefly, peripheral blood mononuclear cells (PBMCs) are initially cultured in expansion media containing different growth factors for approximately 1 week, and then the erythroid precursors are transferred to differentiation culture media containing recombinant human erythropoietin (rhEPO) and grown under reduced oxygen concentrations.

In 1998, Malik, P. et al.[16] developed a single-step liquid culture system *in vitro* for the production of human RBCs from normal HS/PCs using recombinant growth factors to promote terminal erythroid differentiation. Morphologically, the RBCs derived *in vitro* ranged from early polylobulated forms resembling normal reticulocytes to smooth biconcave discocytes. The hemoglobin pattern in the *in vitro*-derived RBCs mimicked the *in vivo* adult or postnatal pattern of β-globin production, with negligible γ-globin synthesis. Almost at the same time, Panzenbock, B. et al.[17] reported an efficient culture system for the amplification and differentiation of erythroid progenitors from UCB cells. The medium was modified from the growth medium established previously for chicken erythroid progenitors. By adjusting the culture conditions and cytokines, such progenitor cells effectively undergo terminal erythroid differentiation in culture. Remarkably, this culture system can also be used for human PB- and BM-derived HS/PCs amplification and differentiation *in vitro*.

In 2002, Migliaccio, G. et al.[18] described a new two-step culture method for mass production of erythroid cells *in vitro* from PB-derived HS/PCs. The culture method involved an early proliferative phase and a subsequent differentiative phase. More than 10 million erythroblasts were generated for each milliliter of blood collected from normal donors or thalassemic patients on day 7 of the proliferative phase. The erythroblasts were composed mostly of $CD45^{low}/GPA^{neg}/CD71^{low}$ cells (90% of erythroblasts). After being transferred to differentiation medium containing EPO and insulin, they progressed to mature erythroblasts with the expression of adult β-globin but without enucleation. At the same time, Douay, L. et al.[19] also described a three-step procedure for the *ex vivo*

expansion of UCB-derived HS/PCs into a pure erythroid precursor population by the sequential application of specific combinations of growth factors in a serum-free culture medium. Extraordinarily, when they were injected into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, the cultured erythroblasts were capable of proliferation and terminal differentiation into mature enucleated RBCs. Three years later, by mimicking the bone marrow microenvironment with cytokines and stromal feeder cells, the same group[19,20] achieved substantial amplification of HS/PCs of diverse origins (almost 2 millions-fold) and more than 95% terminal differentiation into functional mature RBCs, and these cells could survive in NOD/SCID mice. Furthermore, comparing the characteristics of RBCs obtained from HSCs of diverse origins, their results showed that the proliferation capacity of HS/PCs from BM and PB sources was greater than that of HS/PCs from UCB sources. In addition, cultured RBCs (cRBCs) derived from PB and BM mainly contained adult hemoglobin (HbA, 94% ± 2% and 95% ± 10%, respectively), while fetal hemoglobin (HbF, 64% ± 13%) accounted for most of the hemoglobin in UCB-derived cRBCs.[20] In 2006, Miharada, K. et al.[21] developed a four-passage culture protocol that included VEGF and IGF-II in the culture medium to improve upon existing methods for producing enucleated RBCs from UCB-derived HS/PCs. In addition, their improved method produced enucleated RBCs efficiently (with enucleation rates of up to 77.5% ± 2.8%) in the absence of feeder cells.

Later, some groups[22–24] also developed an HS/PC expansion and differentiation procedure to efficiently produce enucleated RBCs by optimizing the use of growth factors and cytokines. Using the optimized culture system, An, X. et al.[25] discovered that BFU-E and CFU-E are characterized by $CD45^{+}GPA^{-}IL\text{-}3R^{-}CD34^{+}CD36^{-}CD71^{low}$ and $CD45^{+}GPA^{-}IL\text{-}3R^{-}CD34^{-}CD36^{+}CD71^{hi}$ phenotypes, respectively. The use of band 3 and a4-integrin enabled us to isolate erythroblasts at specific developmental stages from primary human bone marrow.[23] Transcriptome analyses of terminal erythroid differentiation have shown global patterns and have given us a deeper understanding of the mechanisms of erythroid development.[26,27] These findings provide the standard dynamic molecular profiles for the erythroblast regeneration both *in vitro* and in *vivo*.

3. From hESCs to erythrocytes

hESCs can be propagated and expanded *in vitro* indefinitely, providing a potentially inexhaustible and donorless source of cells for human therapy. As early as 2001, Kaufman, D. S. et al.[28] successfully differentiated hESCs into erythroid progenitor cells by coculturing them with the mouse bone marrow stromal cell line S17 and the mouse yolk-sac endothelial cell line C166. Subsequently, several research groups have clarified that the hematopoietic differentiation of hESCs progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development, and the resulting primitive erythroblasts express embryonic ε- and fetal γ-globin but do not enucleate.[29,30]

In 2008, Ma, F. et al.[31] and Lu, S. J. et al.[32] succeeded in differentiating hESCs to form enucleated erythroid cells with adult β-globin expression upon further maturation *in vitro*. The researchers cocultured hESCs with mouse fetal liver-derived stromal cells (mFLSCs) or mitomycin C treated mouse embryonic fibroblasts (MEFs) to obtain enucleated ripening RBCs that have oxygen carrying capacity, express adult β-globin and have sufficient glucose-6-phosphate dehydrogenase activity.[31] CD81, GPA and CD71 were coexpressed in the hESC-derived erythroid cells. The differentiation of hESCs into enucleated mature RBCs was also successfully achieved using a stromal-free culture system.[32] Although cRBCs have similar oxygen-carrying capacities as those of native RBCs, they mainly express embryonic globin.[32] This suggests that hESCs can be induced to differentiate into adult-type RBCs, but not fully mature RBCs.

4. From hiPSCs to erythrocytes

The development of hiPSCs has been considered a major ethical and technological breakthrough. The hiPSC technology reduces the need to obtain and study hESCs.[33,34] Somatic cells from humans can be used as cell sources for hiPSC generation without ethical problems. In addition, gene therapy using hiPSCs can also be conducted relatively easily. In recent years, hiPSCs have also been manipulated to differentiate into hematopoietic cells.[35] The differentiation of hiPSCs into RBCs has also been reported. In 2010, Lapillonne, H et al. first reported the complete differentiation of hiPSCs derived from human fetal (IMR90) and adult (FD-136) fibroblasts into definitive erythrocytes capable of maturing into enucleated RBCs (~4%).[8] By using hiPS cells derived from sickle cell disease (SCD) patients, the population contained 20–26% RBCs and 74–80% Ortho-E after cultured in the erythroid differentiation media for 25 days.[36]

However, the obtained RBCs all expressed typically produce ε- and γ-globin rather than β-globin.[8,36] hiPSCs from various somatic cells can also produce a large number of erythroid cells with embryonic and fetal-like characteristics.[37] Fujita, A et al.[38] then generated β-globin-expressing definitive erythroid cells through two fibroblast-derived hiPSC lines. In this work, primitive erythropoiesis gradually turned into definitive erythropoiesis during prolonged ES sac maturation, concurrent with the emergence of hematopoietic progenitor cells. GPA and CD34 expression were used as selection markers for primitive and definitive erythroid progenitor cells within the ES sacs before erythroid differentiation. This experimental strategy represents an important step toward the *in vitro* erythroid cell production systems from pluripotent stem cells. hiPSCs from various somatic cells can also produce a large number of erythroid cells with embryonic and fetal-like characteristics. And Uchida, N. et al.[39] successfully simulated erythropoiesis *in vitro* with a system that can efficiently produce β-globin-expressing erythroid cells by using hiPSCs derived from bone marrow stromal cells (BMSCs) and peripheral blood erythroid progenitors (PBEPs) from sickle cell disease (SCD) patients who need reliable production of β-globin. This system will now allow comprehensive testing of genetic strategies aimed at correcting the SCD mutation. Therefore, this important finding will enable the eventual clinical translation of the strategy. Small molecules (isobutyl methyl xanthine (IBMX) and StemRegenin (SR1)) can also be used to increase the β-globin expression of erythroid cells derived from hiPSCs.[40]

To our knowledge, although some hiPSCs can produce erythroid cells with substantial adult β-globin expression, the lack of or low enucleation of blast cells and the low yield of functional RBCs still creates a bottleneck for RBC transfusion.

5. From transformed cell lines to erythrocytes

In addition to the aforementioned progenitors, human dermal fibroblasts can be reprogrammed by expressing OCT4 (also called POU5F1). OCT4-transduced fibroblasts exhibit similar OCT4 expression levels to those of established iPSCs and exclusively give rise to hematopoietic-like $CD45^{+}$ cells.[41] The study uniquely demonstrated the direct conversion of transformed fibroblasts into multipotent cell types. Zhang S. et al. showed that transformed fibroblasts can be induced to differentiate toward erythroid lineages expressing hemoglobin both *in vitro* and *in vivo*.[42] In this process, the hypoxia-mimic chemical cobalt chloride ($CoCl_2$) plays a critical role. Polyploidy giant cells (PGCs) induced from immortalized

fibroblasts express stem cell markers. Most researchers first transform fibroblasts into iPSCs and then induce iPSCs to differentiate into multiple cell types.

By enforcing OCT4 gene expression and cytokine stimulation, human hair follicle mesenchymal stem cells (hHFMSCs) can be transdifferentiated into erythrocutes,[43] even though with low transformation efficiency (1.55% $CD34^+$ cells).

Nouri M et al. showed another way of reprogramming human peripheral blood monocytes to erythroid lineage,[44] just by blocking PU-1 gene expression. PU-1 downregulation lead GATA-1 and hemoglobin overexpression. This strategy may provide another way of erythroid regeneration. These studies described above showed that direct conversions can also be induced through unique strategies.

6. From immortalized human cell lines to erythrocytes

Another strategy to generate erythrocytes is through immortalized erythroid progenitor cell lines. Currently, immortalized human cell lines are useful in studying erythropoiesis because they can be genetically manipulated and infinitely expanded. Erythroid cell lines derived from human primary HS/PCs have been established for studying erythropoiesis and for the large-scale production of RBCs. In 2013, Hirose, S. et al.[45] immortalized erythroblasts by transduction of c-MYC and BCL-XL into multipotent hematopoietic progenitor cells derived from pluripotent stem cells, called imERYPCs, which enabled large-scale erythrocyte production, but the imERYPC-derived erythrocytes mainly express γ-globin with little expression ε- and β-globin and have a very low enucleation ratio.

Kurita, R. and colleagues[46] also established immortalized cell lines that express erythroid-specific markers by immortalizing UCB-derived HS/PCs and hiPSCs using a lentiviral vector containing the Tet-inducible expression system for HPV16-E6/E7. One of the cell lines, the human umbilical cord blood-derived erythroid progenitor (HUDEP-2) cell line, can produce enucleated RBCs with functional hemoglobin expression after induction of differentiation in erythroid differentiation media. However, the efficiency of enucleated RBC production is too low for the large-scale production of RBCs.

By utilizing a similar approach, Trakarnsanga, K. and colleagues[47] immortalized adult bone marrow-derived early erythroblasts, generating a stable line named BEL-A (Bristol Erythroid Line Adult). BEL-A cells can be steadily maintained in the pro-to early basophilic erythroblast phase in expansion media and efficiently differentiated into mature, functional reticulocytes with an enucleation rate of up to 30%. Remarkably, the BEL-A-derived erythroid cells and reticulocytes show no substantive differences compared to those derived from adult PB $CD34^+$ cultures. The BEL-A line demonstrates the great potential of immortalized erythroid lines for the manufacture of novel red cell products for clinical use and as a research tool for the study of erythropoiesis in health and disease, with exciting applications in other research fields.[47]

Further research is needed to determine the side effects of viral transduction related to these newly developed methods of erythrocyte production. In addition, low enucleation rates may be another critical problem that needs to be solved.

7. Discussion

Enucleation is an important biological activity in erythropoiesis *in vivo*, and it is also a rate-limiting process that must be performed to induce various stem cells or erythroid precursors to differentiate into mature RBCs *in vitro*. Low efficiency of enucleation is a very common problem, especially for sources derived from ES and iPSCs, so it is very important to elucidate the regulatory mechanism of enucleation to enable increases enucleation ratio. Understanding of the molecular mechanism of enucleation in late erythroblasts is still in the hypothesis stage. There are several hypotheses, including the apoptosis theory,[48,49] the asymmetric division theory, the bubble transport theory, the signal network regulation theory[50] and the macrophage mediation theory.[51–53] Researchers have used mouse models to demonstrate that the expression of macrophage-specific adhesion molecules plays an important role in mediating enucleation.[52,53] Lodish, H.F. et al. found that there is a substantial difference between human and mouse erythroid development[54]; therefore, elucidating the mechanism of enucleation in human late red blood cells will require much further study. Recent studies have reported that KLF1,[55] dynein,[56] and survivin/EPS15/clathrin[57] play a critical role in human erythroid enucleation.[58] Our unpublished study shows that downregulation of GATA1 can reduce the efficiency of enucleation and that GATA1 rescue can increase enucleation efficiency. We infer that overexpression of key transcription factors in orthochromatic erythroblasts can enhance enucleation efficiency.

In this review, we discussed advances in research seeking to determine sources of erythrocytes and how to regenerate mature and functional red blood cells. Even though the researchers already made several achievement, the production of erythrocytes *in vitro* still needs further fundermental study and functional test. A recent study has revealed that humanized NSGW41 mice show improved human erythropoiesis and platelet formation.[59] This research suggests that the overexpression of certain molecules may contribute to the progression of erythropoiesis. Erythroid regeneration from different sources is regulated by multiple molecules, multiple stages and multiple layers. Autophagy, iron metabolism regulation, and specific cellular organelles are all very important for erythroid regeneration. Effective blood transfusion, drug delivery and hematological disorder treatment strategies are waiting for breakthroughs in erythroid regeneration.

Conflicts of interest

The authors declare no conflict of interest.

Authorship

J. L designed the manuscript, S. S and Y. P collected and analyzed the references, S. S and Y. P wrote the paper, J. L revised and edited the paper.

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