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REVIEW ARTICLE

Recent updates of stem cell-based erythropoiesis

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Abstract
Blood transfusions are now an essential part of modern medicine. Transfusable red blood cells (RBCs) are employed in various therapeutic strategies; however, the processes of blood donation, collection, and administration still involve many limitations. Notably, a lack of donors, the risk of transfusion–transmitted disease, and recent pandemics such as COVID-19 have prompted us to search for alternative therapeutics to replace this resource. Originally, RBC production was attempted via the ex vivo differentiation of stem cells. However, a more approachable and effective cell source is now required for broader applications. As a viable alternative, pluripotent stem cells have been actively used in recent research. In this review, we discuss the basic concepts related to erythropoiesis, as well as early research using hematopoietic stem cells ex vivo, and discuss the current trend of in vitro erythropoiesis using human-induced pluripotent stem cells.

Keywords Red blood cell · Erythropoiesis · Blood · Hematopoietic stem cell · Induced pluripotent stem cell

Introduction

The transfusion of red blood cells (RBCs) is a crucial therapeutic intervention that saves lives under various circumstances. However, blood supplies depend heavily on donations and are affected by several social issues, such as low birth rates and potential population decreases; therefore, a shortage of RBC is likely [1]. A promising solution for overcoming these issues is the generation and production of clinical-grade transfusion-quality RBCs. Artificial blood has already been vigorously studied worldwide on the laboratory scale; however, generated RBCs must have equivalent quality and function to native RBCs for subsequent clinical application [2].

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Generally, previously reported protocols mimic the bone marrow microenvironment by applying specific growth factors/cytokines with or without co-culture on stromal cells. However, the ideal outcome of such research is the significant amplification of stem cells with perfect terminal differentiation into fully mature functional RBCs [3]. Additionally, enucleation remains a critical obstacle to overcome to achieve in vitro RBC synthesis. Currently, the majority of cultured RBCs consist of orthochromatic erythroblasts, with only approximately 5–10% of generated cells being enucleated RBCs [4]. Thus, the global goal is to achieve a compatible quality of 100% enucleated in vitro RBCs in future. In this review, we discuss the basic concepts related to in vivo erythropoiesis and previous attempts to generate RBCs in vitro. Furthermore, we focus on in vitro erythropoiesis using induced pluripotent stem cells (iPSCs).

Overview of erythropoiesis and red blood cells

During embryonic development, definitive erythropoiesis shifts from the fetal liver to the spleen, whereas the site of hematopoiesis switches from the spleen to the bone marrow around the time of birth [5]. Erythropoiesis is a complex process that occurs in the bone marrow and involves the differentiation of multipotent hematopoietic stem cells (HSCs) to mature enucleated erythrocytes [6]. Erythropoiesis is characterized by the movement of erythroid lineage-committed

cells through three compartments: erythroid progenitors, precursors, and mature RBCs (Fig. 1) [7, 8]. The first step of erythropoiesis involves a commitment or engagement phase, in which HSCs differentiate into a more specific type of erythroid progenitor cell. Erythroid progenitors consist of a burst-forming unit erythroid (BFU-E) and colony-forming unit erythroid (CFU-E) cells. During this step, myeloid progenitors transition to a megakaryocytic-erythroid progenitor and eventually differentiate into BFU-Es, which are the first progenitor cells solely committed to the erythroid lineage [9]. These cells further differentiate into CFU-E and CFU-E progenitor cells that undergo terminal differentiation or RBC maturation, giving rise to the second step of erythropoiesis.

The second step of erythropoiesis is erythroid expansion and maturation, where nucleated precursor cells differentiate from proerythroblasts to basophilic, polychromatophilic, and orthochromatic erythroblasts [6]. Proerythroblasts (14–19 µm) have a large nucleus with fine chromatin and nucleoli, and the cytoplasm is reduced and basophilic [10]. During precursor expansion and maturation, the size and RNA content of cells decrease with progressive nuclear condensation, which ultimately leads to enucleation [11]. In addition, hemoglobin synthesis and accumulation gradually increase during this stage. Proerythroblasts differentiate into basophilic erythroblasts (12–17 µm), which have a slightly smaller nucleus with slight chromatin condensation and increased cytoplasm with no granules or nucleoli [10]. Next, the cells differentiate into polychromatophilic erythroblasts (12–15 µm) that show moderately condensed chromatin with light and grayish cytoplasm [10]. The nuclei of polychromatophilic erythroblasts are condensed with slightly accumulated hemoglobin and are intensely basophilic with coarse heterochromatin granules, giving a characteristic checkerboard appearance [10].

Orthochromatic erythroblasts expel the nucleus to form reticulocytes that, through the final step of erythroid differentiation, mature into erythrocytes by final enucleation [12]. Orthochromatophilic erythroblasts (8–12 µm) have a dark, opaque nucleus with gray–red cytoplasm, and the nucleus has a pyknotic morphology with abundant acidophilic hemoglobin [10]. In some cases, in the process of extrusion, the cells become reticulocytes [13]. Reticulocytes (7–10 µm) have a reddish–pale blue cytoplasm with an extruded nucleus and with RNA still present. Finally, the cells terminally differentiate into erythrocytes (7–8 µm) that have no nuclei or RNA and an orange-red cytoplasm [14]. Erythrocytes acquire a typical biconcave shape during this phase by undergoing extensive membrane remodeling [15]. The generation of circulating reticulocytes and mature erythrocytes, or "RBCs," is the final product of the erythropoietic process. The matured cells then circulate in the bloodstream until they are removed by macrophages within the reticuloendothelial system [16].

Another interesting aspect of the process of erythroid terminal differentiation or maturation is that it occurs in anatomic niches known as erythroblastic islands (EBIs), which is unique to mammalian erythropoiesis [17]. EBIs consist of central macrophages surrounded by approximately 30 erythroid cells at varying degrees of EBI maturation. The cells close to macrophages range from CFU-Es to enucleating erythroblasts; this is also the site of hemoglobin synthesis by terminally differentiating erythroblasts [18]. The central macrophage anchors erythroblasts within the island and provides cellular interactions that are necessary for erythroid differentiation and proliferation [19]. Central macrophages also regulate the rate of erythropoiesis through positive or negative feedback mechanisms [20]. Cytokines secreted from macrophages, such as insulin-like growth factor-1, reportedly promote erythroid proliferation and maturation. Furthermore, macrophages phagocytose the extruded nucleus from terminally differentiated erythroblasts and direct the transfer of iron to erythroid progenitors for heme synthesis [21, 22].

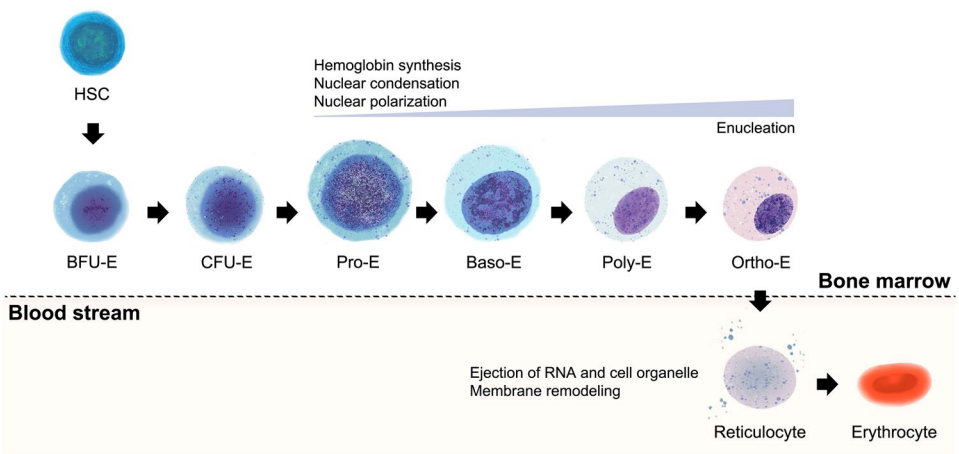

Fig. 1 Stages and cell types of in vivo erythropoiesis from hematopoietic stem cells. *HSC* hematopoietic stem cell, *BFU-E* burst-forming unit erythroid, *CFU-E* colony-forming unit erythroid, *Pro-E* proerythroblast, Baso-E basophilic erythroblast, *Poly-E* polychromatic erythroblast, *Ortho-E* orthochromatic erythroblast

Approximately two million RBCs are generated in the human body every second [6]. Erythropoiesis typically occurs at a steady, low basal rate, with approximately 1% of circulating erythrocytes cleared daily and replaced by new cells [23]. Fully mature RBCs remain in circulation for approximately 120 days; during their lifespan, they are continuously surveyed by resident macrophages within the liver and spleen. Macrophages within the spleen then detect unwanted, damaged, or aged RBCs and remove them [24].

Previous ex vivo erythropoiesis research using hematopoietic stem/progenitor cells

Early research on the ex vivo derivation of HSCs and erythroid cells was performed using stem cells derived from bone marrow, cord blood, and adult peripheral blood [25]. The main source material for in vitro RBC production was originally HSCs derived from various sources, with HSCs isolated from blood containing abundant CD34 + cells [26]. CD34 + cell proliferation and amplification are 10 times higher in cord blood-derived cells than in peripheral blood-derived cells [27]. Therefore, owing to its reduced amplification potential, less research has been performed using peripheral blood.

Methods for the ex vivo production of RBCs using multipotent hematopoietic stem/progenitor cells (HSPCs) have shown substantial progress in recent decades [28]. With the help of public or private clinical blood banking systems that routinely collect cord blood and peripheral blood, there are many opportunities to investigate laboratory-scale RBC production methods using HSPCs [29]. RBC generation from cord-blood-derived HSCs was first reported by Neidez-Nquyen et al. [30], who showed that cord blood-derived HSPCs could generate 200,000 times the number of erythroid cells. However, the limitation of this study was the low enucleation efficiency, which was only ~ 4%. The same group reported terminal maturation in NOD/SCID mice after in vivo transfusion of the generated erythroid precursors. They also established a similar differentiation protocol, which included the use of feeder stromal cells to mimic the bone marrow environment. The ex vivo terminal maturation process of erythroblasts derived from cord blood or peripheral blood HSPCs via co-culture with murine MS-5 stromal cells showed a significant increase in efficacy of approximately 90% [31].

Giarratana et al. also attempted to differentiate autologous RBCs from peripheral blood cells, then infused them into healthy adult subjects [32], confirming the in vivo functionality of the infused cells and enucleation rate. The half-life of the infused in vitro differentiated RBCs was similar to that of native RBCs, with 90% of infused RBCs achieving enucleation. Theoretically, studies suggest that > 500 units of cultured RBCs can be expanded from one unit of cord blood using the appropriate protocol; however, it is not yet clear whether this is sufficient for future clinical use [27, 32, 33].

Growth factors for erythropoiesis

Various growth factors are linked to erythropoiesis. The major growth factors regulating in vivo erythropoiesis are the stem cell factor (SCF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-(IL-)3, IL-6, IL-11, and erythropoietin (EPO) [34]. The addition of these growth factors is inevitable during ex vivo erythropoiesis.

EPO is the most critical and unique ingredient for RBC generation that plays a pivotal role during the final stages of erythroid maturation [3]. EPO is a humoral cytokine synthesized primarily by peritubular cells in the adult human kidney [35]. It is also synthesized in the fetal liver during early development [36]. EPO is secreted into the bloodstream where it targets erythroid progenitor cells in the bone marrow, with CFU-E as the primary target [37]. Besides its role in erythropoiesis, EPO also inhibits the apoptosis of erythroid progenitor cells in the bone marrow and neural cells [38, 39]. In vertebrate erythropoiesis, EPO binds to its receptor, EPOR, which is expressed on the surface of early erythroid progenitor cells [40]. Through binding, cell survival, differentiation, and proliferation are promoted [41].

Additional factors, such as SCF and IL-3, synergistically work with EPO to regulate erythropoiesis [42]. IL-3 and SCF reportedly increase the BFU-E population in cultured cells [43]. However, IL-3 also functions independently at the BFU-E stage and may regulate the self-renewal ability of cells [44]. IL-3 supports the proliferation of HSCs and is involved in other cell activities, such as cell growth, differentiation, and apoptosis [45]. These roles are mediated by G-CSF and GM-CSF, which are two growth factors with established roles in hematopoiesis and clinical practice that are reported to regulate myeloid cell production, differentiation, and activation [46]. Recent studies have shown that other factors may also be related to erythropoiesis. For example, TGF-β reportedly exhibits pro-differentiation effects during the early stages of erythropoiesis [9]. Moreover, transforming growth factor-beta 1 (TGF-β1) is known to accelerate terminal erythroid differentiation by delaying cells in the G_1 phase [47].

Mimicking erythropoiesis using induced pluripotent stem cells

Pluripotent stem cells for in vitro erythropoiesis

Pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are capable of unlimited proliferation while maintaining their

self-renewal and differentiation abilities into various cell types [48]. Considering several controversial issues regarding ESCs, iPSCs, which were first developed in 2006 by Shinya Yamanaka, represent a pioneering alternative to ESCs that can overcome these issues [49, 50]. Since then, many groups have attempted to differentiate various tissue-specific cells, including blood cells, using iPSCs [51–53]. The use of iPSCs in blood cell generation is also expected to relieve issues related to supply shortages. That is, because iPSCs are also capable of self-renewal, they represent an unlimited cell source for RBC expansion in vitro [4, 54, 55].

Human iPSCs have attracted attention as a potential cell source for RBCs [56, 57]. To avoid the ethical issues related to human ESCs, similar protocols are now being attempted by scientists using iPSCs [4, 58–62]. Moreover, assuming the use of appropriate protocols, iPSCs may be capable of resolving issues related to the shortage of RBCs. In addition, constructing an iPSC-based cell bank with various genetic varieties, such as human leukocyte antigen, could provide a new platform for transplantable RBC banks with disease-free and immune rejection-free cells [63, 64].

In vitro hematopoietic stem/progenitor cell differentiation using induced pluripotent stem cells

In many studies on RBC differentiation, cells pass through an intermediate stage of CD34 + or CD43 + HSC generation before in vitro erythropoiesis [65, 66]. The development of a novel protocol to generate a defined population of HSCs using pluripotent stem cells is important for the eventual generation of RBCs (Fig. 2). Three protocols are typically used to differentiate iPSCs into HSCs: (1) a method utilizing co-culture with bone marrow stromal cells, such as the OP9 cell line. (2) a two-dimensional (2D) method using a monolayer culture platform, and (3) a three-dimensional (3D) method using the generation of embryonic bodies (EBs) (Table 1) [65].

To mimic the bone marrow environment, several studies have attempted hematopoietic differentiation via co-culture with feeder cells [67]. The OP9 cell line is a mouse bone marrow stromal cell line, and human iPSCs can be characterized as CD34 + and CD45 + hematopoietic cells when OP9 is used as a feeder cell [68]. A 2009 study confirmed that the co-culture of iPSCs and OP9 improved the differentiation of iPSCs into CD34 + and CD45 + hematopoietic progenitors, which eventually successfully formed all types of hematopoietic colonies [69]. The use of feeder cells showed significant results; however, differentiation using feeder cells such as OP9 may limit large-scale HSC induction [70]. In addition, to develop cell therapeutics that can be used in actual patients, many researchers have attempted to increase HSC differentiation efficiency in feeder- and serum-free HSC differentiation conditions to avoid safety issues [71, 72].

Paes et al. provided a simple and efficient HSC culture method using an inexpensive in-house prepared medium that is both feeder-free and serum-free [73]. They concluded that the homegrown medium produced more CD34 + CD45 + HSPCs and erythroid colonies than STEMdiff APEL 2 [73]. Moreover, Nafria et al. provided a detailed procedure to generate CD45 + CD34 + RUNX1C + HSCs from spin EB that mimic intraembryonic aorta-gonad mesonephros hematopoiesis [59, 74]. Furthermore, it may be possible to model childhood leukemia using this method. Additionally, Netsrithong et al. established a feeder-free and serum-free monolayer differentiation method to generate multipotent hematopoietic progenitors, which produced 54% of CD34 + and CD43 + HSPCs from iPSCs. This novel protocol inhibits GSK3 signaling using CHIR99021 during the early stage of differentiation to induce mesoderm specification [75].

Furthermore, Tursky et al. directly compared the efficacy of serum-free and feeder-free hematopoietic differentiation protocols using 2D and 3D culture platforms [76]. In both culture platforms, a simple culture protocol containing only a few steps was compared to a "multistep" differentiation strategy. The authors aimed to optimize the culture conditions to produce erythro-myeloid progenitor-like definitive progenitors with higher efficiency and reduced production times and costs. Aryl hydrocarbon receptor (AhR) hyperactivation was utilized to induce HSPC expansion. Using the 2D-multistep culture platform with AhR modulation, iPSCs generated large numbers (a seven-fold increase) of CD34 + , CD34 + CD45 + , and CD34 + CD43 + hematopoietic progenitor cells. This protocol also accomplished balanced production of CD41 + , CD235a + , and CD41 + CD235a + erythroid cell populations, and was able to reduce the hands-on time by 40% using only half the cost of reagents per well compared to the original AhR activation protocol conducted by Smith et al. [77]. Tursky et al. also identified disease phenotypes by modeling hereditary hematopoietic disorders using iPSCs derived from patients with Down's syndrome and β-thalassemia [76].

AhR is hypothesized to be a critical transcriptional regulator of several primitive or definitive hematopoietic development pathways [78, 79]. Modulation of AhR plays a critical role in the emergence, proliferation, lineage commitment, and differentiation of HSPCs. Smith et al. demonstrated that AhR hyperstimulation strongly enhances the production of megakaryocyte–erythroid progenitors from iPSCs and that continued AhR stimulation eventually induces erythroid differentiation [77]. In contrast, cells seem to undergo a megakaryocytic lineage when AhR is withdrawn during this step.

While current differentiation protocols for various cell types using iPSCs focus on 3D culture or organoid culture,

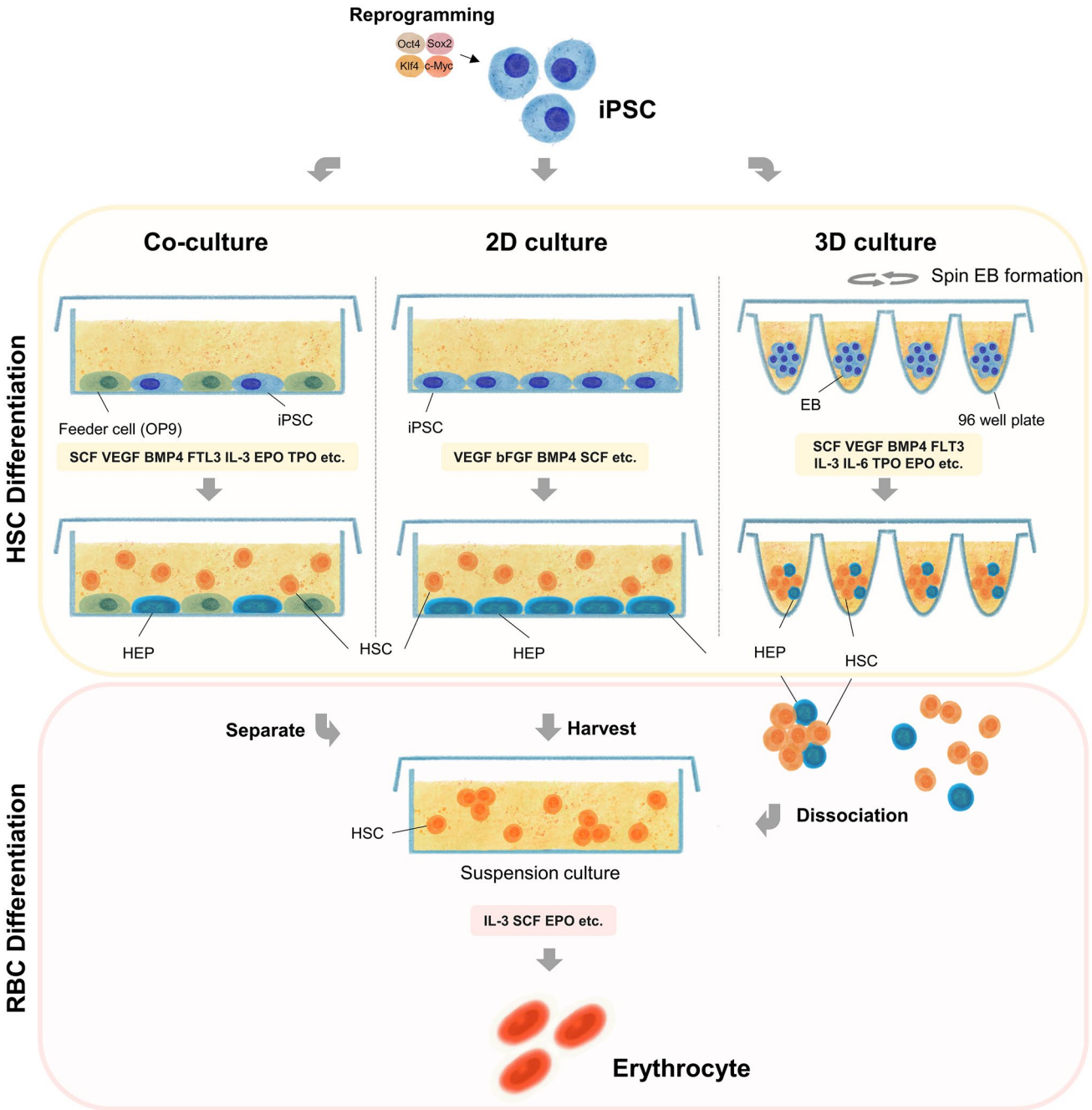

Fig. 2 Summary of erythrocyte differentiation methods using induced pluripotent stem cells (iPSC). Human iPSCs were differentiated into hematopoietic stem cells (HSCs) using three different protocols. 2D culture used a monolayer system, 3D culture used spin-formed EBs, and co-culture used a feeder cell such as OP9 line. iPSC-derived HSCs were matured into erythrocytes in a suspension culture system. *iPSC* induced pluripotent stem cell, *EB* embryoid body, *HSC* hematopoietic stem cell, *HEP* hematoendothelial progenitor, *RBC* red blood cell

many studies have utilized this platform to generate blood cells. For example, in a recent study by Tellechea et al., HSC differentiation was induced using iPSC-derived EBs with growth factors, including vascular endothelial growth factor (VEGF), bone morphogenetic protein 4 (BMP4), SCF, activin A, FMS-like tyrosine kinase 3 ligand (FLT3L), IL-3, IL-6, and G-CSF, for 13 days in floating suspension culture [80]. The hematopoietic differentiation potential was compared between healthy iPSCs and iPSCs derived from patients with aplastic anemia [80, 81]. The hematopoietic differentiation potential of AA-iPSCs was significantly reduced, both quantitatively and qualitatively, compared

Table 1 Summary of erythrocyte differentiation protocols from iPSCs and their advantages and disadvantages

Culture method	Cell source	Cytokines/chemicals used		Culture period (days)		Final cell type	Reference	Advantages and disadvantages	
		HSC differentiation	RBC differentiation	iPSC to HSC	HSC to RBC				
Co-culture (feeder cells)	hiPSC derived from BM-MSCs from severe combined immunodeficiency patient	SCF, TPO, FLT3L, BMP4, ATRA, Dex, etc.	–	12	–	HSC	[67]	Advantages	• Feeder cells help mimic the bone marrow microenvironment
	hiPSC (BC1, JHU181i) hESC (H1)	BMP4, VEGF, FGF2, SB431542, etc.	EPO, etc	13	30	RBC	[68]		
	hiPSC (IMR90, foreskin, SK46) hESC (H1, H7, H9, H13, H14)	–	–	8	–	HSC	[69]		
	hiPSC (SK46)-M4-10 and Foreskin-1, hESC (H1)	–	EPO, SCF, TPO, IL-3, IL-6, Dex, FLT3L, etc.	7–8	20–90	RBC	[58]	Disadvantages	• Limitation of large-scale induction • Unstable cell differentiation • Safety issues due to the feeder cells
	hiPSC derived from BM-MSCs and peripheral blood erythroid progenitors from sickle cell disease patients hESC (H1)	VEGF, SCF, TPO, IL-3, EPO, BMP4, FLT3L, etc.	SCF, EPO, etc	15–17	13	RBC	[62, 89]		
	iPSC from healthy donor PBMC	BMP4, WNT3a, Activin A, b-FGF, VEGF, etc.	SCF, IL-3, EPO, etc	11	21	RBC	[87]		
2D culture (attachment)	hiPSC from MUSIi001-A, MUSIi011-A, PBiPSC1	CHIR99021, VEGF, FGF2, SB431542, etc.	SCF, IL-3, EPO, transferrin, etc	8–12	20	RBC	[75]	Advantages	• Feeder-free and serum-free protocols are available • More simple and less demanding culture method compared to 3D and co-culture methods
	hiPSC derived from Down syndrome or β-thalassemia human fibroblasts	BMP4, VEGF, WNT3a, FGF2, SCF, FLT3L, TPO, IL-6, EPO, FICZ, etc.	SCF, EPO, IGF-1, etc	16	–	HSC	[76]		
	hiPSC derived from fibroblasts from systemic sclerosis patient	BMP4, VEGF, WNT3a, FGF2, SCF, FLT3L, IL-6, TPO, EPO, FICZ, etc.	EPO, etc	10–15	15–45	RBC	[77]	Disadvantages	• Limitations in mimicking in vivo environment
	hiPSC derived from healthy donor PBMCs	BMP4, VEGF, bFGF, WNT3A, WNT5A, Activin A, GSK3b inhibitor VIII, SCF, β-estradiol, IGF2, TPO, SB431542, IBMX, UM171, PDGF, ANGPTL5, CCL28, G-MCSF, G-CSF, etc.	SCF, EPO, IBMX, Dex, RU486, etc	10–24	20	RBC	[85]		

Table 1 (continued)

Culture method	Cell source	Cytokines/chemicals used		Culture period (days)		Final cell type	Reference	Advantages and disadvantages	
		HSC differentiation	RBC differentiation	iPSC to HSC	HSC to RBC				
3D culture (EB formation)	hiPSC derived from sickle cell anemia patient PBMCs	BMP4, VEGF, b-FGF, SCF, TPO, etc.	SCF, IL-3, EPO, transferrin, etc	8–14	10–15	RBC	[73]	Advantages	• Feeder-free and serum-free protocols are available • Provides more realistic and nessesary microenvironment that can mimic the in vivo system • Can induce enhanced maturity of differentiated cells
	hiPSC derived from aplastic anemia patient PBMCs or dyskeratosis congenita patient fibroblasts	VEGF, BMP4, SCF, Activin A, FLT3L, IL-3, IL-6, G-CSF, etc.	–	13–16	–	HSC	[80, 81]		
	hiPSC (7F3955-pMXs#1)	BMP4, b-FGF, SCF, IGF-1, FLT3L, VEGF, IL-3, IL-6, TPO, etc.	–	12	–	HSC	[82]		
	hiPSC derived from CD34+ cord blood cells (CD34-iPSCs) or CD36+ erythroblasts (PEB-iPSCs)	IL-3, SCF, EPO, etc.	transferrin, SCF, IL-3, EPO, etc	18	18	RBC	[84]		
	hiPSC(CD34-4F_iPS, CD34-2F_iPS, NSC-2F-iPS, NSC-1F-iPS, Fib-iPS) hESC (H1)	SCF, TPO, FLT3L, BMP4, VEGF, IL-3, IL-6, EPO, etc.	IL-3, SCF, EPO, etc	20–21	25–26	RBC	[4, 61, 86]	Disadvantages	• More complex and demanding culture method
	hESC (RC9, RC11, H1, H9) hiPSC (33D6), hiPSCs derived from blood group O RhD- donor fibroblasts, hiPSCs derived from peripheral blood	BMP4, VEGF, WNT3A, Activin A, GSK3b inhibitor VIII, IGF2, SCF, TPO, IBMX, β-estradiol, FGFα, etc.	SCF, FLT3L, BMP4, IL-3, IL-11, EPO, IBMX, hydrocortisone, IGF-1, etc	10	16	RBC	[60, 88]		
	hiPSCs derived from five O D-positive donor PBMCs and two patient PBMCs with rare blood types (D– and Jr(a-)) hESC (H9)	BMP4, VEGF, WNT3A, Activin A, GSK3b inhibitor VIII, FGFα, SCF, β-estradiol, IGF2, TPO, IBMX, SR1, etc.	SCF, IL-3, EPO, F68, etc	11	16	RBC	[90]		

HSC hematopoietic stem cell, *RBC* red blood cell, *hiPSC* human-induced pluripotent stem cell, *hESC* human embryonic stem cell, *EB* embryonic body, *BM-MSC* bone marrow mesenchymal stem cell, *EPs* blood erythroid progenitors, *PBMC* peripheral blood mononuclear cell, *SCF* stem cell factor, *TPO* thrombopoietin, *FLT3L* FMS-like tyrosine kinase 3 ligand, *BMP* bone morphogenetic protein, *ATRA* all-trans-retinoic acid; *Dex* dexamethasone, *VEGF* vascular endothelial growth factor, *FGF* fibroblast growth factor, *EPO* erythropoietin, *IL* interleukin, *WNT3a* wingless type 3a, *FICZ* 6-formylindolo[3,2-b]carbazole, *IGF* insulin-like growth factor, *IBMX* isobutylmethyl xanthine, *PDGF* platelet-derived growth factor, *ANGPTL5* angiopoietin-like 5, *CCL28* chemokine ligand 28, *G-MCSF* granulocyte–macrophage colony-stimulating factor, *G-CSF* granulocyte-stimulating factor, *SR1* StemRegenin 1

with that of healthy iPSCs. Takahashi et al. produced EBs with iPSCs and treated them with BMP4 and Fibroblast growth factor 2 (FGF2) in suspension for up to four days [82]. Then, from days 5 to 12, differentiation into HSPCs was established using SCF, VEGF, IL-4, IL-6, FLt3L, thrombopoietin (TPO), Insulin-like Growth Factor 1 (IGF-1), and FGF2 by attaching the EBs onto gelatin-coated dishes. The authors also studied the effect of exposure to magnetic fields on HSC differentiation, and found no effect on the differentiation of human HSPCs [82].

In vitro erythropoiesis using induced pluripotent stem cells

Although HSPC differentiation from iPSCs is a substantial hurdle to overcome, a defined protocol to differentiate HSPCs into an erythroid lineage is also required. Various previous studies have shown that iPSCs can differentiate into erythroid lineages in vitro, similar to ESCs [58]. In early studies using ESCs, Lu et al. achieved approximately 60% enucleated erythroblasts in vitro [83]. The produced RBCs expressed fetal and embryonic hemoglobin and showed comparable oxygen delivery to normal RBCs. As well as achieving a high differentiation rate, the development of an "economically friendly" protocol is the focus of many studies [75, 84, 85]. Considering the inevitable large-scale production of RBCs for future use, a protocol that reduces the use of expensive recombinant growth factors (especially EPO) or that employs a replacement strategy is strongly encouraged.

To generate RBCs or erythrocytes, progenitor blood cells are typically differentiated by exposing them to growth factors, such as SCF, IL-3, and EPO, in suspension [4, 61, 86, 87]. However, current methods are still hindered by low efficiency, with current research attempting to increase RBC differentiation efficiency [62, 76, 88]. Furthermore, xeno-free, serum-free, and feeder-free culture methods are being developed for clinical use. For example, Bernecker et al. described a simple feeder-free culture system for erythrocyte differentiation using iPSCs [84]. The authors proceeded with RBC differentiation using a small number of cytokines, such as SCF, IL-3, and EPO, and confirmed RBC production with enucleation rates close to 40%. Moreover, Oliver et al. reported high-efficiency, serum-free, and feeder-free erythroid differentiation using small molecules from iPSCs. By day 31 of differentiation, the cells expanded into 50,000–200,000 RBCs with the additional use of hydrocortisone [60].

In 2009, Olivier et al. developed an albumin-free, low-transferrin, chemically defined RBC differentiation protocol based on a self-made medium and the differentiation of iPSCs into erythrocytes, which reduced the cost of economical production [85]. Although transferrin is also an important ingredient for RBC production, 10 to 20 times less transferrin was used because iron was provided to the differentiating erythroblasts through small amounts of recombinant transferrin supplemented with FeIII-EDTA, which is an iron chelator that allows transferrin recycling to occur in cell cultures. Moreover, the previously mentioned study by Netsrithong et al. proposed a simple and inexpensive method to differentiate RBCs from iPSCs [75]. The feeder-free and serum-free monolayer differentiation method used to generate multipotent hematoendothelial progenitors produced 54% of CD34 + and CD43 + HSPCs from iPSCs by adding a GSK3 inhibitor to reinforce early-stage mesoderm differentiation. With the generated HSPCs, erythrocyte differentiation was attempted using SCF, IL-3, and EPO. RBCs were reportedly expanded up to 40-fold. This study showed that the produced HSPCs can differentiate into erythrocytes, making the previously complex in vitro erythrocyte protocols more efficient and less expensive.

RBC-related disease modeling has also been attempted using these protocols. For example, in vitro differentiation of iPSCs from patients with sickle cell disease (SCD) or rare blood and healthy iPSCs was studied by Haro-Mora et al. [89] The generated SCD-iPSC genes were corrected by performing viral vector-free gene editing and differentiated into an erythroid lineage through a process that mimics yolk sac formation. RBC differentiation was performed using factors, such as SCF, IL-3, EPO, estradiol, dexamethasone, and transferrin. The resulting genetically corrected SCD-iPSCs showed normal DNA and protein levels of β-globin. Park et al. also demonstrated erythroid differentiation in O D-positive blood and rare blood type (D–and Jr(a-)) iPSCs using a serum-free, xeno-free erythroid differentiation protocol [90]. Thus, the authors suggested the potential for medical advancement of iPSC-based regeneration and the production of iPSC-derived RBCs for transfusion.

Although the quality of in vitro RBC differentiation protocols is improving, the efficiency of RBC differentiation varies according to several factors, including the origin of iPSCs. The use of MSC-derived iPSCs results in more efficient β-globin erythropoiesis than the use of peripheral blood erythroid progenitor-derived iPSCs [62]. In addition, the developmental stage of hematopoiesis may be an important contributor to hematopoietic output [76]. That is, in vivo hematopoiesis occurs in temporally and spatially distinct waves (sequentially, as primitive yolk sac, fetal, and bone marrow), whereas existing in vitro hematopoietic differentiation protocols using pluripotent stem cells do not typically exhibit a specific developmental stage [76, 91]. Erythropoiesis in mammals involves two differentiation processes: (1) primitive erythropoiesis initiated in the yolk sac that generates a large population of nucleated erythroblasts and (2) cells development into smaller enucleated erythrocytes in the fetal liver through definitive erythropoiesis [83]. Therefore, future research through the discovery and use of various signaling modulators to distinguish the developmental

stages of hematopoiesis or induce more mature forms will help characterize and produce the expected cell type with higher efficacy.

Red blood cell maturation

Erythroblastic islands

EBIs were first observed by Marcel Bessis in 1958 [92] and are predominantly found in bone marrow during steady-state erythropoiesis [93, 94]. During stress erythropoiesis, however, EBIs extend to the fetal liver and adult spleen, which allows rapid production of erythrocytes in response to stress conditions such as inflammation [94–96]. During differentiation, proerythroblast-to-orthochromatic stages occur within the EBI (Fig. 3) [94]. The connection between adhesion molecules in macrophages and erythroblasts promotes erythroblast proliferation and induces the expression of proerythroblast characteristics [97, 98]. Central macrophages then secrete growth factors and cytokines that promote enucleation in erythroblasts and provide iron for heme synthesis [99, 100].

EBI composition varies between species, with rat EBIs containing approximately 10 erythroblasts per island, but human EBIs containing up to 30 erythroblasts surrounding the central macrophage [94]. During terminal differentiation, the ejected nuclease from the orthochromatic erythroblast is phagocytosed by the EBI macrophage, and the resulting reticulocytes additionally expel other remaining organelles and enter circulation [10, 101]. Finally, a biconcave shape is generated by membrane remodeling [102]. The co-culture of erythroblasts and macrophages was first reported in 1979, and is now a widely used method following the ex vivo culture of EBIs [98, 103, 104]. Experiments later revealed that the adhesion proteins responsible for the attachment between cells are critical for island integrity [105, 106]. α4β1 integrin was one of the first identified proteins and reportedly binds vascular cell adhesion protein 1 (VCAM-1) [105, 107, 108]. When interaction between these two proteins is blocked by monoclonal antibodies, erythroblast–macrophage attachment is significantly impaired [107]. However, the in vitro reconstitution of EBIs is limited by culture conditions; thus, it is difficult to mimic the bone marrow environment, which limits the confirmation to only a few cell types [94]. In

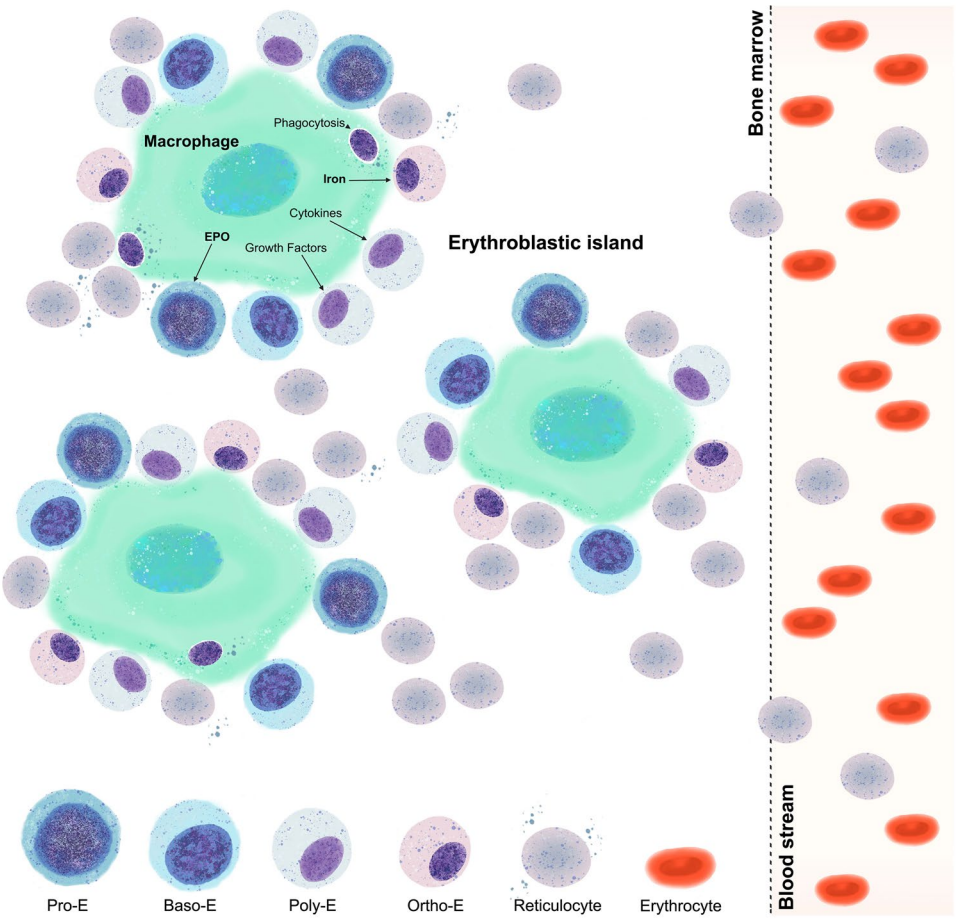

Fig. 3 Schematic of erythroblastic islands in bone marrow. Cells from proerythroblasts to reticulocytes are attached to central macrophages, which supply the critical ingredients required for differentiation. Reticulocytes migrate to the bloodstream and undergo the final process to differentiation into erythrocytes or red blood cells. *Pro-E* proerythroblast, *Baso-E* basophilic erythroblast, *Poly-E* polychromatic erythroblast, *Ortho-E* orthochromatic erythroblast

addition, it is unclear how in vitro EBIs relate to the actual characteristics of in vivo EBIs.

Enucleation

The final step in RBC generation is the enucleation process, which occurs in orthochromatic erythroblasts. Synchronization of cell cycle arrest, chromatin, nuclear condensation, and nuclear polarization is important for successful enucleation [109]. As RBCs mature, chromatin condenses and transcription is suppressed [110]. After the final cell cycle, the nuclei of orthochromatic erythroblasts are polarized to one side of the cell. Through this process, reticulocytes and pyrenocytes are produced, and the nucleus exits the cells by generating the pyrenocyte, which is the nucleus surrounded by a thin layer of cytoplasm and plasma membrane, leaving the reticulocyte mostly consisting of cytoplasm [111]. Pyrenocytes are typically immediately removed by the residing macrophages of the EBI as phosphatidylserine expressed on the plasma membrane acts as an "eat me" signal [101].

Theoretically, there are two prominent models of enucleation: (1) apoptosis and (2) asymmetric cytokinesis [12]. The presence of partial karyolysis and leakage of nuclear material into the cytoplasm, as visualized by electromicroscopy, indicates an apoptosis model [112]. Additional evidence in lens epithelial cells and keratinocytes has hinted that a mechanism similar to the prevailing model of programmed cell death eliminates their nuclei [113, 114]. To confirm this theory, Carlie et al. conducted siRNA-mediated caspase knockdown on enucleation and observed a 50% decrease in enucleation in knockdown cells [115]. However, because blocking was performed at the stage between proerythroblasts and basophilic erythroblasts, the suggested role of caspases may have been involved at an earlier stage of erythroblast development. In addition, treatment of enucleating erythroblasts with pan-caspase inhibitors does not block the enucleation process [101]. Collectively, these findings suggest that apoptosis does not involve enucleation.

Technical challenges for future applications

Enucleation is a critical step in in vitro RBC differentiation [12]. Although current protocols are achieving relatively high rates of enucleated cells, as discussed in the previous section, a 100% fully enucleating protocol is still strongly recommended because of safety issues; thus, further investigation of a defined protocol or new strategy is essential. Notably, Migliaccio et al. suggested that immature erythroblasts may serve as an alternative erythroid cell transfusion product in future [116]. Immature erythroblasts generate 8–16 RBCs after maturation, which can reduce the number of cells required as a useful transfusion product. Moreover, erythroblasts are thought to be more advantageous than RBCs for treating patients with chronic anemia [116]. Unlike transfused RBCs, erythroblasts use iron during maturation rather than increasing iron levels. In addition, RBCs naturally generated by transfused erythroblasts will have younger characteristics, which could lead to a longer survival rate in vivo.

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Data availability All data that support the findings of this study are included within the article.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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