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ARTICLE

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Red cell manufacturing using parallel stirred-tank bioreactors at the final stages of differentiation enhances reticulocyte maturation

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Abstract

The aim of this study was to develop a robust, quality controlled, and reproducible erythroid culture system to obtain high numbers of mature erythroblasts and red blood cells (RBCs). This was achieved using a fully controlled stirred-tank bioreactor by the design of experiments (DOE) methods in the serum-free medium by defining the appropriate culture parameters. Human cord blood CD34+ cells were first cultured in static flasks and then inoculated to stirred-tank bioreactors. Cell diameter was gradually decreased and final RBC yields were significantly higher when cells were inoculated at sizes smaller than 12 μm. The larger immature cells in the basophilic stage did not survive, while smaller mature erythroid cells were successfully expanded at high agitation speeds, demonstrating that appropriate seeding timing is critical. A high inoculation cell density of 5×10^6 cells/ml was achieved reaching 1.5×10^7 cells/ml. By using DOE analysis fitted to precise stages of erythropoiesis, we were able to acquire the optimal culture parameters for pH (7.5), temperature (37°C), dissolved oxygen, agitation speed (500 rpm), inoculation timing (cell diameter 12–13 μm), media feeding regimen, and cell seeding density (5×10^6 cells/ml). The final pure RBCs showed appropriate functions compared with fresh donor RBCs, confirming that manufacturing mature RBCs with reproducibility is possible.

KEYWORDS

bioreactor, blood, design of experiments, erythrocyte, in vitro cell culture

So Yeon Han and Eun Mi Lee contributed equally to this study.

1 | INTRODUCTION

A promising alternative to the current system of donor-dependent blood transfusion is in vitro manufacturing of transfusable red blood cells (RBCs; Choe & Baek, 2015; Giarratana et al., 2005). Most research for in vitro erythropoiesis has been investigated in static culture systems. Giarratana et al. (2005) demonstrated that in vitro production of pure RBCs is possible in static culture flasks. Griffiths et al. (2012) and Kupzig et al. (2017) generated RBCs in stirrer flasks after using static flasks. Heshusius et al. (2019) produced reticulocytes using gas-permeable static culture flasks.

A number of papers have focused on RBC production using various bioreactors. In one study, the final cell product showed highly variable red cell sizes and a very large mean corpuscular volume (MCV) of 133 fl (Timmins et al., 2011), while the reference range for MCV was 80–100 fl (Rodak et al., 2013). In another study using a hollow fiber-type bioreactor, the erythroid cell-specific marker glycophorin A (GPA) showed only 45.5% positivity in the harvested cells, and intact RBC morphology was not shown (Housler et al., 2012). Ratcliffe et al. (2012) used a stirred-tank bioreactor (ambr®) and inoculated hematopoietic stem cells (HSCs) to get parameters for the scale-up of erythroid cell culture. However, the final cell yield was very low, with a less than 8.5-fold increase during 16 culture days. Glen et al. (2013) expanded CD34+ hematopoietic progenitor cells using an ambr® bioreactor but without a design of experiments (DOE) and enucleation rates remained low (up to 12%). Recently, Bayley et al. (2018) showed an improved experimental design using ambr® by seeding CD34+ cells to set up erythroid differentiation parameters using DOE. However, the yield was very low, with an increase of only 12-fold during 25 culture days. Therefore, the reason for the low RBC yield using bioreactors should be further searched.

Erythroid cells show very unique characteristics depending on their maturation stages. In the bone marrow, immature cells are not exposed to fluid shear stress while enucleated reticulocytes enter the bloodstream (Bresnick et al., 2018; Manwani & Bieker, 2008). The shear stress that is inevitable in bioreactors would be stressful to immature erythroid cells. This is supported by the result of the limited proliferation of erythroid cells in bioreactors compared with the static culture (Bayley et al., 2018; Ratcliffe et al., 2012).

In addition to the shear stress, the oxygen supply and nutrient consumption are different during erythropoiesis as erythroblastic islands continuously move toward the sinusoids as erythroid cells mature (Manwani & Bieker, 2008). Considering these stage-specific requirements, the manufacturing process needs to be improved by optimizing culture parameters such as pH, dissolved oxygen (DO), temperature, agitation speed, and media feeding regimens along with cell inoculation timing and cell density for each maturation stage.

The amount of RBCs required for even one dose for transfusion is much higher (2×10^{12} cells per unit) compared with cells required for other cell therapeutics such as immune cells or mesenchymal stem cells (10^7–10^9 cells; Hay & Turtle, 2017). For scale-up, optimized manufacturing parameters using multiparallel bioreactors are essential. However, there has been little understanding of maturation-dependent manufacturing parameters using fully controlled automated bioreactors. Therefore, reproducible technologic advances are needed to develop a precise, optimized RBC manufacturing process. Such a process should be based on a bioreactor system using DOE, which is high-throughput experimentation and statistical technique useful for process optimization of the culture conditions (Eaker et al., 2017).

In this study, optimal process parameters were measured using a fully controlled stirred-tank bioreactor. Our data demonstrate for the first time that viability, cell proliferation, and RBC yield are significantly dependent on cell inoculation timing during maturation, and optimal culture parameters are different among maturation stages. We expect that our data will provide detailed information for the systematic production of RBC products using robust and cost-effective strategies enabling the efficient production of RBCs.

2 | MATERIALS AND METHODS

2.1 | Erythroblast expansion from hematopoietic stem cells before bioreactor inoculation

Umbilical cord blood was obtained from healthy pregnant women after obtaining written informed consent. This study was approved by the Institutional Review Board of Hanyang University (HYI-16-178). Human umbilical cord blood CD34+ cells were isolated as previously reported (Han et al., 2018) via positive selection using CD34 antibody-labeled microbeads (Easysep Human CD34 Positive Selection Kit; Stemcell Technologies) according to the manufacturer's instructions and then cryopreserved in liquid nitrogen until required. Thawed cells were cultured by following a three-stage protocol as described previously (Kim et al., 2019). Briefly, CD34+ cells were cultured in serum-free medium (Stemline® II Hematopoietic Stem Cell Expansion Medium; Sigma-S0192; Sigma-Aldrich) supplemented with 150-μg/ml holo-transferrin (Sigma-Aldrich), 90-ng/ml ferric nitrate, 30.8-μM vitamin C, 160-μM 1-thioglycerol, 50-μg/ml insulin (Sigma-Aldrich), 4-μml glutamine, 2-μg/ml cholesterol, 0.05% pluronic F-68 (Gibco), and 0.5 μl/ml chemically defined lipid mixture 1 (Sigma-Aldrich). Media were supplemented with erythropoietin (6 U/ml; R&D Systems), stem cell factor (100 ng/ml; R&D Systems), and interleukin-3 (10 ng/ml; Sigma-Aldrich) for 0–7 days of culture, erythropoietin (3 U/ml), and stem cell factor (50 ng/ml) for 8–13 days, and erythropoietin (2 U/ml) for 14–18 days. The cells were inoculated in 15 ml/75 cm^2 at 1×10^5 cells/ml and maintained at pH 7.5 in 37°C, 5% CO_2 before inoculation to bioreactors, after which cells were either maintained in control static culture using T75 flasks and then T175 flasks or transferred to the stirred-tank bioreactor system (ambr® 15 cell culture; Sartorius; automated bioreactor system for 24 parallel cultivations) as in Figure 1. One-half of the medium was changed every 2 days unless indicated otherwise (Kim et al., 2019).

In some experiments, CD34+ cells and differentiated erythroid cells were cultured in Stemline II (80% volume) mixed with either Cellartis DEF-CS 500 Basal Medium 20% (Takara), which is a proprietary chemically defined medium, or Cellartis DEF-CS 500

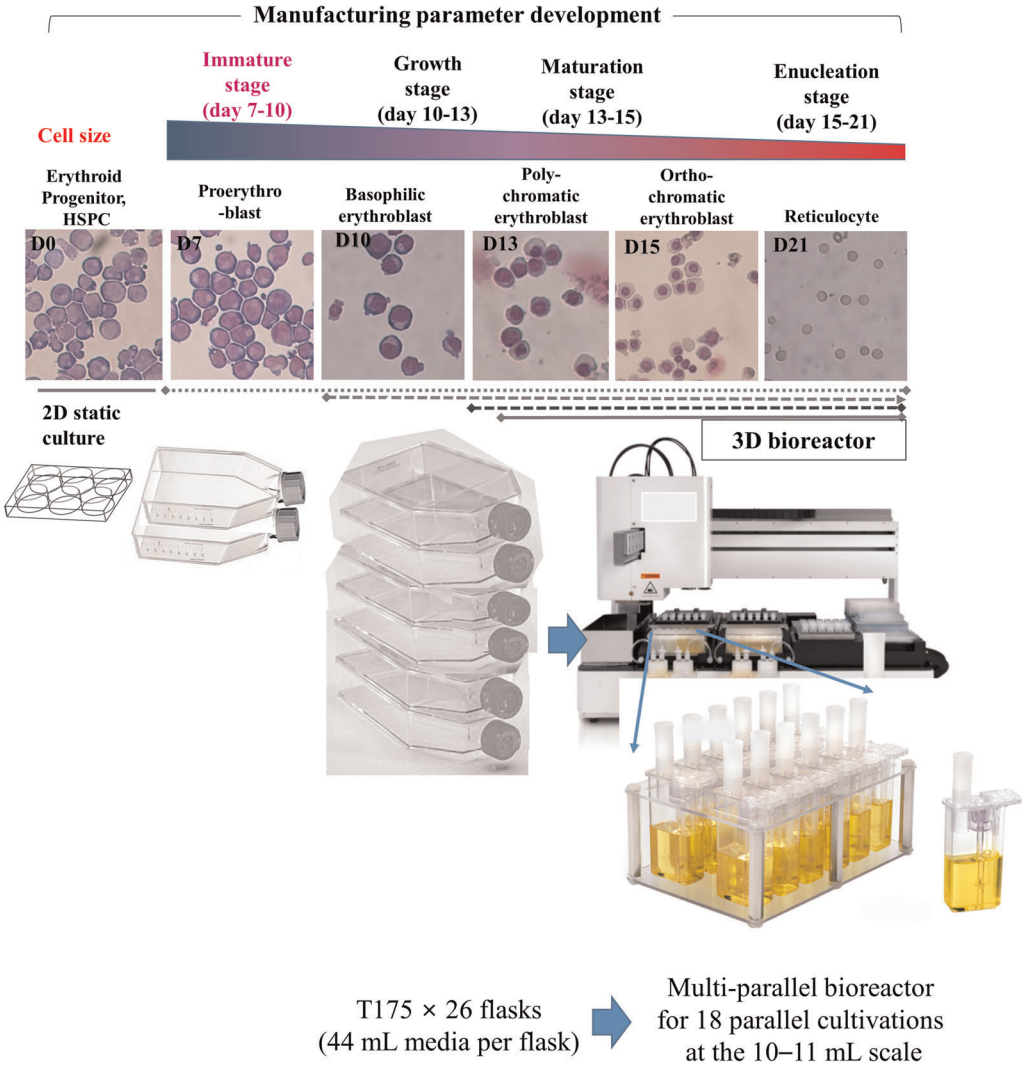

FIGURE 1 Experimental design. An experimental scheme is shown. Cells were cultured at least for 7 days in two-dimensional (2D) flasks without media agitation and then inoculated into automated stirred-tank bioreactors at different culture days (marked as dotted lines) to identify the appropriate culturing parameters for red blood cell production depending on each maturation stage. For 2D culture, hematopoietic stem and progenitor cells (HSPC) were seeded in two six-wells and then 75-T flasks and followed by 24–26 × T175 flasks. In the design of experiments 1–3, cells were inoculated to 16–18 culture vessels at the 10–11 ml media volume scale per vessel [Color figure can be viewed at wileyonlinelibrary.com]

Xeno-Free chemically defined GMP Grade Basal Medium 20% (Takara); these combinations showed very excellent results for erythropoiesis and were called OMM and OMM (GMP), respectively (Kim et al., 2019). The additives in the commercial media package were added following the manufacturer's protocols except for GF3 in DEF-CS, which is used for adherent cell culture. The other culture protocol, supplement set, and cytokines were added as in the control condition.

2.2 | Controlled stirred-tank bioreactor culture

Bioreactor experiments were carried out in a volume of 10–11 ml. Bioreactors were preconditioned for one day before inoculation at 37°C, DO level of 50%, and pH 7.5, and cultured using these conditions if not otherwise stated. Culture vessels were gassed by overlay gassing via the vessel headspace. Temperature, impeller speed, pH, DO, seeding density, and media feeding regimens were varied as specified in Table 1. N_2 gas flow was constantly maintained at 0.15 ml/min. pH was controlled by CO_2 gas infusion and 1-M sodium bicarbonate. Media replacement was conducted every 48 h unless otherwise indicated. The rpms of 300, 400, 500, and 600 are equivalent to impeller tip speeds of 0.18, 0.24, 0.30, and 0.36 m/s, respectively, in the ambr® bioreactor. More information is available at the manufacturer's website and articles (Bresnick et al., 2018; Goldrick et al., 2017; Sewell et al., 2019). Viable cell density (VCD), viability, and cell diameter were measured using a Vi-Cell XR (Beckman Coulter), which measures 4000–5500 cells per each

TABLE 1 Experimental plans for RBC manufacturing in a bioreactor

	‡DOE-1			DOE-2			DOE-3		
	Factor variables								
Run no.	Agitation (rpm)	Inoculation day	Cell density ($\times 10^6$ cells/ml)	Media exchange ratio (%)	DO (%)	pH	Temp.(°C)	DO (%)	pH
1	300	12	0.5	20	10	7.3	30	10	7.1
2	300	12	2.75	20	10	7.4	30	10	7.5
3	300	12	5	20	10	7.5	30	10	7.5
4	300	13	0.5	20	30	7.3	30	40	7.272
5	300	14	0.5	20	50	7.3	30	70	7.1
6	300	14	2.75	20	50	7.4	30	70	7.5
7	300	14	5	20	50	7.5	30	70	7.5
8	400	12	0.5	50	10	7.3	33.5	10	7.1
9	400	12	2.75	50	10	7.4	33.5	10	7.3
10	400	13	5	50	30	7.5	33.5	40	7.5
11	400	14	0.5	50	50	7.3	33.5	70	7.1
12	500	12	0.5	80	10	7.3	37	10	7.1
13	500	12	5	80	10	7.5	37	10	7.3
14	500	13	2.5	80	30	7.39	37	10	7.5
15	500	14	0.5	80	50	7.3	37	40	7.1
16	500	14	5	80	50	7.5	37	70	7.1
17							37	70	7.3
18							37	70	7.5

Note: For DOE-1, The cells cultured in DO 25%, 37°C, pH 7.4 with a media exchange ratio of 50% every 2 days.
Abbreviations: DO, dissolved oxygen; DOE, design of experiments; RBC, red blood cell; Temp., temperature.

counting using a trypan blue dye exclusion method. For media changes, cell suspensions were centrifuged at 300*g* for 5 min and removed old media, and reseed in fresh media.

2.3 | Metabolite analysis

Concentrations of glucose, lactate, glutamine, glutamate, and ammonium in the medium were determined by a Cedex Bio HT Analyzer (Roche Diagnostics GmbH). pH, pCO_2, and pO_2 were analyzed using a RAPIDLab 348EX blood gas system (Siemens).

2.4 | Flow cytometry analysis of lineage and maturation markers

Flow cytometry for erythroid lineage markers was performed after each expansion using CD235a (GPA)-FITC (BioLegend) or -PerCP cy5.5 (BioLegend), CD71-PE (BD Biosciences), and NucRed Live 647 ReadyProbes (Invitrogen), CD233 (band 3-PE; Miltenyi Biotec), integrin-alpha-FITC (BD Pharmingen), and NucRed Live 647 ReadyProbes were used. Isotype controls were used in every experiment to exclude nonspecific binding of the antibody. After washing, the samples were analyzed by flow cytometry as previously described (Han et al., 2018). For hemoglobin staining, cells were fixed, permeabilized, and tagged with fetal hemoglobin (Hb-F)-FITC (BD Biosciences), hemoglobin beta (HBB; Santa Cruz Biotech), GPA-PerCP cy5.5 (BioLegend), and NucRed Live 647 (Invitrogen). After washing, the acquisition and analysis of the samples were performed in an Accuri C6 flow cytometer (Becton Dickinson Biosciences).

2.5 | Cell morphology analysis

Every other day, cell morphology was observed after staining for cell maturation stages. Cells were centrifuged using a Cytospin (Cellspin; Hanil Science Industrial) at 900 rpm for 3 min onto a glass slide and stained with Wright–Giemsa stain (Sigma). Maturation status and dysplastic cells were observed under a light microscope (Nikon Eclipse TE2000-U) in a blinded manner by two experts with 6 years of experience in erythropoiesis research (Kim et al., 2019).

For three-dimensional (3D) cell morphology, RBCs were labeled with CFSE (Invitrogen) and evaluated by confocal laser scanning microscopy.

2.6 | Functional analysis

Pure RBCs were isolated by filtering cells in a deleukocyte reduction filter for RBC (Sepacell; Asahi Kasei Medical). An elongation index (EI) was measured by RheoScan-D200 (Korea). Oxygen equilibria were measured by a Hemox-Analyzer Model B (TCS Scientific) according to the manufacturer's recommendations. RBC ABO grouping was also determined (Kim & Baek, 2012). For storability of bioreactor manufactured RBCs, pure filtered RBCs were plated in 24-well plates and stored in OMM (GMP) media supplemented with 5% fetal bovine serum (FBS) in a 4°C refrigerator.

2.7 | DOE design

We sequentially performed three experiments to identify the appropriate parameters using response surface methodology. Response surface framework is the standard in experiments for manufacturing, research, and development (Myers et al., 2004). Once the conditions of the parameters were optimized, we confirmed significant differences in the primary outcomes, such as viability, VCD, and RBC yields, at the optimized condition in comparisons with those at other combinations of the parameters.

2.8 | Statistical analysis

The various culture conditions were compared with combinations of the parameters at $\alpha = .05$ based on Kruskal–Wallis tests. Bonferroni's correction was performed for multiple comparisons. The goodness of the fitness for the final model at each stage was tested with adjusted R^2 and p values of F-statistic. DOE was analyzed using JMP® 12.0 (SAS Institute Inc.), and all other analyses were analyzed using SAS 9.4 (SAS Institute Inc.). p Values less than .05 were considered statistically significant.

3 | RESULTS

3.1 | Optimization of immature erythroblast cultivation parameters in a stirred-tank bioreactor from Day 7 in various media

CD34+ cell-derived erythroid cell production usually occurs in three- or four-stage strategies with different cytokines and additives (Kim et al., 2019). As the effects of shear stress on more immature erythroblasts are not clear, we examined how to seed erythroblasts to a bioreactor. CB CD34+ cells were cultured in 2D culture plates for 7 days in Stemline media with 5% FBS and other cytokines and additives for the first stage (Figure 1). Then, the proerythroblasts and basophilic erythroblasts at Day 7 (D7 + 0) were seeded to the bioreactor. However, erythroid cells at 300 or 600 rpm at 4 days after seeding (D7 + 4) were not viable, and other lineage cells including macrophages and myeloid cells were the only cells to survive (Figure 2a–c).

To evaluate whether the poor erythroblast viability was due to the culture media condition, we next compared media for the same period of cells (>85% of basophilic erythroblasts in the three different media for culture Day 7). As cell density is important for cell growth, the effects of cell seeding density were compared in three media samples. Cells in Stemline II + DEF-CS of GMP grade (denoted as OMM [GMP]) showed higher VCD and viability than cells in OMM (Figure 2d). These results indicate that manufacturing RBCs in a serum-free GMP-grade media is possible. However, immature cells that were observed on Day 7 + 6 were almost dead in all conditions and only other lineage cells were increased on Day 7 + 7 (Figure 2e). While more erythroid cells were detected by flow cytometry using erythroid cell surface markers of GPA and CD71, fewer erythroid cells were observed on stained slides because the apoptotic and damaged cells were fragile and showed as smudge or ghost cells on slides (Figures 2e and S1). These results show that proerythroblasts and basophilic erythroblasts cannot resist shear stress in our culture conditions and are therefore not appropriate for culturing in a stirred-tank bioreactor during the immature stage.

3.2 | Culturing erythroblasts at the maturation stage by inoculation at Day 12

To evaluate whether mature erythroblasts are adaptable in a stirred-tank bioreactor, we cultured CD34+ cell-derived erythroblasts in 2D plates until Day 12 and then seeded them in various media. After inoculation to bioreactors, the cells were cultured at 37°C, DO 25%, pH 7.4, and an inoculation cell density of 1.5×10^6 cells/ml in 10 ml working volume. One-half of the medium was replenished every 2 days.

The VCD was slightly higher in OMM media with similar viability compared with OMM (GMP) (Figure 3a,b). The cell diameter gradually decreased during culture days (Figure 3c). The enucleation increased from Day 4 after inoculation and was maintained until the final culture day, reaching up to 95% in OMM (Figure 3d,e). The erythroid cell-specific surface markers were analyzed by flow cytometry, showing GPA positivity up to 93% (Figure S2). These results show that the stirred-tank bioreactor is appropriate for culturing mature erythroblasts during the maturation stage for higher RBC yields. The OMM media were used in the following experiments using DOE methods.

3.3 | Inoculation timing of erythroblasts during the growth stage (from Day 10)

From previous results for inoculation at Days 7 and 12, we set up experiments to define the optimal inoculation timing to bioreactors between culture Days 10 and 13 to acquire higher RBC yields. After inoculation into bioreactors at Day 10, erythroid cells with a mean

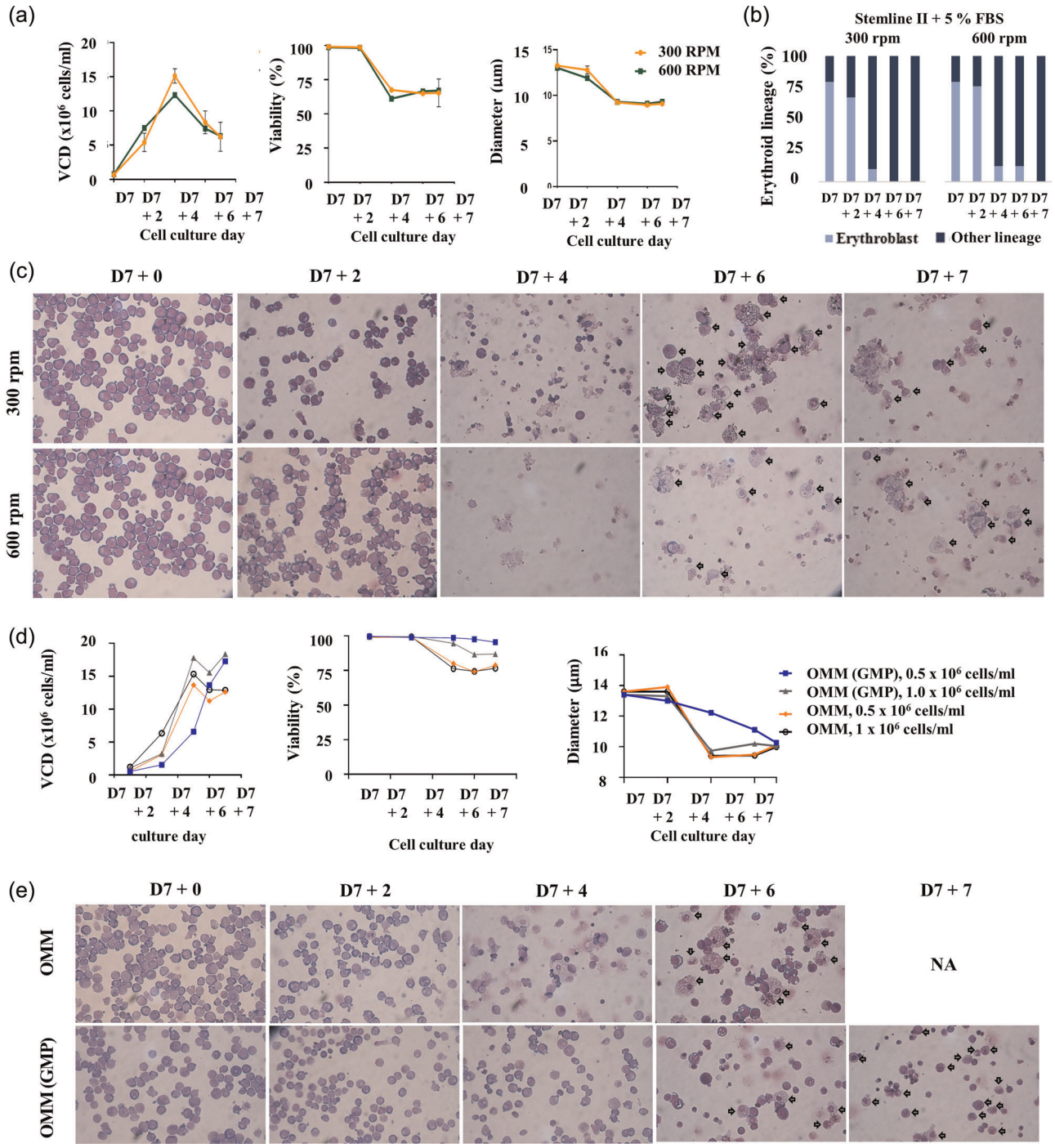

FIGURE 2 Culturing erythroid cells at the immature stage in a stirred-tank bioreactor. Erythroid cells differentiated from CD34+ cells were cultured in a static plate for 7 days in Stemline media supplemented with 5% fetal bovine serum, cytokines, and other additives, followed by inoculation to 11-ml stirred-tank bioreactors at a density of 0.5×10^6 cells/ml. While culturing for the subsequent 7 days at agitation rates of 300 and 600 rpm, (a) viable cell density (VCD), viability, and cell diameter were determined using an automatic cell counter during culture in a bioreactor ($n = 5$ for 300 rpm, $n = 1$ for 600 rpm). (b and c) The Erythroid cell lineage and other cells such as macrophages were counted, showing increased macrophages from Day 4 after inoculation into bioreactors. Cytospun cell morphology by Wright–Giemsa staining, magnification ×200. (d) Basophilic erythroblasts were cultured in OMM or OMM (GMP) media at a cell inoculation density of 0.5×10^6 and 1×10^6 cells/ml at 300 rpm in a bioreactor and VCD, viability, and cell diameter were evaluated. (E) In OMM, more immature erythroid cells were observed at Day 7 + 6 with many nonerythroid cells such as macrophages and myeloid cells (black arrows). No intact erythroblasts were observed on Day 7 + 7. Some orthochromatic cells and nonerythroid cells were detected in OMM (GMP). NA, not applicable due to few viable cells. Black arrows, nonerythroid cells. Wright–Giemsa stain, magnification ×200 [Color figure can be viewed at wileyonlinelibrary.com]

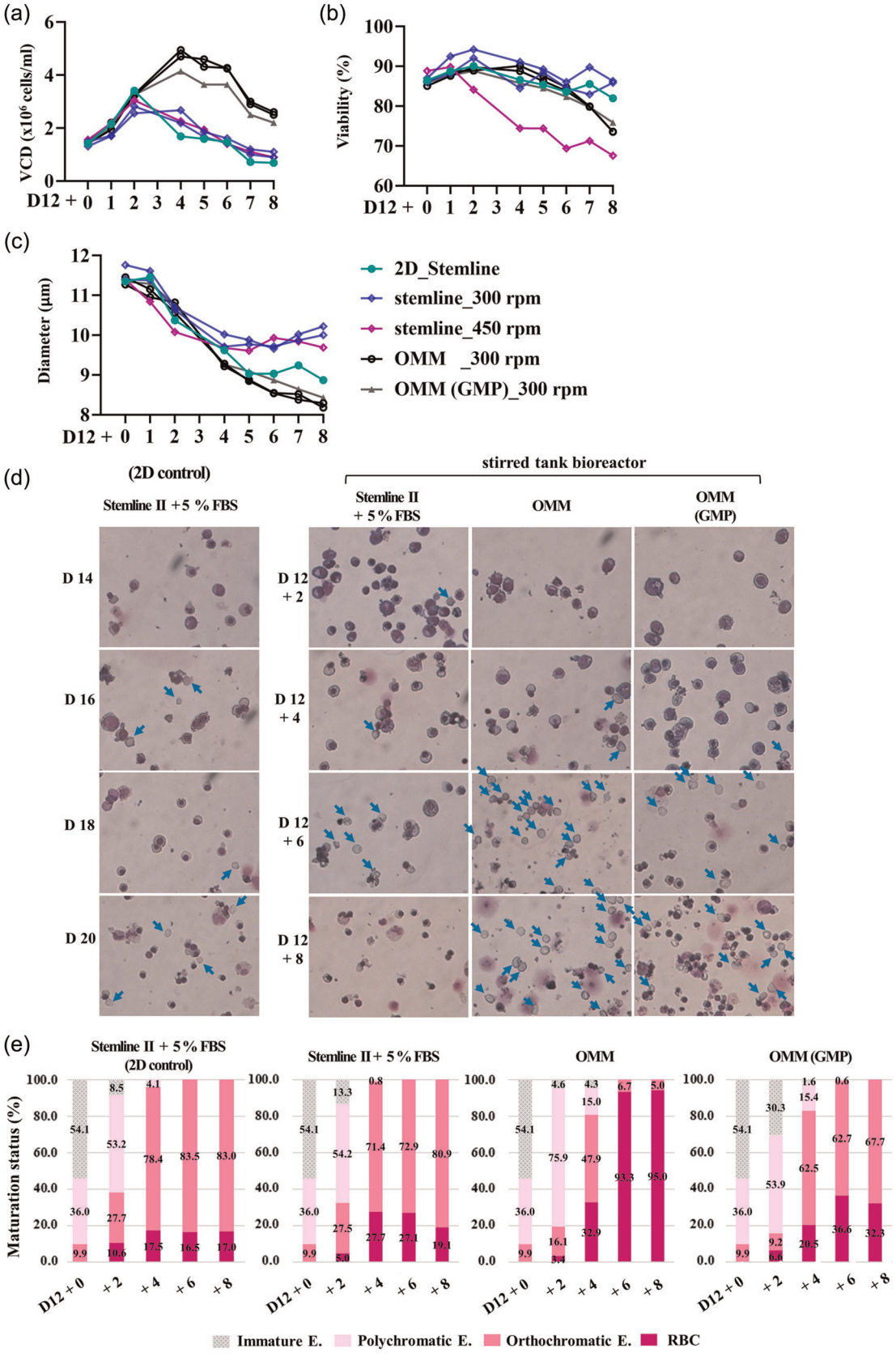

FIGURE 3 (See caption on next page)

cell size of 13.14 μm at Day 10 showed rapidly decreased cell size in 1 day (Figure 4a) along with decreased viability (Figure 4b), demonstrating that the large immature cells were affected (Figure S3). In 2D plates before inoculation, cell sizes were decreased as shown in the previous results (Figure 4c). The similar cell size on Days 11 and 12 demonstrates that the cells at this culture period were in the same maturation stage of basophilic erythroblasts (Figure 4c). The enucleation rate was significantly higher in the groups inoculated at Day 12 compared with those inoculated at Day 10 (Figure 4d). The proliferation-fold was the highest in the groups inoculated at Day 13 (Figure 4e). Therefore, the RBC yield that was calculated as the erythroid cell number × enucleation rate was significantly higher in the groups inoculated at Day 12 compared with others (Figure 4f; analysis of variance analysis, Kruskal–Wallis test, $p < .05$). As immature erythroblasts inoculated at Days 10 and 11 were dead soon after inoculation, the relative proportion of other lineage cells exceeded the erythroid cells (Figure 4g). The enucleated RBCs at the final culture day were increased in conditions inoculated at Day 12 or 13, demonstrating the importance of seeding timing for higher RBC yields (Figure 4h).

3.4 | Effect of agitation speed, inoculation density, and inoculation timing at Days 12–14

We next evaluated the optimal agitation speed and inoculation density along with the inoculation day for the late growth and maturation phase (DOE-1 in Table 1). As the erythroid cell size gradually decreases along with maturation, cell size was evaluated as a key parameter for deciding cell inoculation timing to acquire optimal RBC yields.

Desirability function in JMP software predicted the optimal values as rpm = 459.3, inoculation day = 12.6 day, and inoculation density = 5×10^6 cells/ml for VCD and erythroid cell numbers (Figure 5a). The desirability function for the RBC yield predicts the optimal values as rpm = 500, inoculation at 12.6 day, and inoculation density = 0.5×10^6 cells/ml (Figure 5b). These models showed well-predicted plots (Figure S4). The high-RBC yield at high agitation speed in Figure 5b is consistent with the previous results that agitation speed increases the enucleation rate (Bayley et al., 2018). The VCD was maintained very high (>5×10^6 cells/ml) in many experimental runs, reaching 1.5×10^7 cells/ml (Figure 5c), indicating that manufacturing at the higher cell inoculation density up to 5×10^6 cells/ml was possible. The RBC yield was significantly higher at the higher cell inoculation density (5×10^6 cells/ml) than the lower (0.5×10^6 cells/ml) in Figure 5d. Moreover, theoretically, the low-density condition of 0.5×10^6 cells/ml requires 10 times greater media volume and 10 times more bioreactor machines than the higher condition of 5×10^6 cells/ml. In terms of RBC yield, cost, and volume efficiency, we selected the high-cell density condition as much more efficient for large-scale production. The agitation speed did not affect viability at the final culture Day 21 (Figure 5a). The cell sizes were significantly different on Days 12–13 and Day 14 (12.79 ± 0.13 and 10.68 ± 0.13 μm, respectively; mean ± SD; unpaired *t*-test, $p < .0001$) in Figure 5e. The manufactured RBC morphology was generally good with even cell size and intact membrane as evaluated by Wright–Giemsa stain (Figure S5). Together, these results indicate that cell inoculation to the bioreactors with shear stress is optimal when the erythroid cell size is within 12–13 μm for higher RBC yields. Therefore, these findings confirm that hydrodynamic shear stress due to mechanical agitation can be harmful to immature erythroid cells.

3.5 | Effect of feeding regimens, DO, and pH at a high-cell seeding density on erythroblast growth and metabolism

Using the identified optimal conditions (inoculation timing at Day 13, a high-cell density of 5.0×10^6 cells/ml, and agitation speed of 500 rpm), we evaluated the appropriate culture parameters to overcome the extremely large media volume required for RBC production. Day 13 cells were inoculated in a working volume of 11 ml per vessel at 37°C and 500 rpm with DO 10%–50%, pH 7.3–7.5, and media exchange ratio from 20% to 80% every 48 h (DOE-2 in Table 1).

Starting from 2×10^6 CD34+ cells, the total cultured cell number reached up to 4.5×10^{10} cells (Figure 6a). This corresponds to 1.13×10^{11} erythroid cells from one unit of cord blood, which contains approximately 5×10^6 CD34+ cells. The optimal parameters for VCD seeded with a high-cell density were 20% media exchange ratio per 48 h, 10% DO, and pH 7.481 at Day 19 (Figure 6b). In contrast, the optimal parameters for erythroid cell numbers and enucleation at Day 19 were different from that for VCD (Figures 6c,d and S6). Moreover, the predicted plot for enucleation did not show a good summary of fit in the model, requiring further experimental design focusing on final enucleation. The viability was higher when media volume was frequently changed with DO 37.7%, and a pH of 7.3

FIGURE 3 Culturing erythroid cells at the growth stage in a bioreactor. Erythroid cells cultured in static plates were inoculated to a stirred-tank bioreactor on Day 12 and evaluated for 8 days in two types of media. (a–c) Viable cell density (VCD), viability, and cell diameter were determined during culture in bioreactors (in Stemline II + 5% fetal bovine serum [FBS], OMM, and OMM (GMP) media) or static plates (two-dimensional [2D] in Stemline II + 5% FBS). (d) From 6 days after inoculation, enucleated red blood cells (RBCs; blue arrows) frequently appeared, especially in OMM media in a bioreactor at 300 rpm. Representative cell morphology after cytospinning. Wright–Giemsa staining, magnification ×200. (e) Erythroid cell subpopulation ratio of cultured cells in four different types of media, showing high enucleation ratios up to 95% in OMM [Color figure can be viewed at wileyonlinelibrary.com]

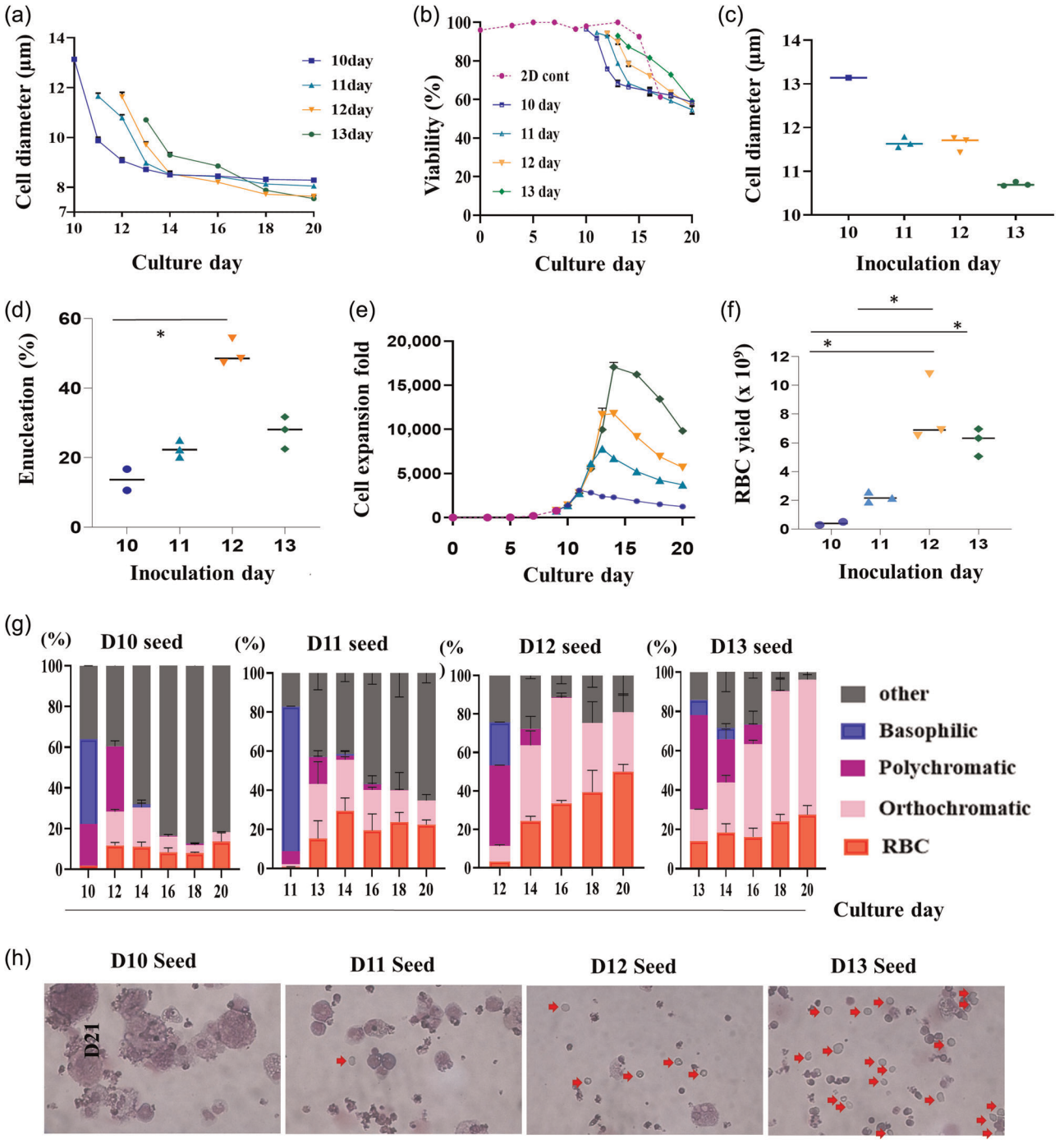

FIGURE 4 Appropriate inoculation timing of erythroblasts to a bioreactor during the growth stage. The erythroid cells were cultured in a static culture until Day 10 and inoculated serially to a bioreactor during Days 10–13. (a) The cell diameter and (b) viability are shown along with the culture days. (c) The cell diameter at the time of inoculation to a bioreactor are shown. Bar indicates a mean value. (d) Enucleation rates at culture Day 21 are shown depending on the inoculation days. (e) Cell expansion fold for 21 days starting from one CD34+ cell at Day 0 are shown. (f) Final red blood cell (RBC) yields from 1×10^6 CD34+ cells. (g) Cell subpopulation changes depending on the cell inoculation timing to a bioreactor. When seeded in earlier days, other cell lineages such as macrophages and granulocytes were increased. Data are mean ± SD. *$p < .05$. (h) Representative cell image at the final culture day. Wright–Giemsa stain, magnification ×400 [Color figure can be viewed at wileyonlinelibrary.com]

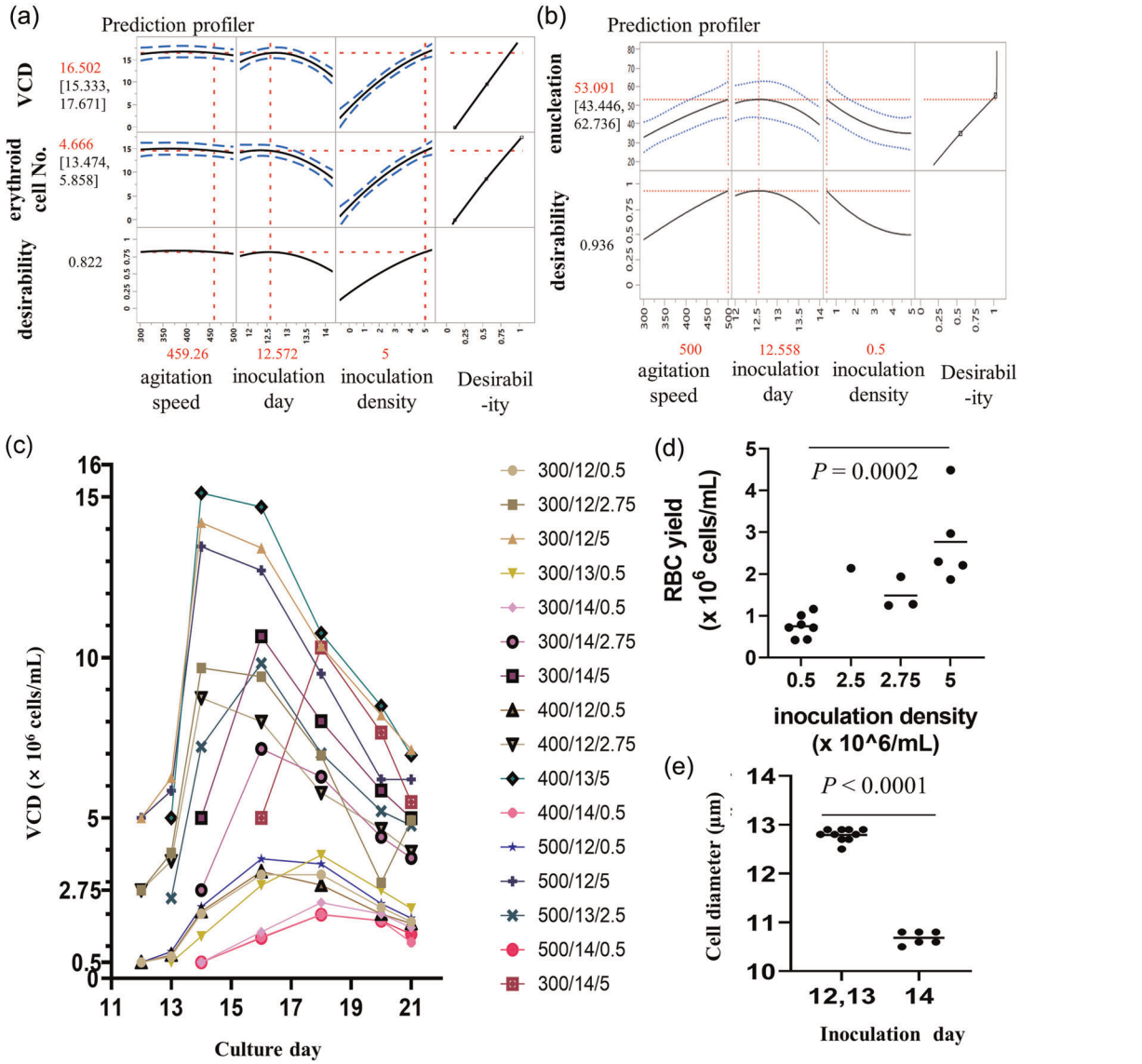

FIGURE 5 Optimal parameters of agitation speed, inoculation density, and inoculation timing at the growth stage. Cells were serially inoculated to bioreactors during culture Days 12–14 as in the design of experiments-1, Table 1. (a and b) *x*-Axes show three controlled culture parameters in the bioreactor, and *y*-axes show the culture results. The most appropriate results and parameters are shown as the red-line cross points. The blue dotted line shows the predicted value of each *Y* variable for the current values of the *X* variables. Black lines within the blue plots indicate how the predicted value changes with *X* variables. (c) Viable cell density (VCD) changes during culture days in bioreactors. The labels indicate rpm/inoculation day/inoculated cell density. (d) Red blood cell (RBC) yield depending on seeding density at the final culture day of 21. (e) Cell diameter at the time of inoculation to a bioreactor. Each dot indicates the mean value of the cells of each experimental run [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 6e). On Day 17, when the erythroid cell numbers were the highest, the optimal media exchange ratio was 55.9% (Figure 6f). The fitness of the final model for VCD was good, confirmed with $R^2 = .91$, and $p = .0013$. The fitness of the final model was not excellent determined by $R^2 > .85$ and $p < .05$ for Day 19 erythroid cells and by $R^2 = .82$ and $p = .0956$ for RBCs at Day 19 from F-statistic, requiring more sophisticated DOE for the final enucleation phase.

As the media exchange ratio, DO, and pH affect metabolic functions, we evaluated their effects on cell metabolites in cultured media supernatant throughout the culture period using an automatic analyzer. In experiments adjusting the medium replacement schedule (20%–80% every 48 h), significant effects were observed on most metabolites except lactate, which was mainly dependent on DO (Table 2). All concentrations of measured metabolites in DOE-2 showed significant differences between culture days, indicating that metabolism changes considerably as erythropoiesis progresses (Table 2).

The metabolite results on Day 21 were analyzed in Figure S7. The lactate level significantly increased at DO 50% compared with levels at DO 10%. However, lactate concentration was always less

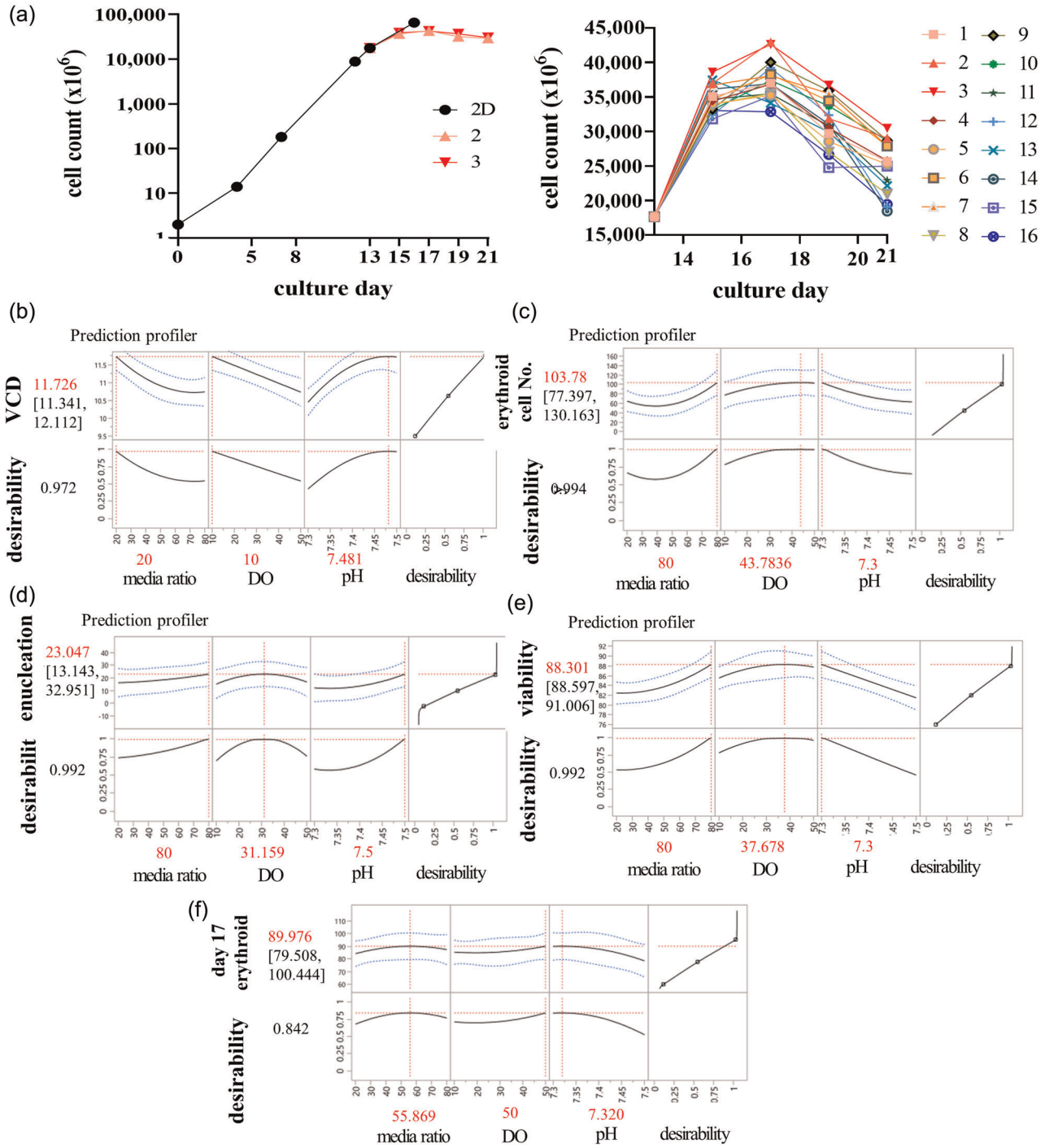

FIGURE 6 Optimal parameters of the media exchange ratio, dissolved oxygen (DO), and pH at the maturation stage. Erythroid cells were inoculated to bioreactors at culture Day 13 and evaluated the optimal culture parameters as in the design of experiments (DOE)-2, Table 1. DOE outcome reporting the optimal parameter configuration obtained maximizing the desirability for erythroid cell expansion, enucleation ratio, viable cell density (VCD), and viability. (a) Viable erythroid cell expansion during culture days with representative lots of run number 2 and 3 compared with two-dimensional (2D) plate culture. (b–e) Using JMP software, the desirability function predicts the optimal values of the media exchange ratio, DO, and pH for VCD, the erythroid cell number, the enucleation ratio, and viability [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 The effects of culture conditions and culture days on metabolites in DOE-2

Source	Factors	F[a]	*p* Value
Glucose	Media exchange ratio	8.60	.0048[b]
	DO	0.30	.5870
	pH	0.01	.9167
	Days	83.25	<.0001[b]
Lactate	Media exchange ratio	3.34	.0727
	DO	51.04	<.0001[b]
	pH	0.36	.5534
	Days	81.11	<.0001[b]
Glutamine	Media exchange ratio	5.61	.0213[b]
	DO	0.18	.6704
	pH	0.02	.8969
	Days	14.34	<.0001[b]
Glutamate	Media exchange ratio	127.19	<.0001[b]
	DO	1.87	.1769
	pH	0.35	.5579
	Days	9.29	<.0001[b]
Ammonia	Media exchange ratio	133.74	<.0001[b]
	DO	0.04	.8367
	pH	0.46	.4983
	Days	19.52	<.0001[b]

Abbreviations: Days, culture days in a bioreactor; DO, dissolved oxygen; DOE, design of experiments.

[a]F-statistics which tests whether there are significant differences in the mean values of "Source" by the change of different "Factors."

[b]Statistical significance at $\alpha = .001$.

than 1801.8 mg/L (20 mol/L), above which lactate is reported to inhibit cell proliferation in other mammalian cells (Figure S7a; Buchsteiner et al., 2018; Patel et al., 2000). During culture with a low media exchange ratio of 20% per 48 h, glucose concentration decreased to low levels (1189.2 ± 599.4 mg/L at Day 19 and 18.0 ± 47.7 mg/L at Day 21), suggesting that the medium replacement schedule should be more frequent than 20% per 48 h. The 50% medium change schedule caused a decrease in glucose concentration but maintained acceptable levels for proliferation (1962.0 ± 496.2 mg/L at Day 19 and 694.1 ± 521.4 mg/L at Day 21). Ammonia, which is a derivative of glutamine metabolism, has a toxic effect on mammalian cells and can inhibit cell growth at a level higher than 4 mM. With a higher media exchange ratio, ammonia level was significantly decreased, although ammonia levels did not reach 4 mM until the final culture day (Figure S7b). The change of pH did not significantly affect metabolite concentrations or gas levels (Figure S7c). These results suggest that a medium replacement strategy is critical for RBC production. A partial media change of 50% every 48 h until Day 21 allowed for an adequate supply of nutrients and maintain waste concentrations at acceptable levels.

3.6 | Investigation of the culture parameters for the enucleation stage

Focusing on the enucleation stage, we examined the optimal culture conditions. Following the best protocol acquired by DOE-1 and DOE-2, mature erythroblasts were inoculated at the high density of 5.0×10^6 cells/ml to bioreactors at Day 13 when the mean cell size decreased to less than 12 μm (11.24 ± 3.03 μm). The erythroid cell markers analyzed by flow cytometry were presented in Figure S8. Cells were cultured until Day 15 at 37°C, DO 10%, and pH 7.5, 500 rpm. The media change ratio was 20% every 2 days until Day 15, and then 50% exchange until the last day. The cell culture parameters of temperature, DO, and pH were changed during Days 15–21 as in DOE-3 in Table 1.

Generally, viability, enucleation rates, and RBC yield showed high degrees of convolution effect in response to changes in pH, DO, and temperature (Figure 7a). Viability was significantly higher at lower temperatures, while the enucleation rate and RBC yield were significantly higher at higher temperatures (Figure 7b,c). A prediction profile showing the relative desirability values demonstrated that the final erythroid cell and enucleated RBC (%) at Day 21 were the best at 37°C, DO 40.2%, and pH 7.5 (Figure S9).

Concentrations of glucose, lactate, glutamine, glutamate, and ammonium in cultured media supernatant showed no significant changes depending on the three variables. In contrast, all concentrations of measured metabolites in DOE-3 in Table 1 showed significant differences between culture days, indicating that metabolism changes considerably as erythropoiesis progresses (Table S1). Overall, waste metabolite concentrations were usually under growth-inhibiting levels and roughly constant during culture days.

3.7 | Function analysis of manufactured RBCs

At culture Day 21, bioreactor RBCs in DOE-3 in Table 1, except the first figure on top which used RBCs cultured in OMM (GMP) in Figure 3, were harvested and filtered using a deleukocyting filter and then evaluated for RBC functions compared with fresh adult peripheral blood (PB)-RBCs. The manufactured RBCs showed very consistent sizes and healthy cell membrane in Wright–Giemsa stained slides and confocal microscopy after CFSE staining (Figure 8a). For the deformability, EI at pressure 3 Pa showed comparable results at 0.31% for fresh adult PB-RBCs and 0.29% for manufactured RBCs at a bioreactor, confirming that the deformability is similar (Figure 8b). Moreover, the manufactured RBCs showed similar deformability to PB-RBCs than RBCs cultured in 2D plates. In the oxygen-carrying capacity analysis, similar oxygen equilibrium curves between fresh adult PB-RBCs (p50 = 31.2%) and manufactured RBCs (p50 = 22.0%) were observed (Figure 8c).

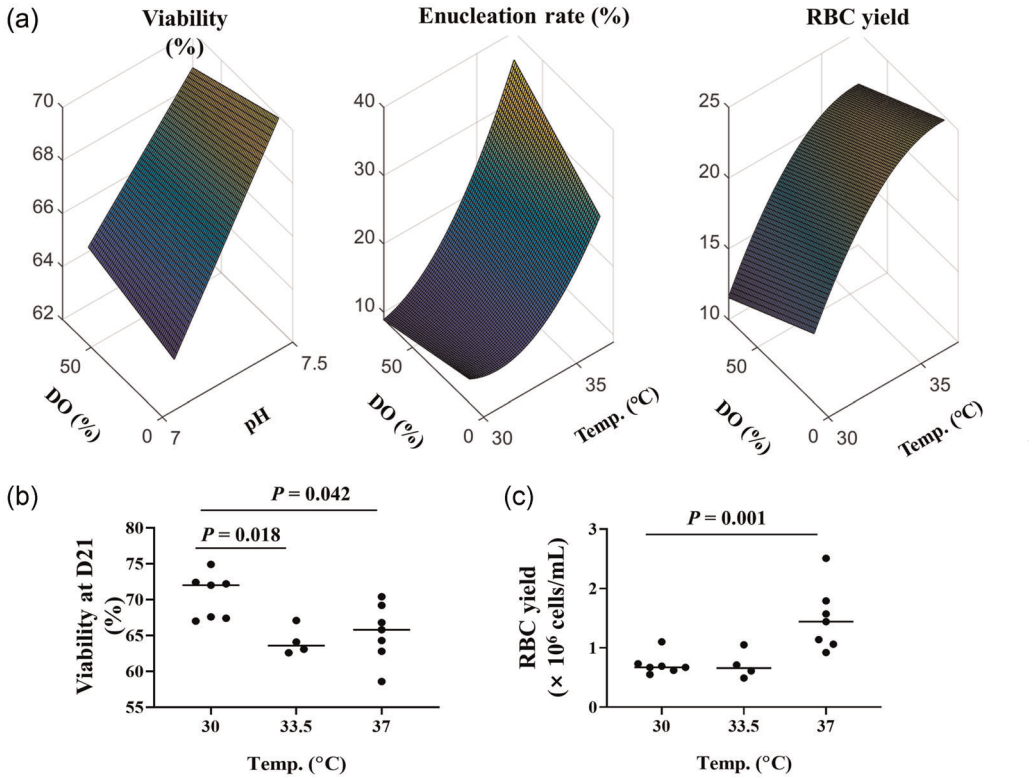

FIGURE 7 Response surfaces and optimal parameters of pH, dissolved oxygen (DO), and temperature at the enucleation stage. Erythroid cells were inoculated to bioreactors at Day 13 and cultured as in the design of experiments-3, Table 1. (a) Surface profiles of the viability, enucleation rates, and red blood cell (RBC) yield, related to the DO, pH, and temperature are illustrated (red: maximum efficiency; green: medium efficiency; blue: low efficiency). (b) Viability and (c) RBC yields at culture Day 21 depending on temperature [Color figure can be viewed at wileyonlinelibrary.com]

Even though a previous study showed that RBCs stored in a refrigerator showed dysmorphic morphology due to ATP decrease and increased calcium, the manufactured RBCs stored in a 4°C refrigerator showed intact morphology by microscopy comparable to freshly withdrawn PB-RBCs for 21 days, confirming storability and cell intactness (Figure 8d). The manufactured pure RBCs showed appropriate blood grouping results with anti-A, anti-B, and anti-D antibodies (Figure 8e). The bioreactor RBCs showed mature erythrocyte cell surface markers (CD71−/nuclei−/GPA+) comparable to fresh PB-RBCs as analyzed by flow cytometry (Figure 8f). The bioreactor RBCs expressed both adult Hb (Hb-alpha) and fetal Hb (Hb-gamma) as analyzed by flow cytometry (Figure 8g).

4 | DISCUSSION

The present study established the optimal manufacturing process for RBC generation from the late-stage erythroid cells in a fully controlled stirred-tank bioreactor. Our results demonstrate that the optimal parameters are different depending on the erythroblast maturation stage, highlighting that the seeding timing to a bioreactor plays a critical role in the final RBC yield. The decrease in RBC yield is caused by damage of immature erythroblasts, which are very weak in shear stress, revealed by the marked decrease in viability. The final cell product manufactured in the stirred tank showed a more mature phenotype of reticulocytes, which can be induced by circulatory shear stress (Moura et al., 2018).

We also identified for the first time the maturation stage-specific culture parameters of pH, DO, temperature, cell inoculation density, and agitation speed which can be used for further scale-up for automatic manufacturing of mature reticulocytes and RBCs. Furthermore, our method using DOE designing and multivariate analysis provides very reliable, reproducible, and scalable culture parameters. As the manufacturing process was performed with a working volume of 10–11 ml using up to 18 vessels simultaneously in parallel, our data provide very precise and reproducible results with the capability of evaluating numerous cells in each run. Our results demonstrated the feasibility of successfully generating mature erythroid cells at a high-cell density of 1.5×10^7 cells/ml, reaching cell expansion as high as 1.125×10^{11} erythroid cells if we start from 5×10^6 CD34+ cells.

In contrast to the immature erythroid cells before culture Day 12, more mature cells were resistant to shear stress at up to 600 rpm, showing more evenly sized erythrocyte diameters. Interestingly, the generated cells showed a biconcave shape and negativity for the CD71 cell surface marker, which are the characteristics

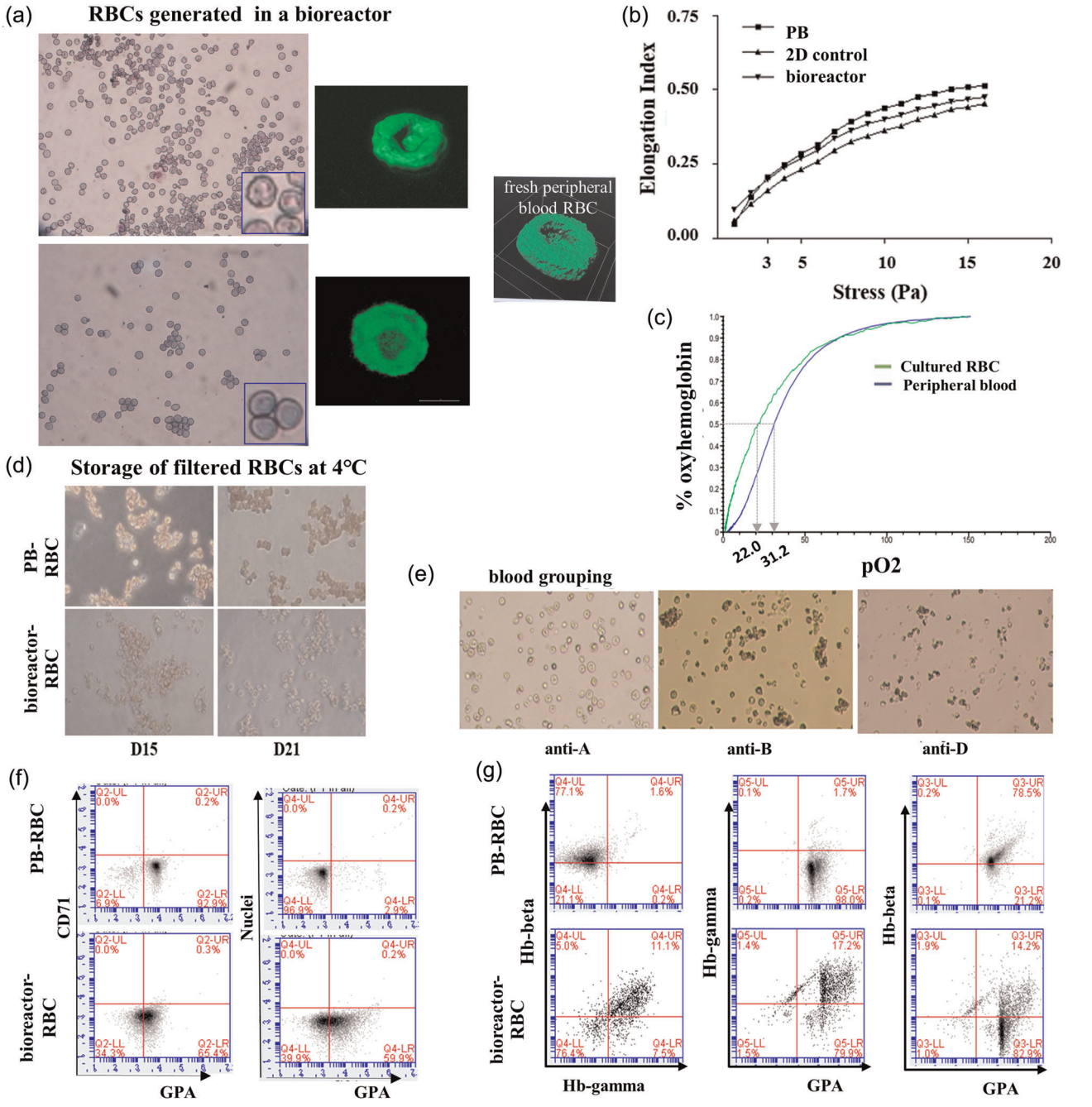

FIGURE 8 Evaluation of manufactured red blood cell (RBC) functions. (a) Morphology of RBCs manufactured in a bioreactor (bioreactor-RBC). Representative morphology of two independent cases. Wright–Giemsa staining, magnification ×200 (left). RBC morphology after staining with CFSE and examined with confocal laser scanning microscopy (right). (b) RBC deformability test by elongation index (EI). (c) Oxygen equilibrium curves were measured by a Hemox analyzer (a representative image from two cases). (d) Fresh peripheral blood (PB) RBCs and manufactured RBCs were stored for 21 days in a 4°C refrigerator and observed using phase-contrast microscopy. (e) The manufactured RBCs were tested for ABO grouping, revealing appropriate blood clumping. Representative images of one case of blood group B RhD+. (f and g) The manufactured RBCs were evaluated for the erythrocyte-specific cell surface markers of CD71−/nuclei−/glycophorin A (GPA)+ and hemoglobin (Hb)-beta and Hb-gamma compared with PB-RBCs [Color figure can be viewed at wileyonlinelibrary.com]

of mature erythrocytes, not reticulocytes. The final mature RBCs contained adult hemoglobin.

In the only previous article that studied RBC culture parameters using DOE methods, CB CD34+ cells were cultured in 2D culture plates for 3 days and then inoculated to stirred-tank bioreactors (Bayley et al., 2018). The final RBCs showed good morphology, but erythroid cell proliferation was very low at 12-fold for 25 days of culture. In both the study by Bayley and our study, the DO value was the important culture factor; the low value of DO (25%) produced higher cell yields than higher DO. Our data in DOE-2, however,

suggest that the erythroid cell number, reticulocyte yield, and viability were higher at DO 30%–40%, while pVCD was higher when DO was low at 10%. While they used agitation speeds of 300 or 450 rpm, our results showed that mature cells were able to endure the high impeller speed from 300 to even 600 rpm. In addition, our data provide more experimental sets covering various parameters such as media exchange ratio and temperature, which all resulted in very significant effects on cell proliferation and final RBC yields.

A report using a wave bioreactor (Cultibag®; Sartorius) resulted in RBCs with no intact cell shape and low erythroid cell-specific (GPA) positivity (Timmins et al., 2011). They also suggested that differentiation increases in agitated cultures compared with static controls. The authors argued that this may be due to the altered dynamics of cell–cell interactions and substrate adhesion in agitated culture. However, in view of our results of marked decreases in cell sizes and low viability, the reason may be because the immature cells could not survive and only mature cells remained. Their data with high-cell proliferation (1.68×10^{10}-fold expansion) at Day 55 but lack of red color and low GPA positivity might be due to cell death of erythroid cells. The final cells at day 55 might be mono-macrophage lineages that can proliferate for several months (Timmins et al., 2011). Recently, Trakarnsanga et al. (2017) succeeded in the establishment of erythroblast cell lines and cultured the cell lines in an agitated bottle. However, the precise culture condition was not mentioned.

High-cell density is harmful to the cell culture due to the shortage of nutrients and accumulated metabolic wastes (Buchsteiner et al., 2018). In our study, the higher media exchange rate of 80% per 48 h was good for viability. However, the lower exchange rate of 50% also showed acceptable RBC yields and is an attractive choice for the optimal protocol because of the tremendously high-RBC cell numbers to fit transfusion requirements and the expensive costs of manufacturing. In addition, the low media exchange conditions were sufficient to maintain cell density over 10^7 cells/ml until day 21 of cell culture, suggesting that effective media supply is possible for scale-up. Generating RBCs from PB or CB CD34+ cells in tissue culture flasks at 15 rpm has been shown to reach $10–60 \times 10^5$ cells/ml in the final maturation stage (Griffiths et al., 2012; Kupzig et al., 2017). PB MNCs were also cultured in a stirred bioreactor but showed 1/10-fold expansion (Heshusius et al., 2019). This was probably due to the damage of immature cells by shear stress compared to the static culture as like in our study. This is because there may be a shock when the cells are transferred suddenly to the stirred condition. However, this possibility was considered here. Even with good data in previous reports, DOE experiments using fully controlled automatic bioreactors remain necessary for scale-up to minimize the risk of large-scale culture.

In comparison to the former process, our stage-specific processes markedly enhanced the proliferation and RBC yields and provided a very efficient generation of healthy and functional RBCs. Using our results, the timing of transferring from static to stirred-tank bioreactors can be automatically determined using cell size without manual intervention by an operator.

The limitation of our study is that culturing immature cells in other types of bioreactors and transferring the cells into the stirred-tank bioreactor should be realized to make the entire manufacturing process automatic. Another limitation is the scale-up of total culture volume. The highest RBC yield was 4.5×10^6 cells/ml in DOE-1, 3.0×10^6 cells/ml in DOE-2 (Day 17; Figure 6c,d), and 2.51×10^6 cells/ml in DOE-3 (Figure 7c). The volume of the reactor would be 440 L (2×10^{12} divided by 4.5×10^6 cells/ml = 0.44×10^6 ml). Therefore, a substantial volume of bioreactors is still required to substitute for the current donor blood.

To confirm the consistency of the RBC production process, we used one donor for each DOE set. Even though the manufacturing system for clinical use requires very consistent yield, there can be donor-dependent variations. However, we found that the cell size is the key to decide on the inoculation timing as the patterns of the decreasing tendency of cell sizes were similar among different donor cells (Figure S10).

In this study, the method of shifting from static cultures to stirred-tank bioreactors was established using DOE methods. According to the results, combining the static cultures of immature erythroid cells and stirred-tank bioreactors for mature erythroid cells would be effective to obtain a large number of functional mature RBCs. The optimized setting resulted in significantly increased productivity and significantly decreased metabolic waste levels compared with other conditions. To our knowledge, this is the first report of cultivation to simultaneously improve RBC yields and cell proliferation by specifically optimizing the manufacturing process using real-time monitored bioreactors. This method suggests an efficient approach to manufacturing RBC yields while considering cell characteristics during maturation.

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CONFLICTS OF INTEREST

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

Designed research: Eun Jung Baek and Won-Seok Choi. Performed research: Eun Mi Lee, So Yeon Han, Janghan Lee, and Hyosang Lee. Designed the DOE experiments and analyzed the data: Janghan Lee, Hyosang Lee, and Won-Seok Choi. Statistical analysis: Amy M. Kwon. Wrote the manuscript: Eun Jung Baek.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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REFERENCES

Bayley, R., Ahmed, F., Glen, K., McCall, M., Stacey, A., & Thomas, R. (2018). The productivity limit of manufacturing blood cell therapy in scalable stirred bioreactors. *Journal of Tissue Engineering and Regenerative Medicine*, *12*(1), e368–e378. https://doi.org/10.1002/term.2337

Bresnick, E. H., Hewitt, K. J., Mehta, C., Keles, S., Paulson, R. F., & Johnson, K. D. (2018). Mechanisms of erythrocyte development and regeneration: Implications for regenerative medicine and beyond. *Development*, *145*(1):dev151423. https://doi.org/10.1242/dev.151423

Buchsteiner, M., Quek, L. E., Gray, P., & Nielsen, L. K. (2018). Improving culture performance and antibody production in CHO cell culture processes by reducing the Warburg effect. *Biotechnology and Bioengineering*, *115*(9), 2315–2327. https://doi.org/10.1002/bit.26724

Choe, W. H., & Baek, E. J. (2015). RBC substitutes: From the past to the future. *ISBT Science Series*, *10*(S1), 150–153. https://doi.org/10.1111/voxs.12153

Eaker, S., Abraham, E., Allickson, J., Brieva, T. A., Baksh, D., Heathman, T. R. J., Mistry, B., & Zhang, N. (2017). Bioreactors for cell therapies: Current status and future advances. *Cytotherapy*, *19*(1), 9–18. https://doi.org/10.1016/j.jcyt.2016.09.011

Giarratana, M. C., Kobari, L., Lapillonne, H., Chalmers, D., Kiger, L., Cynober, T., Marden, M. C., Wajcman, H., & Douay, L. (2005). Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. *Nature Biotechnology*, *23*(1), 69–74. https://doi.org/10.1038/nbt1047

Glen, K. E., Workman, V. L., Ahmed, F., Ratcliffe, E., Stacey, A. J., & Thomas, R. J. (2013). Production of erythrocytes from directly isolated or Delta1 Notch ligand expanded CD34+ hematopoietic progenitor cells: process characterization, monitoring and implications for manufacture. *Cytotherapy*, *15*(9), 1106–1117. https://doi.org/10.1016/j.jcyt.2013.04.008

Goldrick, S., Holmes, W., Bond, N. J., Lewis, G., Kuiper, M., Turner, R., & Farid, S. S. (2017). Advanced multivariate data analysis to determine the root cause of trisulfide bond formation in a novel antibody-peptide fusion. *Biotechnology and Bioengineering*, *114*(10), 2222–2234. https://doi.org/10.1002/bit.26339

Griffiths, R. E., Kupzig, S., Cogan, N., Mankelow, T. J., Betin, V. M. S., Trakarnsanga, K., Massey, E. J., Lane, J. D., Parsons, S. F., & Anstee, D. J. (2012). Maturing reticulocytes internalize plasma membrane in glycophorin A-containing vesicles that fuse with autophagosomes before exocytosis. *Blood*, *119*(26), 6296–6306. https://doi.org/10.1182/blood-2011-09-376475

Han, S. Y., Lee, E. M., Choi, H. S., Chun, B. H., & Baek, E. J. (2018). The effects of plasma gelsolin on human erythroblast maturation for erythrocyte production. *Stem Cell Research*, *29*, 64–75. https://doi.org/10.1016/j.scr.2018.03.001

Hay, K. A., & Turtle, C. J. (2017). Chimeric antigen receptor (CAR) T cells: Lessons learned from targeting of CD19 in B-cell malignancies. *Drugs*, *77*(3), 237–245. https://doi.org/10.1007/s40265-017-0690-8

Heshusius, S., Heideveld, E., Burger, P., Thiel-Valkhof, M., Sellink, E., Varga, E., Ovchynnikova, E., Visser, A., Martens, J. H. A., von Lindern, M., & van den Akker, E. (2019). Large-scale in vitro production of red blood cells from human peripheral blood mononuclear cells. *Blood Advances*, *3*(21), 3337–3350. https://doi.org/10.1182/bloodadvances.2019000689

Housler, G. J., Miki, T., Schmelzer, E., Pekor, C., Zhang, X., Kang, L., Voskinarian-Berse, V., Abbot, S., Zeilinger, K., & Gerlach, J. C. (2012). Compartmental hollow fiber capillary membrane-based bioreactor technology for in vitro studies on red blood cell lineage direction of hematopoietic stem cells. *Tissue Engineering Part C: Methods*, *18*(2), 133–142. https://doi.org/10.1089/ten.TEC.2011.0305

Kim, H. O., & Baek, E. J. (2012). Red blood cell engineering in stroma and serum/plasma-free conditions and long term storage. *Tissue Engineering Part A*, *18*(1-2), 117–126. https://doi.org/10.1089/ten.tea.2010.0711

Kim, S. H., Lee, E. M., Han, S. Y., Choi, H. S., Ryu, K. Y., & Baek, E. J. (2019). Improvement of red blood cell maturation in vitro by serum-free medium optimization. *Tissue Engineering Part C: Methods*, *25*(4), 232–242. https://doi.org/10.1089/ten.TEC.2019.0023

Kupzig, S., Parsons, S. F., Curnow, E., Anstee, D. J., & Blair, A. (2017). Superior survival of ex vivo cultured human reticulocytes following transfusion into mice. *Haematologica*, *102*(3), 476–483. https://doi.org/10.3324/haematol.2016.154443

Manwani, D., & Bieker, J. J. (2008). The erythroblastic island. *Current Topics in Developmental Biology*, *82*, 23–53. https://doi.org/10.1016/S0070-2153(07)00002-6

Moura, P. L., Hawley, B. R., Mankelow, T. J., Griffiths, R. E., Dobbe, J. G. G., Streekstra, G. J., Anstee, D. J., Satchwell, T. J., & Toye, A. M. (2018). Non-muscle myosin II drives vesicle loss during human reticulocyte maturation. *Haematologica*, *103*(12), 1997–2007. https://doi.org/10.3324/haematol.2018.199083

Myers, R. H., Montgomery, D. C., Vining, G. G., Borror, C. M., & Kowalski, S. M. (2004). Response surface methodology: A retrospective and literature survey. *Journal of Quality Technology*, *36*(1), 53–77. https://doi.org/10.1080/00224065.2004.11980252

Patel, S. D., Papoutsakis, E. T., Winter, J. N., & Miller, W. M. (2000). The lactate issue revisited: Novel feeding protocols to examine inhibition of cell proliferation and glucose metabolism in hematopoietic cell cultures. *Biotechnology Progress*, *16*(5), 885–892. https://doi.org/10.1021/bp000080a

Ratcliffe, E., Glen, K. E., Workman, V. L., Stacey, A. J., & Thomas, R. J. (2012). A novel automated bioreactor for scalable process optimisation of haematopoietic stem cell culture. *Journal of Biotechnology*, *161*(3), 387–390. https://doi.org/10.1016/j.jbiotec.2012.06.025

Rodak, B. F., Keohane, E. M., & Fritsma, G. A. (2013). *Hematology-E-Book: Clinical principles and applications*. Elsevier Health Sciences.

Sewell, D. J., Turner, R., Field, R., Holmes, W., Pradhan, R., Spencer, C., Oliver, S. G., Slater, N. K., & Dikicioglu, D. (2019). Enhancing the functionality of a microscale bioreactor system as an industrial process development tool for mammalian perfusion culture. *Biotechnology and Bioengineering*, *116*(6), 1315–1325. https://doi.org/10.1002/bit.26946

Timmins, N. E., Athanasas, S., Gunther, M., Buntine, P., & Nielsen, L. K. (2011). Ultra-high-yield manufacture of red blood cells from hematopoietic stem cells. *Tissue Engineering Part C: Methods*, *17*(11), 1131–1137. https://doi.org/10.1089/ten.TEC.2011.0207

Trakarnsanga, K., Griffiths, R. E., Wilson, M. C., Blair, A., Satchwell, T. J., Meinders, M., Cogan, N., Kupzig, S., Kurita, R., Nakamura, Y., Toye, A. M., Anstee, D. J., & Frayne, J. (2017). An immortalized adult human erythroid line facilitates sustainable and scalable generation of functional red cells. *Nature Communications*, *8*, 14750. https://doi.org/10.1038/ncomms14750

SUPPORTING INFORMATION

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