Int J Clin Exp Med 2020;13(9):6635-6643
www.ijcem.com /ISSN:1940-5901/IJCEM0111920

Original Article

Construction and evaluation of a highly effective culture system for erythroid differentiation of umbilical cord blood-derived hematopoietic stem and progenitor cells

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Received April 4, 2020; Accepted June 16, 2020; Epub September 15, 2020; Published September 30, 2020

Abstract: Objective: To construct and evaluate a highly effective culture system for the erythroid differentiation of umbilical cord blood-derived CD34$^+$ hematopoietic stem and progenitor cells (HSPCs). Methods: Magnetic-activated cell sorting (MACS) was applied to separate CD34$^+$ HSPCs from umbilical cord blood. Cells were cultured in 3 culture systems (1, 2, or 3), which have the three cell factors including SCF, IL3 and EPO, for 18 days using a 3-stage method to induce erythroid differentiation after *in vitro* proliferation for 4 days. Both cell proliferation ability and cell surface CD235a$^+$CD71$^+$ expression were detected at each differentiation stage. The percentage of burst-forming unit-erythroid (BFU-E) colonies in each system were analyzed on day 12. Results: Cell proliferation in all culture systems peaked between day 10 and day 12, followed by a decreasing trend. Nevertheless, cell proliferation remained higher after day 12 in system 1 than in the other two systems (P<0.05). The expression of the erythroid surface markers CD71 and CD235a reached the highest level on day 18 in culture systems 1, 2, and 3 (89.1%, 79.4% and 88.0%, respectively). Statistical analyses revealed no significant differences between systems 1 and 3, which had the highest percentages of CD71$^+$CD235a$^+$ cells among the three culture systems (P=0.44). Progenitor cells were formed in all culture systems on day 12 of differentiation. The percentage of BFU-E colonies was higher in system 1 than in systems 2 and 3, with approximate percentages of 87.5%, 73.5% and 83.5%, respectively (P<0.05). Conclusion: We successfully constructed and evaluated a high-efficiency erythroid differentiation culture system for umbilical cord blood-derived CD34$^+$ HSPCs based on continuous cytokine supplementation during all stages of erythroid differentiation.

Keywords: Erythroid differentiation, culture system, hematopoietic stem cell, umbilical cord blood

Introduction

With the increasing demand for blood products for clinical use, insufficient sources have become an urgent problem in the clinic, especially for patients with severe trauma and acute massive bleeding. Blood transfusion is sometimes necessary to ensure patient safety [1, 2]. Stem cell research has revealed possibilities for solving the issues with clinical demand for blood products [3, 4]. Hematopoietic stem and progenitor cells (HSPCs), which have the potential for self-renewal and robust differentiation, can differentiate into erythroid cells *in vitro*, and this finding has attracted the attention of researchers, bringing hope as a consistent blood source for clinical use [5, 6]. HSPCs can be derived from peripheral blood or umbilical cord blood. The proliferation rate of HSPCs derived from umbilical cord blood is 4 times that of HSPCs derived from peripheral blood [7, 8]. The regulatory factors in umbilical cord blood related to differentiation are the same as those in peripheral blood. Besides, obtaining umbilical cord blood is convenient and painless, and there are abundant sources [9, 10]. Therefore, it is widely used in basic research and clinical trials. Umbilical cord blood was used as the source of HSPCs in this study.

Currently, culture methods for the erythroid differentiation of HSPCs are still under investiga-

tion and vary from serum to serum-free culture systems [11, 12]. Due to the complex composition of serum-containing culture systems and the relative difficulty in clinical application, serum-free culture systems have received increasing attention. Serum-free culture systems promote the erythroid differentiation of HSPCs through the addition of growth factors, i.e., stem cell factor (SCF), interleukin 3 (IL3) and erythropoietin (EPO) [13]. Based on the previous methods reported by Dever et al [14], we have established a new culture system, system 1 by dislodged the component of lipids and shortening the culture time from 21 days to 18 days. In addition, we also constructed culture system 3 by adding hydrocortisone at the first stage following the methods reported by Giarratana et al [15]. To evaluate the influence of serum in the culture efficacy, we have compared both systems 1 and 3 to system 2 including FBS designed by our laboratory. Our study aimed to construct and evaluate a highly effective culture system for the erythroid differentiation of umbilical cord blood-derived $CD34^+$ HSPCs. Furthermore, we aimed to provide a reference for basic research on the erythroid differentiation of stem cells from umbilical cord blood and a theoretical foundation for clinical application.

Materials and methods

Specimen source

Umbilical cord blood was provided by the Department of Obstetrics and Gynecology at the Third Affiliated Hospital of Guangzhou Medical University. Samples were obtained after informed consent was signed by patients and the protocol was approved by the hospital ethics committee. Each sample consisted of 100-120 mL of cord blood collected using a sterile anticoagulant blood collection bag (4% sodium citrate as the anticoagulant). We used one sample for each experiment, and all the samples were from full-term pregnant women.

Reagents

Ficoll lymphocyte separation solution was purchased from Guangzhou Saiguo Biotech Co., Ltd. The magnetic bead sorting kit for $CD34^+$ collection was purchased from Miltenyi Biotech Co., Germany. Red blood cell lysis buffer was purchased from Sigma, and serum-free expansion medium II (SFEMII) was purchased from Stem Cell. The cytokines; SCF, IL3 and EPO were purchased from PeproTech. Fetal bovine serum (FBS) was purchased from Gibco, and L-glutamine, transferrin and hydrocortisone were purchased from Guangzhou Peipu Biotechnology Co., Ltd. Trypan blue and H4434 semisolid medium was purchased from Stem Cell, and CD235a and CD71 antibodies were purchased from BD.

Extraction of $CD34^+$ cells from umbilical cord blood

Freshly harvested umbilical cord blood from one full-term pregnant woman was mixed with PBS at a volume ratio of 1:1. Mononuclear cells in cord blood were isolated using Ficoll lymphocyte separation with density gradient centrifugation. The volume ratio of Ficoll separation solution and mixed umbilical cord blood was 1:2. After gradient centrifugation, the cells in the white-film layer were carefully collected and washed 3 times with PBS. After erythrocyte lysis, the mononuclear cells were obtained, and one sample yielded approximately 10^8 mononuclear cells. After the cells were counted, they were sorted using CD34-based magnetic beads, and the obtained $CD34^+$ HSPCs were cultured in SFEMII. Usually, 10^6 HSPCs were obtained from 10^8 mononuclear cells, the purity of HSPCs was greater than 90% by flow cytometry analysis, and the viability was over 95% as determined by trypan blue staining (data not shown).

In vitro culture of cord blood-derived $CD34^+$ cells

The sorted $CD34^+$ cells were first cultured in proliferation medium for 4 days and then seeded into 3 different differentiation media (**Table 1**) at a density of 2×10^5 cells, followed by culture at 37°C with 5% CO_2.

Cell counting

Cells were counted on days 2, 4, 6, 9, 12, 15, 18, and 21. After generating a single-cell suspension, 10 μl of the suspension was mixed thoroughly with 10 μL of Trypan blue staining solution, and 10 μL of the mixed solution was added to a cell counting machine. The cell number and survival rate were determined.

Table 1. Components of the 3 culture systems

Culture system	Components	Final concentration		
		Stage 1	Stage 2	Stage 3
System 1	L-Glutamine (mM)	2	2	2
	SCF (ng/ml)	100	100	100
	IL3 (ng/ml)	10	10	10
	EPO (U/ml)	0.5	3	3
	Transferrin (μg/ml)	200	200	1000
System 2	SCF (ng/ml)	10	-	-
	IL3 (ng/ml)	10	-	-
	EPO (U/ml)	2	3	3
	FBS	10%	30%	30%
System 3	SCF (ng/ml)	100	100	-
	IL3 (ng/ml)	5	-	-
	EPO (U/ml)	3	3	3
	Hydrocortisone (μmol/L)	1	-	-

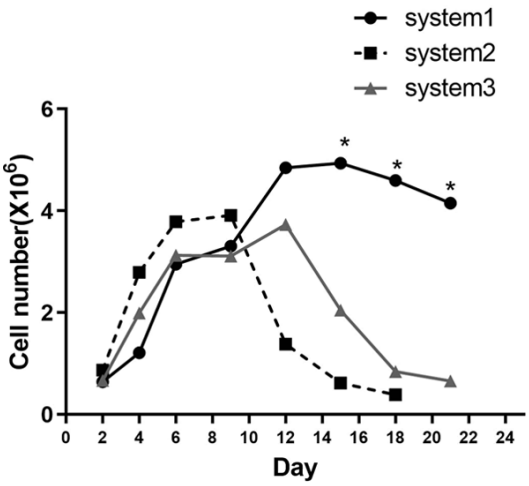


Figure 1. CD34⁺ HSPC proliferation in three culture systems (*P<0.05).

Detection of CD235a and CD71 expression on the cell surface

On days 2, 4, 9, and 18, the cell surface erythroid markers CD235a and CD71 were detected by flow cytometry. Cell suspensions were collected and washed once with PBS. The cells were counted, and 5 × 10^5 cells were analyzed by flow cytometry. After the cells were centrifuged, the supernatant was discarded, and 100 μL of PBS and 5 μL each of CD235a-APC and CD71-FITC antibodies were added. After incubation at 4°C for 30 min, the cells were washed twice with PBS and resuspended in 500 μL of PBS for flow cytometry analysis.

Colony formation assay

On day 9 of differentiation in the 3 different culture systems, the cells were mixed with H4434 semisolid medium and seeded into 24-well plates. Each well was seeded with 500 cells, and each sample was analyzed in 3 replicates. After the cells were cultured at 37°C with 5% CO_2 for 14 days, a colony-forming unit (CFU) count was performed under an inverted microscope. One colony was considered a focus with more than 50 cells, and the percentage of erythroid burst-forming units (BFU-E) to the total number of colonies was calculated.

Statistical analysis

All experimental data were analyzed using SPSS 20.0 statistical software. Measurement data are expressed as the mean ± SD, and statistical analysis was performed using the 2 independent samples *t*-test. Count data are presented as percentages, and statistical analysis was performed using the chi-square test. $P<0.05$ was considered to indicate statistical significance.

Results

Comparison of CD34⁺ HSPC proliferation in different culture systems

Culture systems 1 and 3 were serum-free culture systems with multiple cytokine combinations, and system 2 was a serum-containing culture system. During erythroid differentiation, higher cell proliferation potential is more conducive to subsequent differentiation. During the 18 days of differentiation and culture, the cells cultured in the 3 systems gradually increased in number before stage 2, after which the proliferation decreased in systems 2 and 3 due to the reduction of cytokine levels in stage 3, and the proliferation in system 1 plateaued. After 12 days, the results showed that system 1 maintained a high cell proliferation capacity for a long period time, significantly higher than that for systems 2 and 3 ($P<0.05$, **Figure 1**).

Comparison of the erythroid differentiation ability of CD34$^+$ HSPCs in different culture systems

All cells in the 3 systems were cultured in 3 stages (**Figure 2A**). Flow cytometry was performed on days 2, 4, 9 and 18. The results showed that the cells in system 3 differentiated the fastest during the 18 days of directed differentiation. On days 4 and 9, the percentages of CD71$^+$ and CD235a$^+$ cells were nearly 50% and 70% in system 3, respectively, and these percentages were higher than those in systems 1 (31.5% and 45.6%) and 2 (37.1% and 56.2%) (P<0.05) (**Figure 2B**, **2C**). On day 18 of differentiation, the percentage of CD71$^+$CD235a$^+$ cells was significantly higher in systems 1 (89.1%) and 3 (88%) than in system 2 (79.4%, P<0.05) (**Figure 2B**, **2C**). Therefore, umbilical cord blood stem cells exhibited better erythroid differentiation and maturity in systems 1 and 3.

Comparison of BFU-E colonies derived from CD34$^+$ HSPCs in different culture systems

To further evaluate the ability of the 3 culture systems to induce cord blood CD34$^+$ HSPCs to differentiate into erythroid progenitor cells, cells at day 12 of differentiation were used for hematopoietic colony formation experiments. The results showed that the cells in the 3 culture systems were capable of forming 6 different types of colonies: BFU-E; CFU-erythroid (CFU-E); CFU-granulocyte (CFU-G); CFU-macrophage (CFU-M); CFU-granulocyte, macrophage (CFU-GM); and CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) (**Figure 3**). The percentage of BFU-E in system 1 was nearly 90%, which was significantly higher than that in system 2 (73.5%) and system 3 (83.5%) (P<0.05, **Figure 4**). Therefore, system 1 could more effectively induce cord blood CD34$^+$ HSPCs to differentiate into erythroid progenitor cells and enter the next differentiation stage.

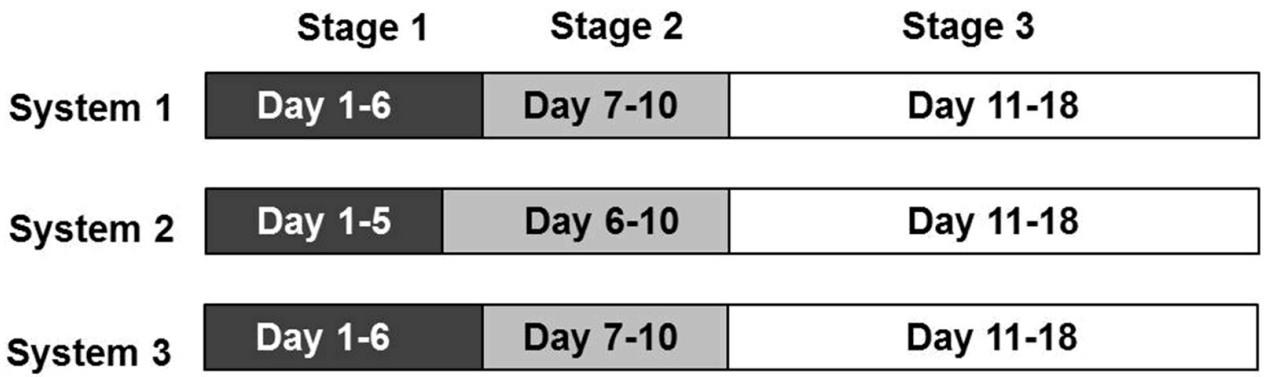
Discussion

Currently, methods for the *in vitro* culture of hematopoietic stem cells from umbilical cord blood include serum-free and serum-containing culture methods. Serum-free culture methods rely on the addition of cytokines to the basal medium to promote cell growth and are the most commonly used methods [16]. SCF, IL3 and transferrin are important cytokines for maintaining the *in vitro* proliferation of HSPCs [17, 18]. EPO facilitates HSPC erythroid differentiation, and dexamethasone can inhibit cell proliferation and promote differentiation at high cell density [19, 20].

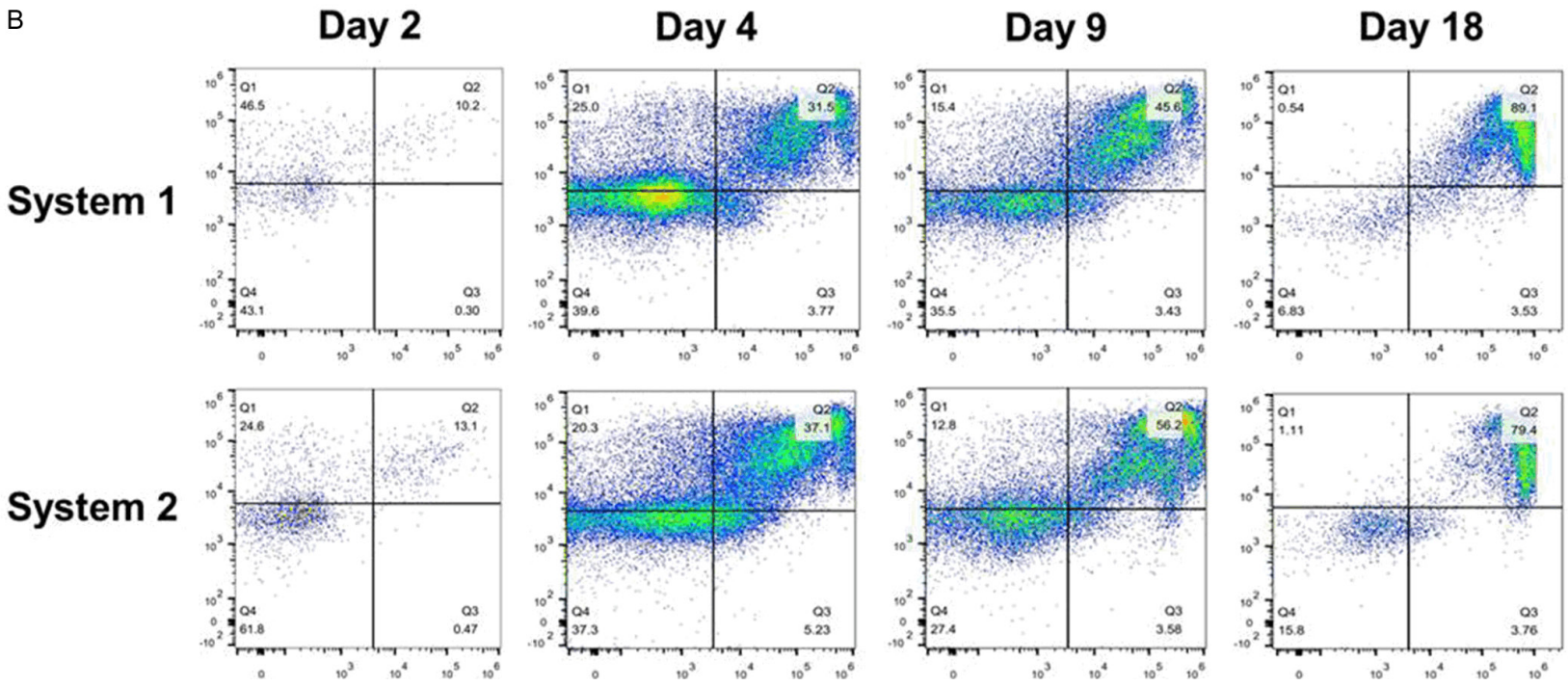
This study analyzed the effect of 3 different culture systems on the differentiation of CD34$^+$ HSPCs from umbilical cord blood. System 1 maintained both the proliferation and the differentiation efficiency of cells and was the most effective of the 3 tested culture systems. Cell proliferation assays showed that cell proliferation in the 3 culture systems gradually increased before stage 2, after which it decreased because of the reduction in cytokines in stage 3. However, the cells in system 1 maintained good growth even after 12 days of culture, which was conducive to entering the next differentiation stage. Flow cytometry analysis showed that the percentage of CD71$^+$ and CD235a$^+$ cells in systems 1 and 3 on day 18 of differentiation was approximately 90%, significantly higher than that in system 2. Because system 3 involves the addition of a large amount of EPO in the first stage of differentiation, erythroid differentiation was faster. On days 4 and 9, the percentages of CD71$^+$ and CD235a$^+$ cells in system 3 were nearly 50% and 70%; however, the corresponding percentages were 31.5% and 45.6% in system 1 and 37.1% and 56.2% in system 2. The colony formation assay showed that cells on the 12th day of differentiation formed 6 different types of colonies and that BFU-E formation was the highest in system 1, at nearly 90%. Therefore, during the erythroid differentiation process, system 1 was the most effective of the 3 culture systems tested.

The 3 culture systems evaluated in this study included the serum-free systems 1 and 3, which included the different cytokines mentioned above, and the serum-containing system 2. The 3 culture systems exhibited differences in cell proliferation, erythroid differentiation, and erythroid colony formation. System 1 maintained a good proliferative state for a longer time, and its BFU-E colony percentage was the highest among the 3 culture systems. Flow cytometry analysis showed that the percentage of CD71$^+$ and CD235a$^+$ cells reached

A


Stage 1
Stage 2
Stage 3
System 1
Day 1-6
Day 7-10
Day 11-18
System 2
Day 1-5
Day 6-10
Day 11-18
System 3
Day 1-6
Day 7-10
Day 11-18

B


Day 2
Day 4
Day 9
Day 18
System 1
Q1 46.5
Q2 10.2
Q4 43.1
Q3 0.30
Q1 25.0
Q2 31.5
Q4 39.6
Q3 3.77
Q1 15.4
Q2 45.6
Q4 35.5
Q3 3.43
Q1 0.54
Q2 89.1
Q4 6.83
Q3 3.53
System 2
Q1 24.6
Q2 13.1
Q4 61.8
Q3 0.47
Q1 20.3
Q2 37.1
Q4 37.3
Q3 5.23
Q1 12.8
Q2 56.2
Q4 27.4
Q3 3.58
Q1 1.11
Q2 79.4
Q4 15.8
Q3 3.76

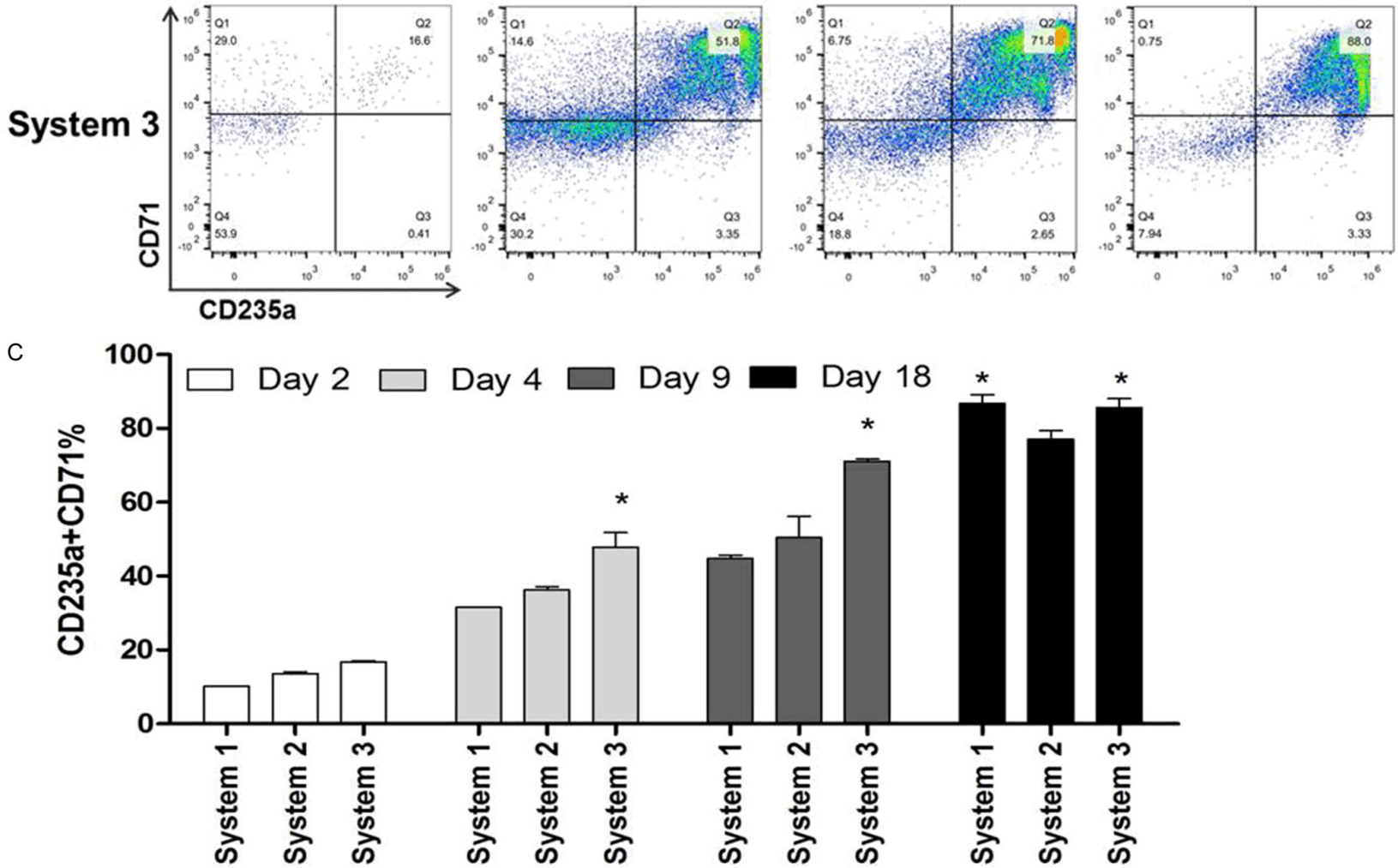


Figure 2. CD34$^+$ HSPC erythroid differentiation in three culture systems. A. Schematic diagrams of the 3 stages. B, C. Expression of the CD71$^+$ and CD235a$^+$ in each culture system (*P<0.05).

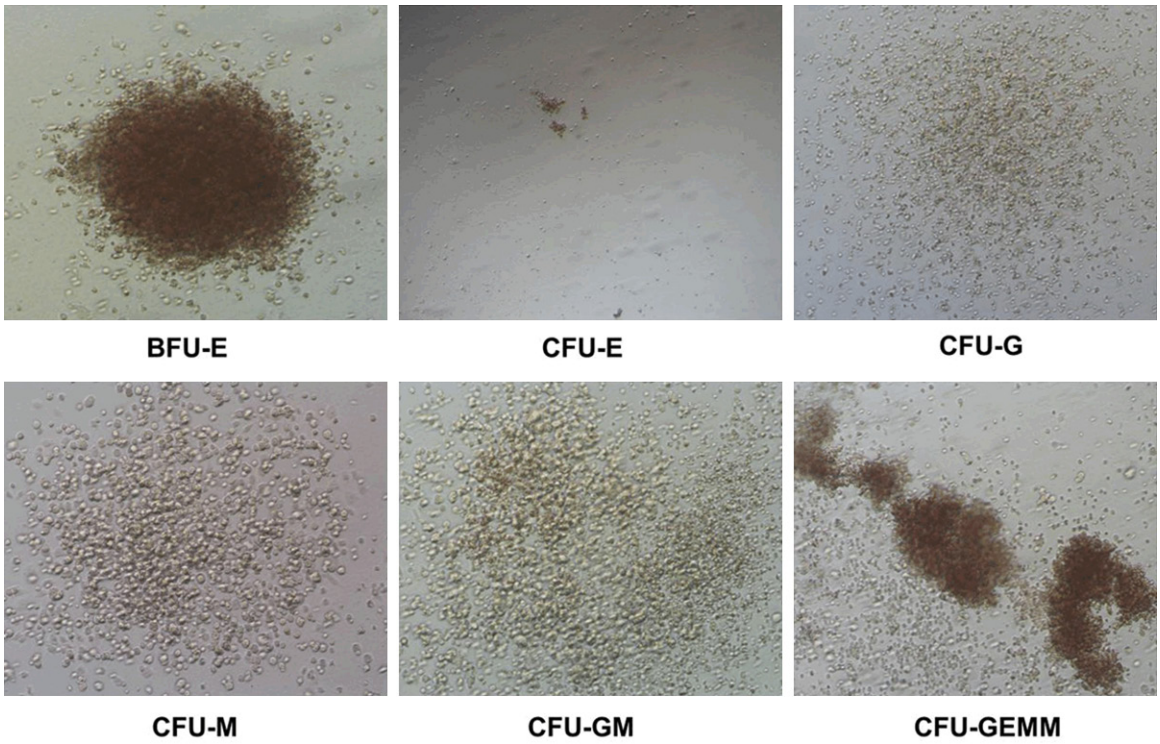


Figure 3. Cell colony formation in the 3 culture systems. BFU-E, erythroid burst-forming units; CFU-E, colony forming unit-erythroid; CFU-G, colony forming unit-granulocyte; CFU-M, colony forming unit-macrophage; CFU-GM, colony forming unit-granulocyte, macrophage; CFU-GEMM, colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte.

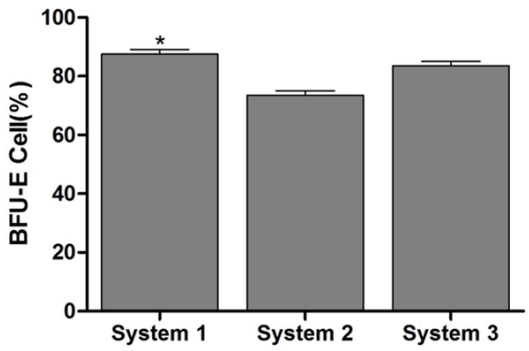


Figure 4. The percentage of BFU-E colonies derived from $CD34^+$ HSPCs in each culture system. *P<0.05.

approximately 90% on day 18. Although cells in system 3 entered erythroid differentiation rapidly, the final $CD71^+$ and $CD235a^+$ percentages were not significantly different from those in system 1, and the ability of system 3 to maintain good cell proliferation was significantly lower than that of system 1.

It has been reported that co-culturing HSPCs with stromal cells can promote the denucleation of differentiated erythroid cells [21, 22]. In this study, we used a suspension culture method without a matrix. We intend to further evaluate coculture methods to promote the denucleation of differentiated erythroid cells to provide experimental bases for obtaining a large number of mature red blood cells and to lay the foundation for clinical transformation.

In summary, serum-free culture system 1 was the most effective and optimal culture system among those tested, and these results provide a reference for future research on the erythroid differentiation of HSPCs.

Acknowledgements

This study was supported by the Guangzhou City Science and Technology Key Topics Project (201904020025), National Natural Science Foundation of China (31872800), Clinical Innovation Research Program of Guangzhou Regenerative Medicine and Health Guangdong Laboratory (2018GZR0201002), Natural Science Foundation of Guangdong Provin-

ce (2014A030312012), Science Foundation of Guangdong Province (2016B030229008), Foundation of Guangzhou Science and Information Technology of Guangzhou Key Project (201803040009), and Science Foundation of Health Commission of Sichuan Province (18PJ459).

Disclosure of conflict of interest

None.

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