Sequential cellular niches control the generation of enucleated erythrocytes from human pluripotent stem cells

Human pluripotent stem cells (hPSC), including human embryonic stem cells (hESC) and human-induced pluripotent stem cells (hiPSC), possess a powerful potential for *in vitro* generation of red blood cells (RBC) to overcome blood supply shortages.[1,2] Previous studies have demonstrated that hPSC have the ability to differentiate into erythroid cells.[3-7] However, the generation of enucleated erythrocytes has still been challenging. The goal of this study is to investigate cellular niche effects on erythroblast generation and their enucleation.

During embryonic development, hemogenic endothelial (HE) cells in the aorta-gonad-mesonephros (AGM) have been recognized as precursors of hematopoietic stem cells (HSC) that give rise to multi-lineage hematopoietic cells.[8,9] Therefore, we firstly initiated hematopoietic differentiation from hPSC by HE generation in a chemically defined monolayer differentiation system (Figure 1A). As previously described, hESC-derived HE cells on day 5 (D5) exhibited previously described HE phenotypes ($CD34^+CD144^+CD43^-CD73^-CD184^-$)[10,11] (*Online Supplementary Figure S1A*). The hematopoietic potential of HE cells through endothelial-to-hematopoietic transition (EHT) was confirmed by culture of the $CD34^+$ cells isolated from the D5 cells (*Online Supplementary Figure S1B-E*). To induce erythroid differentiation, the D5 cells were sub-cultured for eight days in the presence of erythropoietin (EPO) (Figure 1A). Immunostaining analysis of the emerged round-cell clusters on day 2 showed that they co-expressed CD235a erythroid and CD41 megakaryocytic markers (Figure 1B). Flow cytometry analysis further confirmed that the majority of the emerged hematopoietic cells on day 2 were $CD235a^+CD41^+$ cells (>90%) (Figure 1C) and nearly all the $CD235a^+CD41^+$cells expressed CD31, but not CD144 (*Online Supplementary Figure S2*). The $CD235a^+CD41^+$ progenitor cells gradually lost the expression of CD41 megakaryocytic marker (Figure 1C). By day 5 of erythroid differentiation (D5+5), less than 50% of the cells were $CD235a^+CD41^+$ cells (Figure 1C). By day 8 (D5+8), the majority of the cells (>90%) were $CD235a^+CD71^+CD41^-CD34^-CD45^-$ cells, indicating an erythroid commitment (Figure 1 C and 1D). Benzidine staining detected hemoglobin expression in erythroid

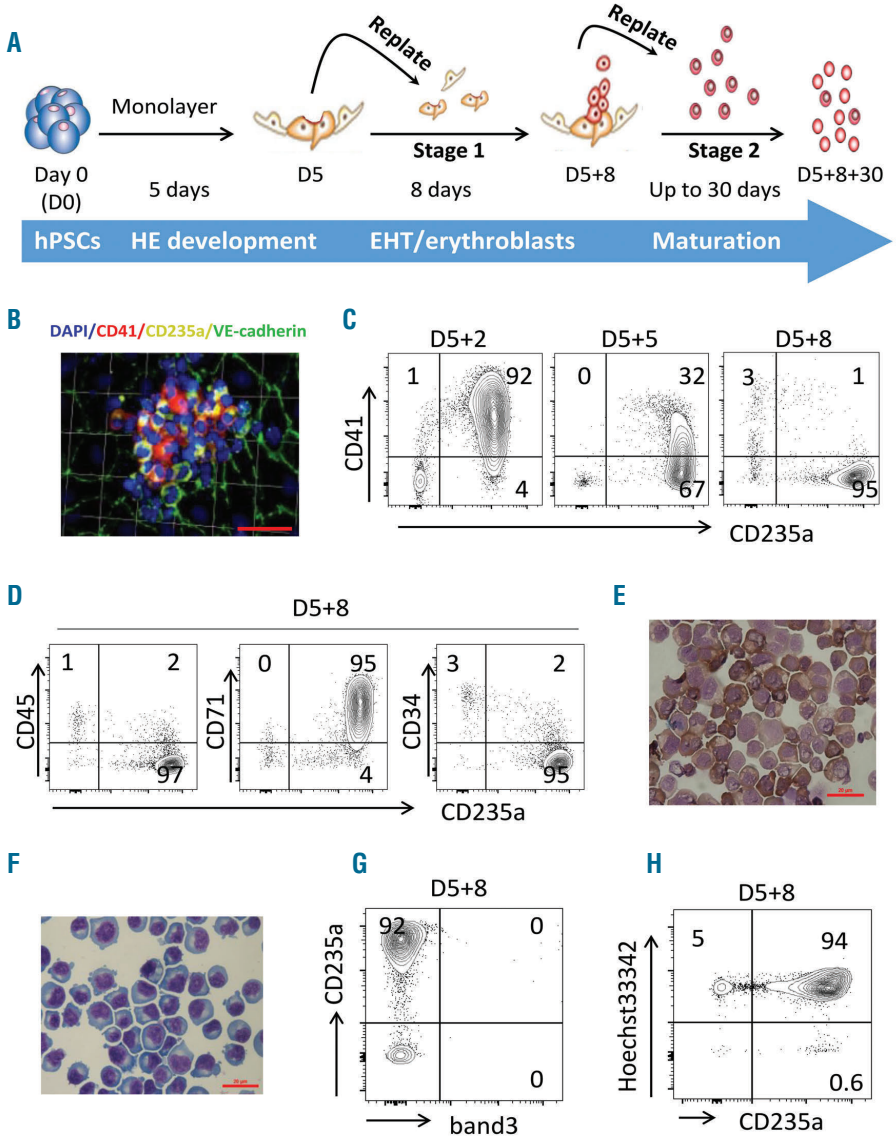

Figure 1. A novel chemically-defined monolayer differentiation system of hPSC for effective generation of erythroid cells. (A) Schematic diagram showing the strategy for the development of HE cells, and HE-derived erythroid differentiation (Stage 1) and maturation (Stage 2). (B) Visualization of emerging hematopoietic clusters in EHT cultures by confocal imaging. Immunostaining was performed with antibodies for the endothelial marker (VE-cadherin in green) and the hematopoietic markers (CD41 in red and CD235a in yellow). DAPI in blue was used for nuclear staining. Scale bar, 100 μm. (C) Representative flow cytometric analysis of the frequency of $CD235a^+$ and $CD41^+$cells after erythroid differentiation of HE cells for two days (D5+2), five days (D5+5) and eight days (D5+8). The suspended and semi-adherent hematopoietic cells, excluding adherent endothelial cells, were collected for flow cytometry analysis. n=3. (D) Representative flow cytometric analysis of the frequency of $CD235a^+$, $CD45^+$, $CD71^+$, $CD34^+$ cells in D5+8 erythroid cells. The suspended and semi-adherent hematopoietic cells, excluding adherent endothelial cells, were collected for flow cytometry analysis. n=3. (E) Benzidine (brown color) for hemoglobin and Wright-Giemsa (nuclear DNA) staining of D5+8 erythroid cells. Scale bar, 20 μm. (F) Wright-Giemsa staining of D5+8 erythroid cells. Scale bar, 20 μm. (G) Representative flow cytometric analysis of the expression of band3 in D5+8 erythroid cells. n=3. (H) Representative flow cytometric analysis of enucleation of D5+8 erythroid cells. n=3. Experiments were performed on H1 hESC unless otherwise indicated.

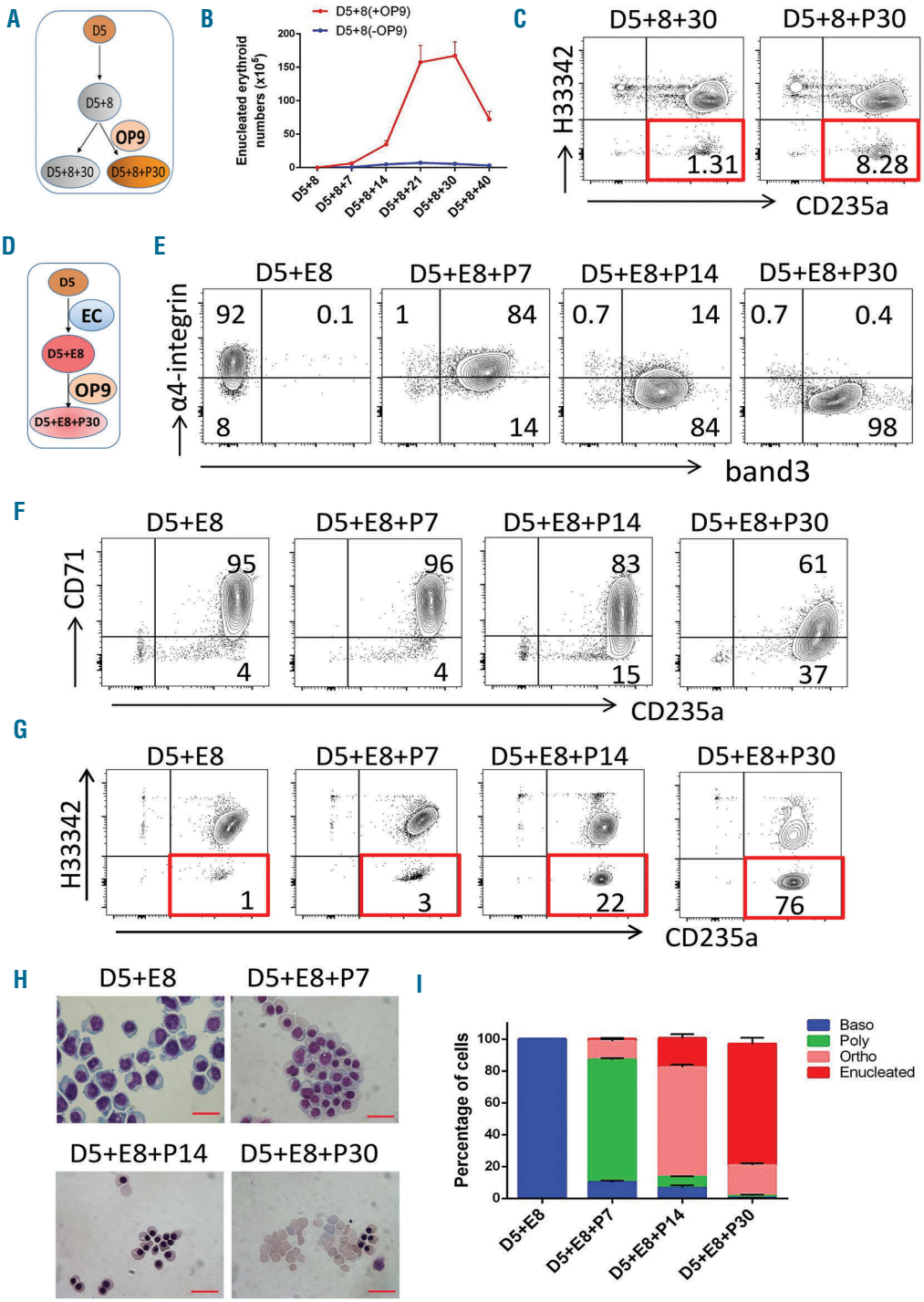

Figure 2. Promoting effect of endothelial niche on the generation of erythroblasts with high potential of enucleation. (A) Schematic diagram showing the strategy of OP9 cellular niches employed to promote the maturation of erythroblasts. (B) The yield of enucleated erythrocytes generated from 6000 cells of H1 hESC under the co-culture with or without OP9 cells in the indicated days. n=3. (C) Representative flow cytometric analysis of enucleation of D5+8 erythroid cells following 30 days of co-culture with OP9 cells or without OP9 cells. n=3. (D) Schematic showing the strategy of sequential EC and OP9 cellular niches employed to promote the maturation of erythroblasts. (E) Representative flow cytometric analysis of band3 and α4 integrin expression in erythroid cells generated by sequential EC and OP9 co-cultures on the indicated days. n=3. (F) Representative flow cytometric analysis of CD71 and CD235a expression in erythroid cells generated by sequential EC and OP9 co-cultures on the indicated days. n=3. (G) Representative flow cytometric analysis of gradual enucleation of erythroid cells generated by sequential EC and OP9 co-cultures on the indicated days. n=3. (H) Progressive morphologic changes of erythroid cells after erythroid differentiation with sequential EC and OP9 co-cultures in the presence of EPO. Cells were stained with Wright-Giemsa on the indicated days. Scale bars, 20 μm. (I) The percentage of basophilic erythroblasts (Baso), polychromatic erythroblasts (Poly), orthochromatic normoblasts (Ortho), and enucleated erythrocytes (Enucleated) on the indicated days after erythroid differentiation with sequential EC and OP9 co-cultures in the presence of EPO. n=3. Experiments were performed on H1 hESC unless otherwise indicated. Data are shown as means ± the standard error of the mean (±SEM).

cells on D5+8 (Figure 1E). Wright-Giemsa staining indicated that D5+8 cells were morphologically basophilic erythroblasts (Figure 1F) that were CD235a$^+$band3$^-$ basophilic erythroblasts (Figure 1G) and contained cellular nuclei (Hoechst33342$^+$CD235a$^+$) (Figure 1H).

OP9, a mouse bone marrow stromal cell (BMSC) line, is often used for supporting hematopoietic differentiation *in vitro*.[12] Considering the ontogeny route that leads to the generation of mature RBC from HSC in adult bone marrow, we determined erythroid maturation potential of D5+8 erythroblasts in the presence of OP9 as a BMSC cellular niche (Figure 2A). Morphologic analysis of the suspension cells after 14 days indicated that OP9 co-culture (D5+8+P14) significantly decreased the number of basophilic erythroblasts and increased the number of orthochromatic erythroblasts that were smaller cells with shrunk nucleus, compared to the control group without OP9 co-culture (D5+8+14) (*Online Supplementary Figure S3A-B*). Flow cytometry analysis confirmed that OP9 co-culture increased the frequency of CD235a$^+$band3$^+$ erythroblasts, and decreased the frequency of CD235a$^+$band3$^-$ basophilic erythroblasts (*Online Supplementary Figure S3C*), indicating the promoting effect of BMSC on a transition from basophilic erythroblasts to orthochromatic erythroblasts. Although Hoechst33342 (H33342) nuclei acid staining indicated that the number of CD235a$^+$H33342$^-$ enucleated erythrocytes was significantly increased with OP9 co-culture (Figure 2B), the generation of enucleated erythrocytes remained inefficient, resulting in ~8% enucleated erythrocytes by 30 days of OP9 co-culture (Figure 2C).

Intrigued by significant yet insufficient enhancement through the OP9 co-culture in erythroid maturation, we hypothesized that the HE-derived erythroblasts lack the intrinsic ability to reach enucleation, and a specific niche is required at an early stage to prime the HE cells to generate erythroblasts with full maturation potential. The endothelial niche platform of E4EC has been reported to deliver a promoting effect on the generation of engraftable hematopoietic stem and progenitor cells (HSPC) from hPSC.[13,14] Considering that HSC emerged from HE cells is in an endothelial microenvironment in AGM, we established a sequential niche system, consisting of endothelial cells (EC) on stage 1 and OP9 on stage 2 (Figure 1A and Figure 2D). After co-culture of D5 HE cells on E4ECs for eight days, the EC-primed erythroblasts (D5+E8) were transferred onto OP9 cells for continuing erythroid maturation. Flow cytometry analysis of band3 and α4 integrin demonstrated that there was a decrease in the α4 integrin^{hi}band3^{low} population and a progressive increase in the α4 integrin^{low}band3^{hi} population during erythroid maturation (Figure 2E), a similar pattern of terminal erythroid differentiation of human primary CD34$^+$ cells.[15] Along with erythroblast maturation, the expression of CD71 decreased gradually (Figure 2F), whereas the frequency of enucleation increased gradually and most of the cells after co-culture with OP9 for 30 days (D5+E8+P30) were CD235a$^+$H33342$^-$ enucleated erythrocytes (~75%) (Figure 2G). Morphological analysis by Wright-Giemsa staining indicated a gradual synchronous transition from basophilic erythroblasts (D5+E8) to polychromatic erythroblasts (D5+E8+P7), to orthochromatic normoblasts (D5+E8+P14), and eventually to enucleated erythrocytes (D5+E8+P30) (Figure 2H-I). To test whether EC-primed erythroblasts continue to undergo terminal maturation *in vivo*, we infused CFSE-labeled erythroblasts (1x10^6) into NOG mice, and assessed their enucleated potential *in vivo*. Our data demonstrated that the CFSE+ cells from D5+E8 gradually decreased in the peripheral blood (PB) of mice, and H33342- enucleated erythrocytes increased from 30% on day 2 to 80% on day 4 in CFSE+ population, compared to undetectable of CFSE+ cells from erythroblasts in the absence of EC (D5+8) (*Online Supplementary Figure S4A-B*).

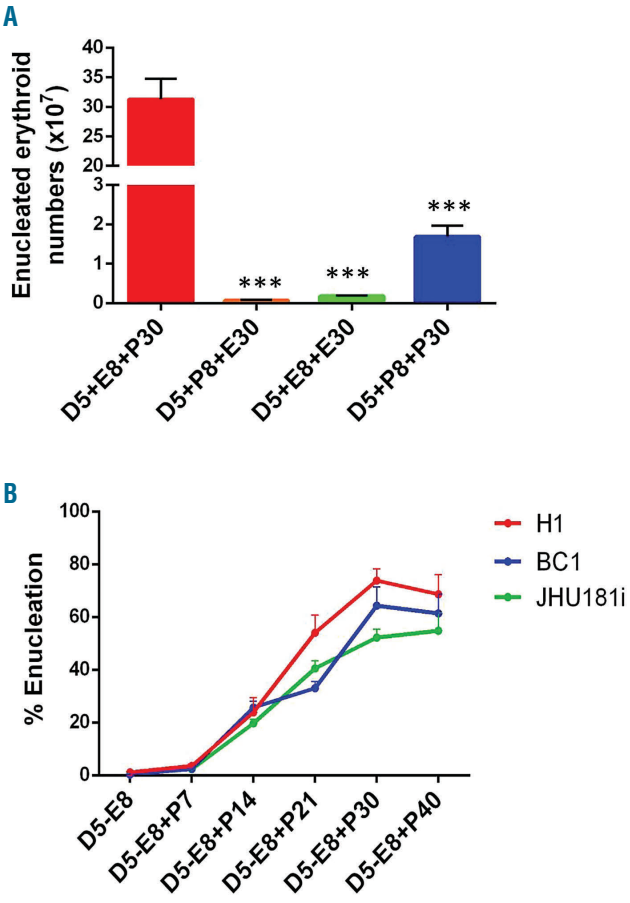

Figure 3. Generation of enucleated erythroid cells from hiPSCs by sequential endothelium and OP9 cellular niche induction. (A) The number of erythroid cells generated by sequential enucleated EC and OP9 co-cultures (D5+E8+P30), by switching the order of OP9 cells (stage 1) and EC (stage 2) (D5+P8+E8), or by prolonging differentiation of HE cells with a single-niche either on EC or OP9 cells (D5+E8+E30 or D5+P8+P30). The total enucleated erythroid cells were generated from 6000 cells of H1 hESCs under different culture conditions. N=3. Data are shown as means ±SEM. Statistical analysis was done by two-tailed Student's *t* test. ***P<.001. (B) The percentage of enucleated erythrocytes generated from hiPSC on the indicated days. BC1-hiPSC and JHU181i-hiPSC were used and H1-hESC were used as control. N=3.

To investigate whether EC and OP9 niches are interchangeable, we induced erythroid differentiation from D5 HE cells with revised orders of EC and OP9. When the D5 cells were first differentiated on OP9 (stage 1) and then on EC (stage 2) (D5+P8+E30), only fewer enucleated erythrocytes were generated (Figure 3A). Furthermore, prolonged differentiation of HE cells with a single-niche, either on EC (D5+E8+E30) or on OP9 (D5+P8+P30), was not permissive to efficiently generate enucleated erythrocytes (Figure 3A). To further test the reliability of EC-OP9 sequential cellular niche system, we applied two hiPSC lines (BC1 and JHU181i) to the sequential platform, and demonstrated that EC-OP9 sequential cellular niche system had the ability to greatly enhance the frequency and yield of enucleated erythrocytes from both hiPSC lines (Figure 3B and *Online Supplementary Figure S5A-B*).

Interestingly, prolonged OP9 co-culture to 40 days (D5+E8+P40) was unable to further increase the efficiency of enucleation (Figure 3B), but resulted in a decreased production of enucleated erythrocytes (*Online Supplementary Figure S5B*). Although the total number of hematopoietic cells reached a plateau by day 21 (*Online Supplementary Figure S5A*), the number of enucleated erythrocytes continued to increase from day 21 to day 30 (*Online Supplementary Figure S5B*), indicating that during this period, erythroid enucleation was still being carried out efficiently.

In summary, we found that EC had the ability to endow erythroid progenitors with high enucleation potential, whereas OP9 BMSC promoted the further maturation of EC-primed erythroblasts to generate enucleated erythrocytes. Omitting either of the cellular niches or reversing the temporal order of cellular niches of EC and BMSC significantly reduced the efficiency of enucleated erythrocyte generation. Our sequential multi-niche model not only presents a platform for further investigation of the molecular and cellular mechanism underline erythroid differentiation and maturation, but also offers a new strategy for the generation of functional HSC from hPSC.

Jun Shen,[1,2] Yaoyao Zhu,[1] Cuicui Lyu,[3] Zicen Feng,[1] Shuzhen Lyu,[1] Yuping Zhao,[1] Dixie L. Hoyle,[2] Guangzhen Ji,[1] Weimin Miao,[1,4] Xiaobing Zhang,[1,5] Linzhao Cheng,[2] Robert A. Brodsky,[2] Tao Cheng[1,6,7] and Zack Z. Wang[2]*

[1]State Key Laboratory of Experimental Hematology, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, China; [2]Division of Hematology, Johns Hopkins University School of Medicine, Baltimore, MD, USA; [3]Department of Hematology, the First Central Hospital of Tianjin, Tianjin, China; [4]Tianjin Key Laboratory of Blood Cell Therapy and Technology, Tianjin, China; [5]Department of Medicine, Loma Linda University, Loma Linda, CA, USA; [6]Center for Stem Cell Medicine, Chinese Academy of Medical Sciences, Tianjin, China and [7]Department of Stem Cell & Regenerative Medicine, Peking Union Medical College, Tianjin, China

Funding: this work was supported by grants from the National Key Research and Development Program of China Stem Cell and Translational Research (2017YFA0103102, 2016YFA0100600, and 2017YFA0103400); the Ministry of Science and Technology of China (2015CB964902); the National Natural Science Foundation of China (81421002); Tianjin Science and Technology Research Program (18PTSYJC00070); Chun Miao Foundation of the First Central Hospital of Tianjin (TFCHCM201808); CAMS Initiative for Innovative Medicine (2016-I2M-1-017) and the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Disease grant R01DK106109 (Z.W).

Acknowledgments: we thank Dr. Xiuli An (Department of Bioengineering, Zhengzhou University, China) for providing band 3 and α4 integrin antibodies.

Correspondence: ZACK Z. WANG/TAO CHENG
zwang51@jhmi.edu/chengtao@ihcams.ac.cn
doi:10.3324/haematol.2018.211664

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Lapillonne H, Kobari L, Mazurier C, et al. Red blood cell generation from human induced pluripotent stem cells: perspectives for transfusion medicine. Haematologica. 2010;95(10):1651-1659.
2. Vanuytsel K, Matte T, Leung A, et al. Induced pluripotent stem cell-based mapping of beta-globin expression throughout human erythropoietic development. Blood Adv. 2018;2(15):1998-2011.
3. Lee E, Sivalingam J, Lim ZR, et al. Review: In vitro generation of red blood cells for transfusion medicine: Progress, prospects and challenges. Biotechnol Adv. 2018;36(8):2118-2128.
4. Qiu C, Olivier EN, Velho M, Bouhassira EE. Globin switches in yolk sac-like primitive and fetal-like definitive red blood cells produced from human embryonic stem cells. Blood. 2008;111(4):2400-2408.
5. Razaq MA, Taylor S, Roberts DJ, Carpenter L. A molecular roadmap of definitive erythropoiesis from human induced pluripotent stem cells. Br J Haematol. 2017;176(6):971-983.
6. Yang CT, French A, Goh PA, et al. Human induced pluripotent stem cell derived erythroblasts can undergo definitive erythropoiesis and co-express gamma and beta globins. Br J Haematol. 2014;166(3):435-448.
7. Kobari L, Yates F, Oudrhiri N, et al. Human induced pluripotent stem cells can reach complete terminal maturation: in vivo and in vitro evidence in the erythropoietic differentiation model. Haematologica. 2012;97(12):1795-1803.
8. Rafii S, Kloss CC, Butler JM, et al. Human ESC-derived hemogenic endothelial cells undergo distinct waves of endothelial to hematopoietic transition. Blood. 2013;121(5):770-780.
9. Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D. Haematopoietic stem cells derive directly from aortic endothelium during development. Nature. 2010;464(7285):108-111.
10. Choi KD, Vodyanik MA, Togarrati PP, et al. Identification of the hemogenic endothelial progenitor and its direct precursor in human pluripotent stem cell differentiation cultures. Cell Rep. 2012;2(3):553-567.
11. Ditadi A, Sturgeon CM, Tober J, et al. Human definitive haemogenic endothelium and arterial vascular endothelium represent distinct lineages. Nat Cell Biol. 2015;17(5):580-591.
12. Uenishi G, Theisen D, Lee JH, et al. Tenascin C promotes hematoendothelial development and T lymphoid commitment from human pluripotent stem cells in chemically defined conditions. Stem Cell Reports. 2014;3(6):1073-1084.
13. Sandler VM, Lis R, Liu Y, et al. Reprogramming human endothelial cells to haematopoietic cells requires vascular induction. Nature. 2014;511(7509):312-318.
14. Gori JL, Butler JM, Chan YY, et al. Vascular niche promotes hematopoietic multipotent progenitor formation from pluripotent stem cells. J Clin Invest. 2015;125(3):1243-1254.
15. Hu J, Liu J, Xue F, et al. Isolation and functional characterization of human erythroblasts at distinct stages: implications for understanding of normal and disordered erythropoiesis in vivo. Blood. 2013;121(16):3246-3253.