

## Chapter 2

# Derivation of Mature Erythrocytes from Human Pluripotent Stem Cells by Coculture with Murine Fetal Stromal Cells

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**Abstract** Transfusion of red blood cells (RBCs) is a requisite cell therapy today, while RBCs supplied by donors cannot match the huge demand of patients. Human pluripotent stem cells (hPSCs) are promising cell sources to obtain RBCs as an alternative transfusion product for clinical application. Several in vitro culture systems have been reported that in which mature erythrocytes can be efficiently generated from hPSCs. However, different efficiency and maturity of hPSC-derived erythrocytes could be obtained when using different culture systems. We still lack a complete understanding of the regulatory pathways controlling human erythrocyte development and maturation, especially the origination of erythrocytes early in the embryo and enucleation at the terminal stage of differentiation. In this chapter, we focus on an efficient method established successfully in our laboratory to derive functionally mature erythrocytes from hPSCs by coculture with mouse fetal stromal cells [aorta–gonad–mesonephros stromal cells (mAGM) and fetal liver stromal cells (mFLSCs), respectively]. The procedures to investigate the characteristics of these hPSC-derived erythrocytes are also introduced, including colony formation assay to detect the hematopoietic potential, flow cytometry assay to detect the phenotypic

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expression pattern, and immuno-staining assay of the Hb components to evaluate the maturity. At the end of this review, several future prospects are also be addressed in this research fields.

**Keywords** Erythrocytes • hPSCs • AGM • Fetal liver • Hematopoiesis

## 2.1 Introduction

Transfusion of red blood cells (RBCs) is an indispensable procedure used in the clinic today. There is a notable imbalance between the demand of patients and the supply of donors, especially in developing countries [1]. The safety and sufficiency of the blood supply are national and international priorities [2]. Immense efforts have been made to enhance the supply of RBCs in vitro as an alternative transfusion product since two decades ago. Studies pioneered by L Douay's group prove that it is possible to produce RBCs from human adult hematopoietic stem cells/hematopoietic progenitor cells (HSCs/HPCs) of cord blood (CB), bone marrow (BM), and peripheral blood (PB) [3–5]. This group reported that the autologous cultured RBCs (cRBCs) derived from peripheral CD34<sup>+</sup> cells were transfused back into the human recipient. The survival of cRBCs was successful as long as natural counterparts do through detecting the 51Cr labeled on cRBCs [6]. This work set up an example of transfusion cRBCs generated from adult HSCs/HPCs in vitro for the first time. However, so far it is still a thorny problem for us to expand adult HSCs/HPCs in vitro [7, 8], and the productivity of RBCs is related to cell sources from different individuals [9]. Thus, to a great extent, the progression of using adult HSCs/HPCs to produce large scale of RBCs for transfusion purpose is hampered.

Human pluripotent stem cells (hPSCs), including human embryonic stem cell (hESCs) [10] and induced pluripotent stem cells (iPSCs) [11], have the ability of unlimited self-renewal and pluripotency. By specific inducing method, large number of hematopoietic cells can be generated from these hPSCs, indicating they might be a potential cell sources to obtain large-scale RBCs for clinical application. cRBCs of specific blood group, rare blood group, or null blood group could be produced from hPSCs by genetically modified; thus, it may satisfy an ideal transfusion for those who with rare blood types. In addition, induced pure cRBCs without other granulocytes and lymphocytes would reduce transfusion reactions and graft-versus-host disease (GVHD) in immunocompromised patients.

Besides as cell source for clinical application, hPSC-derived erythrocytes also offer a subtle model to study human erythropoiesis in vitro. Previously, the mechanism controlling early development of human embryonic/fetal hematopoietic is largely unknown, because there lacks a proper experimental model to mimic the early progress in human ontogeny. Erythrocytes generated from hESCs support an excellent platform to study on the origination, development, and maturation of erythroid cells derived from the earliest endothelial/hematopoietic progenitors and

to uncover important regulating mechanisms at each developmental stage step by step. Since hiPSCs can be established from specific individual patients, they also provide a powerful tool for modeling diseases and supply a new opportunity for patient-tailored therapies. Several reports have referred to the successful establishment of hiPSC lines derived from patients with erythrocytes-associated diseases [12–17].

Schemes of successfully inducing large-scale production of erythrocytes from hPSCs have been reported by several groups, including our laboratory. Generally, hematopoietic progenitors are produced first through the formation of embryonic bodies (EBs) spontaneously by hPSCs [17–23], or coculture with stromal cells isolated from hematopoietic niches [24–29]. Then, hPSC-derived multipotential hematopoietic progenitors are differentiated into erythrocytes directionally (and other blood lineage cells, as well). At last, erythrocytes undergo maturation with or without feeder cells. Based on the establishment of methods to induce erythrocytes from hPSCs in vitro, more and more information relating development of human early erythropoiesis has been accumulated. Yet we are still lacking of a complete understanding of the regulatory pathways controlling erythrocyte development and maturation, especially the origination and initiation of erythrocytes early in the embryo and the enucleation at the terminal stage of differentiation. It has long been known that there are two erythropoietic waves in human. The first wave of primitive erythrocytes, which are originated from the extra-embryonic mesoderm of yolk sac, synthesize Hb Gower I ( $\zeta_2\epsilon_2$ ) and Hb Gower II ( $\alpha_2\epsilon_2$ ) [30]. The second wave of definitive erythrocytes, which are originated from the AGM region of the embryo proper [31], express  $\zeta$ -,  $\epsilon$ -,  $\alpha$ -,  $\gamma$ -, and small amount of  $\beta$ -globin just at the beginning, and then,  $\zeta$ - and  $\epsilon$ -globins are silenced rapidly. Around birth, BM becomes the main site of erythropoiesis and expresses  $\beta$ -globins which almost replaces  $\gamma$ -globin expression [25]. So the hemoglobin (Hb) contents can be used to evaluate the maturity of erythrocytes. Consistent data of Hb contents from different laboratories demonstrate that hPSC-derived erythrocytes from coculture system, which express higher adult  $\beta$ -globin, are more mature than erythrocytes generated from EBs (Table 2.1). Affording appropriate microenvironment is indispensable for the maturation of erythrocytes. The common stromal cells used for stimulating the generation of hematopoietic cells from hPSCs include mouse BM stromal cell lines: OP9, MS-5, S17, mAGM, mFLSCs, FH-B-hTERT (immortalized human fetal liver hepatocyte line), and so on. Although the OP9 cell line, which is used most frequently, has a good ability to induce the derivation of early hematopoietic cells, it is obtained from gene mutation mouse which cannot produce macrophage colony-stimulating factor (M-CSF). So the hematopoiesis induced by coculture with OP9 could not stand for the natural development process.

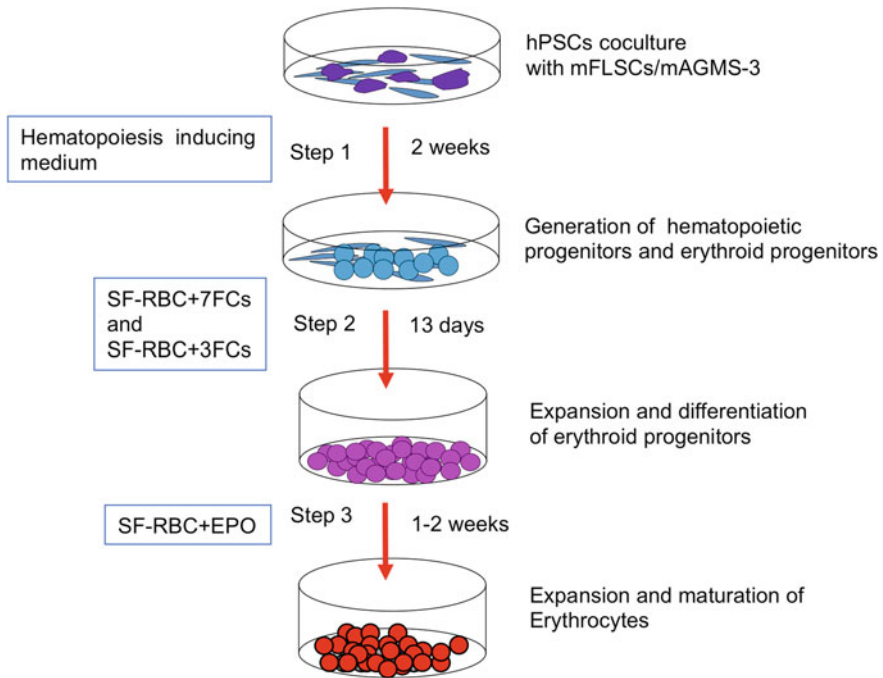
We have established an efficient system by coculture hPSCs with mAGM cell lines [32, 33] and mFLSCs [26, 27] (Fig. 2.1), which were obtained from normal fetal mice, to generate robust growth of HPCs [26]. When coculture with these early definitive hematopoiesis-supporting stromal cells, considerable hPSC-derived E colonies and E bursts could be generated on day 10–14 following hematopoietic

**Table 2.1** Erythrocytes derived from hPSCs in vitro by different methods

|                | Methods   | Expansion folds                  | Expression ratio of Hb-β | Enucleation ratio | Ref.             |
|----------------|---|----------------------------------|--------------------------|-------------------|------------------|
| EB             | Step 1: hESC→EBs, SF/SC   | High (10 <sup>3</sup> -fold)     | Little (2 %) or no       | High (34–48 %)    | [20, 22]         |
|                | Step 2: differentiation to erythrocyte, SF  |                                  |                          |                   |                  |
|                | Step 1: hiPSCs→EBs, SF  | Moderate (10 <sup>2</sup> -fold) | No                       | Low (4–10 %)      | [20, 23]         |
|                | Step 2: differentiation to erythrocyte, SF  |                                  |                          |                   |                  |
| EB + coculture | Step 1: hESC→EBs, SF  |                                  | Low (0–16 %)             | High (30–65 %)    | [18]             |
|                | Step 2: differentiation to erythrocyte, SF  |                                  |                          |                   |                  |
|                | Step 3: enhancing enucleation ratio by coculture with stromal cells (OP-9/MS-5), SF |                                  |                          |                   |                  |
| Coculture      | Step 1: hESC + stromal cells, SC  | Moderate (10 <sup>2</sup> -fold) | High (99.8 ± 0.6 %)      |                   | [24, 25, 27, 28] |
|                | Step 2: expansion of hemangioblasts, SC   |                                  |                          |                   |                  |
|                | Step 3: differentiation to erythrocyte, SC/SF                                       |                                  |                          |                   |                  |
|                | Step 4: enhancing enucleation ratio by coculture with stromal cells (OP-9/MS-5), SF |                                  |                          |                   |                  |

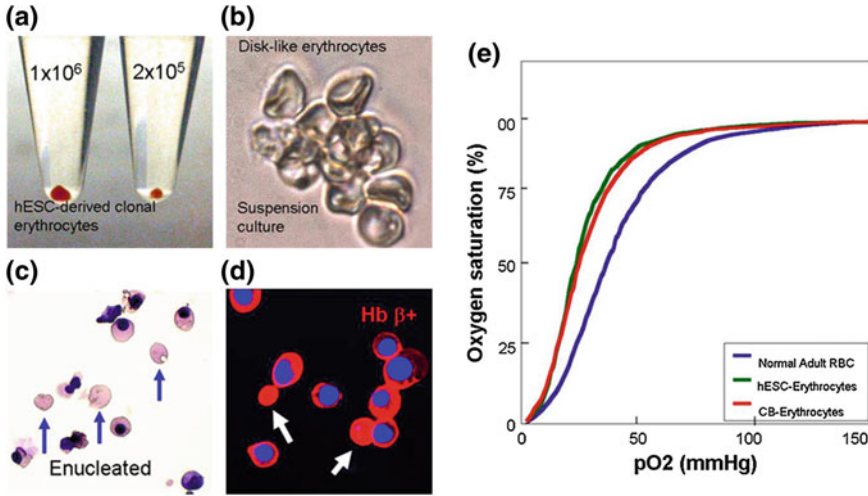
SC Serum-containing culture; SF Serum-free culture

colony culture. By a clone tracing method, we demonstrate that most hESC-derived erythroid colonies expressed adult β-globin and gradually increased to almost 100 % with additional 6 days liquid culture. In addition, the hESC-derived erythrocytes can undergo enucleation and carry and release oxygen functionally [27] (Fig. 2.2). These data indicate that the coculture of hPSCs with definitive hematopoiesis-niche-derived stromal cells is an efficient method to develop hematopoiesis system and generate adult-type erythrocytes with functional maturity. Using this model, we recently have observed the earliest erythrocytes development from hPSCs, which emerge earlier than the definitive hematopoiesis takes place. The phenotypic pattern of hPSC-derived early erythrocytes is distinct from erythrocytes derived from adult HSCs/HPCs. Since there is some difficulty to gain large-scale production of murine fetal liver-derived stromal lines [34], the system of hPSCs coculture with mAGM is superior for looking into the origination of the earliest erythroid cells.



**Fig. 2.1** The illustration of the induction method of erythrocytes derived from hPSCs in our laboratory. The induction procedure of erythrocytes differentiation was divided into three steps. *Step 1* Undifferentiated hPSC colonies were individually picked up from the primary culture manually (routinely  $0.5-1 \times 10^3$  cells/colony). Coculture the colonies with irradiated mFLSCs/mAGMS-3 about 2 weeks to generate multipotential hematopoietic progenitors and erythroid progenitors. *Step 2* To expand and differentiate the erythroid progenitors, treat the cocultures with 0.25 % Trypsin/EDTA and transfer all the harvested cells into suspension culture medium. First with a cocktail of SF-RBC + 7FCs (IMDM + SCF, VEGF, TPO, FL, IL-6, IL-3, EPO) for about 6 days and then in SF-RBC + 3FCs (IMDM + SCF, IL-3, EPO, dexamethasone) for about 7 days. *Step 3* The erythrocytes develop and mature further in the SF-RBC + EPO medium but without any other cytokines

In this chapter, we here introduce a coculture system developed in our laboratory to produce erythrocytes efficiently from hPSCs. Since the derivation of mAGMS and mFLSCs has been described elsewhere [32, 34], we will focus on the methodological processes of generating large number of functionally mature erythrocytes. Our method include three steps: (1) generation of erythroid cells and other hematopoietic cells from cocultures; (2) expansion of erythroid progenitors derived from cocultures in suspension culture; and (3) further maturation of erythrocytes (Fig. 2.1). We also introduce the procedures for assaying the erythroid (and other hematopoietic lineages) differentiation in semisolid culture, detecting phenotypic expression pattern by flow cytometry assay and evaluating the maturity level of derived erythrocytes by Hb immuno-staining assay, respectively. And the pivotal



**Fig. 2.2** Functionally mature erythrocytes derived from hESCs/mFLSCs coculture. **a** Photograph of harvested large BFU-E colony cells from day 16 coculture, showing the *red color* of human erythrocytes. A total of  $2 \times 10^5$  (*right*) and  $1 \times 10^6$  (*left*) erythroid cells were collected from one and five large BFU-E colonies, respectively. **b** Cluster of enucleated erythrocytes derived from hESCs from the same suspension cultures shown in (c) and (d). **c** Sample of hESC-derived erythroid cells from a day 12 + 6 suspension culture (May-Grünwald-Giemsa staining, MGG). *Arrows* indicate enucleated erythrocytes. **d** Immuno-staining for  $\beta$ -globin expression in hESC-derived erythroid cells. *Arrows* indicate  $\beta$ -globin expressing enucleated erythrocytes. **e** Oxygen dissociation curves of day 15 hESC/mFLSC coculture-derived clonal large BFU-E erythroid cells at day 16 of the colony culture compared with hCB-derived BFU-E cells and normal peripheral blood cells. Adapted from Ref. [27]

details during the experimental operation that should pay attention to are described additionally as notes in this protocol. At the end of this chapter, the hampers existed in the process of clinical translation of hPSC-derived erythrocytes and the methodological advantage of our coculture system to resolve these puzzles are also addressed.

## 2.2 Materials

### 2.2.1 hPSCs Lines

1. hESC line, H1, was obtained from WiCell Research Institute (Madison, WI), and KhES-3 was kindly provided by Professor N Nakatsuji at Department of Development and Differentiation, Institute for Frontier Medical Sciences, Kyoto University, Japan.

2. hiPSC 201B7 line was kindly provided by Professor S Yamanaka at Center for iPS Cell Research and Application, Kyoto University, Japan.
3. hESC-/iPSCs-maintaining medium.
  - Dulbecco's modification of Eagle's medium (DMEM; Gibco, Cat. No. 12800-017),
  - F12 (Gibco, Cat. No. 21700-075),
  - KnockOut™ Serum Replacement (KSR; Gibco, Cat. No. 10828-028),
  - 2-mercaptoethanol (2-ME; Sigma, Cat. No. M7522),
  - L-glutamine (Gibco, Cat. No. 25030-081),
  - Nonessential amino acid solution (NEAA; Gibco, Cat. No. 11140-050), and
  - Basic fibroblast growth factor (bFGF; Gibco, Cat. No. 13256-029).
4. Gelatin-coated 10-cm culture dish (Corning, Cat. No. 430167).
5. Scraper (Nunc, Cat. No. 179693).

### 2.2.2 *mFLSCs* (Adapted from Refs. [27, 34])

1. Mice (Pregnant day 14–15, Strain: C57/Black6).
2. Ophthalmology surgery scissors and forceps (Autoclaved before use).
3. Sterile tissue culture dish (Corning, Cat. No. 3296).
4. Gelatin-coated 6-well culture plate (Corning, Costar, Cat. No. 3335).
5. Dulbecco's phosphate-buffered saline without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (D-PBS (-); Wako, Cat. No. 045-29795).
6. Trypsin/EDTA solution [Gibco, Cat. No. 25200-056/0.25 %, diluted to 0.05 % with D-PBS (-)].
7. *mFLSCs* culture medium
  - DMEM medium (Gibco, REF. 31600-034).
  - Fetal bovine serum (FBS; Biowest, Cat. No. S1580-500) 10 % in volume.

### 2.2.3 *mAGMS-3 Cell Line Culture* (Adapted from Ref. [32])

*mAGMS-3* cell line derived from murine AGM region had been established and maintained in our laboratory since 1998 [32] with stable and efficient hematopoiesis-supporting potential.

1. *mAGMS-3* cell line (between passage 30 (P 30) and P 40).
2. Gelatin-coated 10-cm culture dish (Corning, Cat. No. 430167).

3. T225 flask (Nunc, Cat. No. 159934).
4. Cryogenic vial (Nunc, Cat. No. 377267).
5. mAGMS-3 cell-maintaining medium:
  - Minimum essential medium, alpha modified ( $\alpha$ -MEM; Hyclone, Cat. No. SH30265) and
  - FBS (Biowest, Cat. No. S1580-500) 5 % in volume.

### ***2.2.4 Induction of Multipotential Hematopoietic Progenitors***

1. Biological X-ray irradiator (Rad Source Technologies, Inc. RS2000).
2. Gelatin-coated 6-well culture plate (Corning, Cat. No. 3335).
3. Undifferentiated hPSC colonies.
4. Radiated mFLSCs or mAGMS-3 cells (Radiation dose: 25 Gy for mFLSCs and 15 Gy for mAGMS-3 cells).
5. Hematopoiesis-inducing medium in coculture:
  - Iscove's modified Dulbecco's medium (IMDM; Gibco, Cat. No. 12440-053),
  - FBS (Hyclone; 10 % in volume),
  - NEAA (Gibco, Cat. No. 11140-050),
  - Ascorbic acid (AA; Sigma, Cat. No. 1043003-1G),
  - Transferrin (Sigma, Cat. No. T2252),
  - recombinant human Vascular endothelial growth factor (rhVEGF; WAKO, Cat. No. 229-01353), and
  - 2-ME (Sigma, Cat. No. M7522).
6. Y-27632 (CALBOCHEM, Cat. No. 68800).

### ***2.2.5 Suspension Culture in Liquid Medium***

1. Erythroid progenitors expansion medium, named as SF-RBC + 7FCs medium:
  - Serum-free expansion medium (SFEM; Stemcell, Cat. No. 09650),
  - Bovine serum albumin (BSA; Sigma, Cat. No. A-4161),
  - Transferrin (Sigma, Cat. No. T2252),
  - 2-ME (Sigma, Cat. No. M7522),
  - rhVEGF (Wako, Cat. No. 229-01353),
  - rhStem cell factor (rhSCF, Wako, Cat. No. 199-12813),
  - rhInterleukin 3 (rhIL-3, Kirin, Cat. No. MYE0317),



- rhInterleukin 6 (rhIL-6, Wako, Cat. No. 099-04631),
  - rhFlt-3 ligand (rhFL, Wako, Cat. No. 061-04051),
  - rhTrombopoietin (rhTPO, Kirin, Cat. No. NHK0823-SDM), and
  - rhErythropoietin (rhEPO, provided by Kirin Brewery Company, Tokyo, Japan).
2. Erythrocytes expansion medium, named as SF-RBC + 3FCs medium:
    - SFEM (Stemcell, Cat. No. 09650),
    - BSA (Sigma, Cat. No. A-4161),
    - Transferrin (Sigma, Cat. No. T2252),
    - 2-ME (Sigma, Cat. No. M7522),
    - rhSCF (Wako, Cat. No. 199-12813),
    - rhIL-3 (Kirin, Cat. No. MYE0317),
    - rhEPO (provided by Kirin Brewery Company, Tokyo, Japan), and
    - Dexamethasone (Sigma, Cat. No. D4902).
  3. Ultra-low adherent cluster 6-well plate (Corning, Cat. No. COSTAR® 3471).
  4. hPSC-derived hematopoietic cells (after 0.25 % trypsin/EDTA treatment, total harvested cells were plated).

### ***2.2.6 Hematopoietic Colony Culture***

1. Semisolid culture medium:
  - $\alpha$ -Methylcellulose ( $\alpha$ -MTC; Sigma, Cat. No. M0512),
  - $\alpha$ -MEM (Gibco, REF. 41061-029),
  - FBS (Hyclone; 56 °C/30 min heat inactivated),
  - BSA (Sigma, Cat. No. A-4161),
  - 2-ME (Sigma, Cat. No. M7522),
  - rhSCF (Wako, Cat. No. 199-12813),
  - rhIL-3 (Kirin, Cat. No. MYE0317),
  - rhIL-6 (Wako, Cat. No.099-04631),
  - rhFL (Wako, Cat. No. 061-04051),
  - rhTPO (Kirin, Cat. No. NHK0823-SDM),
  - rhEPO (provided by Kirin Brewery Company, Tokyo, Japan), and
  - rhGranulocyte colony-stimulating factor (rhG-CSF, Kirin, Cat. No. NSG0205-G604).
2. hPSC-derived hematopoietic cells (after 0.25 % trypsin/EDTA treatment, total harvested cells were plated).
3. 35-mm Petri dish (Becton Dickinson Labware, Cat. No. 35-1008).
4. 2-mL plastic syringe (Top Surgical Taiwan Corporation).
5. 18-gauge (18G) syringe needle (Terumo, Cat. No. 1838S).

### **2.2.7 Flow Cytometric Analysis of hPSC-Derived Erythrocytes**

1. Trypsin/EDTA solution (Gibco, Cat. No. 25200-056/0.25 %).
2. Sorting medium (SM):
  - D-PBS (-) (Wako, Cat. No. 045-29795),
  - FBS (Biowest, Cat. No. S1580-500) 5 % in volume, and
  - Penicillin–streptomycin (PS; Hyclone, Cat. No. SV30010) 1 % in volume.
3. Antibodies:
  - Mouse antihuman c-kit, APC-conjugated (eBioscience, Cat. No. 17-1179-42),
  - Mouse antihuman glycoporphin A (GPA) or CD235a, PE-conjugated (DAKO Cytomation, Cat. No. R 7078),
  - Mouse antihuman CD34, FITC-conjugated (BD Pharmingen™, Cat. No. 555821),
  - Mouse antihuman CD45, APC-conjugated (BD Pharmingen™, Cat. No. 559864),
  - Mouse antihuman CD47, FITC-conjugated (BD Pharmingen™, Cat. No. 556045),
  - Mouse antihuman CD71, FITC-conjugated (BD Pharmingen™, Cat. No. 555536),
  - Mouse antihuman CD81, FITC-conjugated (BD Pharmingen™, Cat. No. 551108),
  - Mouse antihuman erythropoietin receptor (EPO-R), FITC-conjugated (R&D Systems, Cat. No. FAB307F), and
  - 7-amino-actinomycin D (7-AAD; BD Pharmingen™, Cat. No. 559925).
4. Flow cytometry system (Becton Dickinson Company, FACSCanto™ II).
5. Rabbit serum (Zhongshan, ZLI9025).

### **2.2.8 Immuno-Staining Assay of hPSC-Derived Erythrocytes**

1. Cells harvested on different days were cytopspined on clean glass slides.
2. Antibodies (Abs):
  - Goat antihuman hemoglobin (Hb; Bethyl Laboratories, Cat. No. A80-134A),
  - Mouse antihuman Hb $\epsilon$  (Santa Cruz Biotech, Cat. No. sc70421),
  - Mouse antihuman Hb $\gamma$  (Santa Cruz Biotech, Cat. No. sc-21756),
  - Mouse antihuman Hb $\beta$  (Santa Cruz Biotech, Cat. No. sc-21757),

- Donkey antigoat Cy3-conjugated secondary Ab (Jackson Immuno Research, Cat. No.705-165-003), and
  - Donkey antimouse FITC-conjugated secondary Ab (Jackson Immuno Research, Cat. No. 715-095-150).
3. Liquid marker pen (DAKO, Cat. No. 69932).
  4. Skim milk (SM; BD, REF: 232100).
  5. 4 % paraformaldehyde solution (4 %PFA; Boster, Cat. No. AR1608).
  6. Triton X-100 (Uni-chem, Cat. No. 9002-93-1).
  7. 4',6-diamidino-2-phenylindole (DAPI; Roche, REF. 0 236 276 001).
  8. D-PBS (-) (Wako, Cat. No. 045-29795).
  9. Mounting medium (Vector Laboratories, Cat. No. H-100).
  10. Microcover glass (Matsunami, 24 × 24 mm).
  11. Nail polish.
  12. Cell cytospin machine (Thermo Scientific, Cytospin 4).
  13. Fluorescence microscope (Olympus, BX53).

## 2.3 Methods

In mammals, the hematopoietic system developed during early embryonic development starts in the yolk sac, transfers to intra-embryonic sites, initially to the aorta-gonad-mesonephros (AGM) region, and then processes to fetal liver until birth. Late in gestation, hematopoietic precursors settle in BM, where HSCs predominantly stay and recruit life long [35]. To our experiences, an efficient in vitro generation of multipotential HPCs from hPSCs is mostly depended on the supporting role of the mouse fetal stromal cells. Fetal liver is the dominant region for definitive hematopoiesis before birth. The coculture system of hPSCs with mFLSCs established by us can produce a large quantity of functional mature erythrocytes, and a progressively maturation process of hPSC-derived erythrocytes could be observed [26]. Because only primary mFLCs give rise to efficient hematopoiesis-induction effects in coculture and it is difficult to make large-scale expansion of efficient mFLSC lines, hPSCs coculture with mFLSCs shows some instability when used to gain large-scale experiments for blood cell production. Since the AGM region has been recognized as the earliest microenvironment to support the origination of definitive hematopoiesis [36–38], we then optimize our coculture system by using mAGM cell line as the hematopoiesis-inducing cells. The mAGM-S3 cell line was derived from the murine AGM region of a 10.5 days postcoitum (dpc) mouse embryo and reported previously [32]. In our hPSC-mAGM-S3 coculture system, robust growth of human early hematopoietic stem/progenitor cells could be achieved. By lineage-specific inducing method (such as toward erythroid development), we can harvest large quantity of functionally mature blood cells, including mature erythrocytes and innate immune-related cells

(mast cells, eosinophils, basophilic cells, etc.). Consistent with our data, it has been reported that the mAGM stromal cells show more efficient hematopoietic supporting potential than mFLCs do [39]. Hence, the coculture system using mAGM cells provides a promising platform for us to gain both enough hPSC-derived mature erythrocytes and the understanding how the earliest erythroid cells come out.

### **2.3.1 Maintenance of hPSCs Lines**

In short, the hPSCs lines (H1, H9, KhES-3, 201B7) can be maintained and passaged weekly on irradiated mouse embryonic fibroblast (MEF) feeder cells as described [10, 11].

#### **Notes:**

1. Prepare MEF cells well from 13.5 dpc mouse embryo. Routinely, MEF cells of passage3 (P3) to P5 are suitable for maintaining the undifferentiated hPSCs.
2. For maintaining the hPSCs in a long term, the physical method of scraping the colonies with pipette tips and then pipetting colonies into small pieces mechanically is more beneficial than chemical method of treating the cells with enzyme.
3. Feeder cells are not essential for maintaining the hPSCs. They could be replaced by Matrigel-coated culture dish, though more expensive. But MEF cells contribute to a better long-term maintenance of hPSCs under undifferentiated condition.

### **2.3.2 Establishment of mFLSCs**

mFLSCs are prepared as described, and the procedure has been depicted in detail [26, 27, 34, 40]. We simplify the description of its establishment process here. Briefly, mFL are removed carefully from embryonic day15 (E15) C57/Black6 mice. After triturating with two glass slides carefully, mFL total cells were washed once with D-PBS (-) and plated in a gelatin-coated 10-cm dish at a density of 2–3 fetal livers per dish. After 2 days in culture, floating cells are removed through washing with D-PBS (-) gently, and add fresh mFLSCs culture medium. When reaching a confluence after 4–5 days, the mFLSCs are treated with 0.05 % trypsin/EDTA and replated into a new culture dish at a rate of 1:1.5–1:2. After enough quantity has been gained, harvest the cells and cryopreserve them in liquid nitrogen.

**Notes:**

1. The efficiency of production of cell lines from mFLSCs is low at large, so it should be prepared very carefully.
2. Make sure to tear off the fascia tissue on the fetal livers before triturating.
3. Do not touch the culture dish within the first 2 days. Disturbance before mFLSCs completely adhere to surface of culture dish will damage cells irreversibly.
4. Good quality of P1 mFLSCs is composed of a loosely distributed stromal cell layer. They are not easily to be occupied by narrow and paralleling fibroblastic cells.
5. After P1, the proliferation of the mFLSCs decreases down drastically over the passaging, especially when frozen mFLSCs are thawed and recultured. In this situation, a reduction in area ratio should be considered, and the addition of bFGF (5–10 ng/mL) and increasing the percentage of FBS up to 20–30 % in culture medium are beneficial for the proliferation of mFLSCs.

### 2.3.3 Maintenance of mAGMS-3 Cell Line

The mAGMS-3 cell line has been established successfully in our laboratory [32] and is maintained in liquid nitrogen. It can strongly induce hematopoietic differentiation of hPSCs by coculture with them.

1. Thaw one vial of cells cryopreserved in liquid nitrogen and plated them on gelatin-coated culture dish. mAGMS-3 cells reach confluence by 2–3 days in culture regularly. Passage these cells by treating with 0.05 % trypsin/EDTA and replating in a new culture dish.
2. After passage 3–5 times, a large quantity of mAGMS-3 cells can be harvested. According to the need of experiments, sufficient cells are cryopreserved in liquid nitrogen as working library, at a density of  $1 \times 10^6$  cells per cryogenic vial.
3. Before coculture, mAGMS-3 cells are thawed and passaged one time as described above.

**Notes:**

1. When the mAGMS-3 cells reach confluence in culture, treat them with 0.05 % trypsin/EDTA, and replated into a new culture dish, the appropriate ratio for the passage is about 4–6 in area.
2. When passage, the time of digestion process and the seeding density (20–30 % initial confluence) are important factors to keep the mAGMS-3 cells maintained with primary feature.
3. mAGMS-3 cells are prone to adhere to each other in serum-containing solution. So trypsin solution should be diluted by D-PBS (-) (4 °C) first to avoid

accumulating, and the enzyme should be terminated by serum-containing medium after that.

4. Observed under phase contrast microscope, the naïve cells have clear boundaries and the aging cells become larger and more flat.

### **2.3.4 Coculture of Undifferentiated hPSCs with mFLSCs/mAGMS-3**

1. Before coculture, plate the mFLSCs or mAGMS-3 cells in gelatin-coated 6-well culture plate at  $1-2 \times 10^5$  per 6-well plate. After about 1–2 days, the fetal stromal cells reach a good confluence.
2. Radiate the confluent mFLSCs at 25 Gy and mAGMS-3 cells at 15 Gy. After radiation, the stromal cells can be utilized at anytime within 3 days. The culture medium should be exchanged to hPSCs-maintaining medium 1–2 h right before hPSCs are plated.
3. For inducing hematopoietic cells, undifferentiated hPSCs colonies should be mechanically picked up because the mass size of the colony is very much related with the efficiency of blood cell production. For this reason, the colonies are first cutted into small pieces by using a 200- $\mu$ L pipette tip under a reverse microscope in a clean bench. Individual mass of the undifferentiated hPSC colony was then carefully moved onto the culture plates prepared with radiated mFLSCs/mAGMS-3 cells. In order to obtain high efficiency, routinely each piece of the undifferentiated hESC colony should contain about  $0.5-1 \times 10^3$  cells.
4. Plate the undifferentiated hPSCs-colony pieces onto the irradiated stromal cells at about 20–30 pieces per one 6-well. The medium is hPSCs-maintaining medium containing 5 nmol Y-27632 in order to increase the adherent rate of hPSCs pieces.
5. 1 mL hPSCs-maintaining medium is added lightly into per one 6-well plate on the 1st day, and fresh medium is changed on the 2nd day.
6. The medium is exchanged to hematopoiesis-inducing medium on the 3rd day when the hPSCs colonies are growing bigger and still keep undifferentiated. And it is recorded as day 0 of differentiation. Then, the medium should be changed every 1–2 days.
7. During the first 4–6 days after changing with the hematopoiesis-inducing medium, the hPSCs colonies grow larger and begin to differentiate, with the outer edge of the colonies looking like mesoderm structure.
8. Around days 6–8, the cobble stone-like cells will appear, and the proliferation of the cells accelerates which lead to the pH value change rapidly. To moderate the pH change, the medium should be changed a little more frequently or by reducing FBS concentration in the medium. These means could help to slow

down the acidification process of the medium and provide a stable culture microenvironment which is beneficial for the early hematopoietic differentiation.

9. After 10 days in coculture, the hematopoietic CD34<sup>+</sup> cells amplify rapidly and reach peak on day 14. Then, the hematopoiesis-supporting potential decreases and exhausts gradually after 16 days. In order to further inducing them toward mature blood cells, the HPCs should be shifted to a new environment for further development and maturation.

#### Notes:

1. The maintaining status of mAGMS-3 is one of the crucial factors in determining the hematopoietic efficiency of the coculture system.
2. The confluence of the stromal cells should in harmony with the size of hPSCs pieces. About 85–90 % confluence of the stromal cells and about  $0.5\text{--}1 \times 10^3$  cells in each hPSCs piece are favorable to an efficient hematopoietic differentiation. According to our experiences, the larger and thicker undifferentiated hPSCs colonies have better hematopoietic efficiency when coculture with the stromal cells than small and thin ones do.
3. Use hPSCs-maintaining medium of the first 3 days to help the hPSCs adapt to the new matrix smoothly.
4. Although VEGF (20 ng/mL) in hematopoiesis-inducing medium is not necessary for differentiation, it can improve the generation of CD34<sup>+</sup>CD45<sup>+</sup> cell fraction [41].
5. Components of FBS are crucial for the differentiating efficiency. Thus, the hematopoietic supporting potential of different commercial FBS should be compared through colony formation analysis by human cord blood (hCB) CD34<sup>+</sup> cells or by hPSC-mFLSCs/mAGMS-3 coculture cells. It is found that significant differences of hematopoietic inducing ability exist in even different batches of FBS products.
6. After 10 days of coculture, medium should be added softly and slowly to avoid impairing the microcavity structure where hematopoietic cells originate and proliferate.

### **2.3.5 Colony Formation Analysis of Hematopoietic Progenitors Derived from hPSCs Coculture with mFLSCs/mAGMS-3**

The technique of colony assay was established by Ogawa and Nakahata [42–44]. The potential of hematopoiesis can be evaluated subtly by this method. The individual hematopoietic colony is derived from single cell and specifies a lineage potential at a clonal level. Most of the myeloid cells and erythrocytes can be stimulated in the semisolid colony culture system by adding a cytokine cocktail to favor hematopoietic cell development. Our previous work has showed that

considerable hematopoietic progenitors including erythroid progenitors could be generated in this system [27]. Routinely, a 4 or 5 mL mixture of semisolid cell culture should be prepared for one test. The components needed are listed as below:

- $\alpha$ -methylcellulose (final concentration: around 1 %),
  - BSA solution (final 1 %),
  - FBS (heat inactivated, final 30 %),
  - 2-ME (final  $10^{-4}$  M),
  - rhSCF (final 100 ng/mL),
  - rhIL-3 (final 10 ng/mL),
  - rhIL-6 (final 50 ng/mL),
  - rhFL (final 10 g/mL),
  - rhTPO (final 50 ng/mL),
  - rhEPO (final 4 IU/mL), and
  - rhG-CSF (final 10 ng/mL).
1. The total coculture-derived cells (or sorted fractionated populations) on different culture days (D8–D18) are harvested and plated to colony cultures, at a density of about  $2\text{--}5 \times 10^5$  total cells or  $2\text{--}5 \times 10^3$  CD34<sup>+</sup> cells in 1 mL semisolid culture.
  2. Mix the culture well using a 2-mL syringe with an 18-G needle and then shake it violently to mix further.
  3. The culture should be put away on ice for 20 min before plating to let the bulbs float up.
  4. Lift accurate 1 mL of the culture into one 35-mm Petri dish. The culture is so viscous that only 4 mL aliquots can be made from 5 mL preparation.
  5. Two culture dishes and another with sterile water are put into a 10-cm dish together, and then, they are cultured at 37 °C in a 5 % CO<sub>2</sub> high humidity incubator.
  6. Observation and calculation of colonies should be performed around days 7–14. The criterion for identifying colony types has long been established in our laboratory [27, 42–44].
  7. Pick up large E-burst colonies from the methylcellulose culture, wash them with  $\alpha$ -MEM and then replat the erythroid cells in a suspension culture or cytopspin them on glass slides directly for next detection.

#### Notes:

1. Do not move the cultures until culture for 5 days, shaking may leads to several daughter colonies come from one individual colony. It may effect colony counting and give rise to wrong result at last.
2. Erythroid cell colonies include E colonies, E bursts, and Mix colonies, which could be defined refer to reference [27]. The numbers of E colonies are determined on days 7–10 in culture, while the numbers of E bursts and Mix colonies are counted on days 12–14 in semisolid culture.



### **2.3.6 *Erythrocytes Derived from hPSCs Coculture with mFLSCs/mAGMS-3***

#### **2.3.6.1 Expansion of Erythroid Progenitors**

1. Wash the cocultures by D-PBS (-) twice and treat them by 0.25 % trypsin/EDTA solution.
2. After centrifuge, harvested cells are resuspended in SF-RBC + 7FCs medium and plated in ultra-low adherent culture 6-well plate, including about  $1-2 \times 10^6$  total cells per well.
3. The medium should be changed every 2–3 days, and after 5–7 days, the HSCs/HPCs are expanded looking like grape cluster-like structure, while most other cells died. The culture medium is exchanged to SF-RBC + 3FCs medium.

#### **Notes:**

1. The efficiency of the production of multipotential HPCs in the cocultures overwhelmingly decides the later erythrocyte production.
2. The hematopoietic structure in cocultures is compact. For better digestion, the cocultures should be immersed in D-PBS (-) for 2–3 min to eliminate the remaining serum in the structure and the 0.25 % trypsin/EDTA solution should be prewarmed to 37 °C before use.
3. According our experiences, total harvested coculture cells should be plated into the following culture system, because cells other than hematopoietic lineage in the cocultures might help to promote a better expansion of hematopoietic progenitors.
4. Although cell number of CD34<sup>+</sup> fraction reaches peak on day 14 coculture, the erythroid progenitors emerge much earlier than that. According to our data, the cocultures on day 10–12 have a better erythrocyte production after suspension culture.

#### **2.3.6.2 Differentiation and Maturation of Erythrocytes**

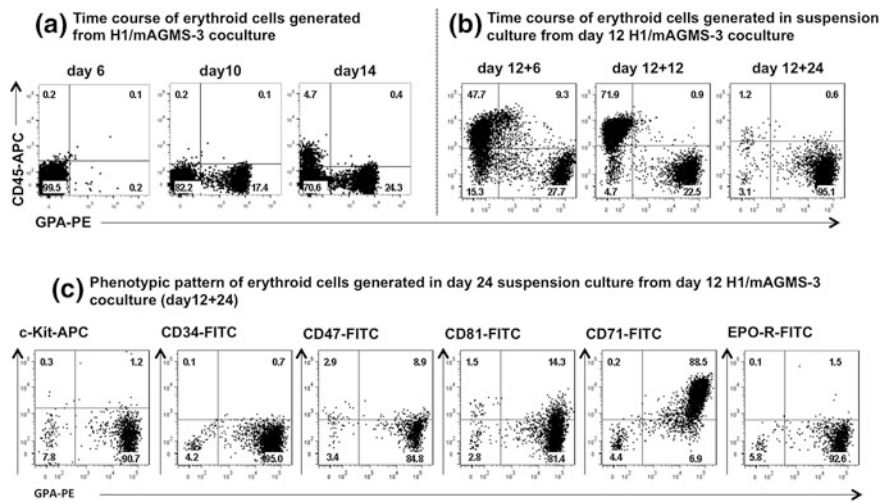
1. The cells in suspension culture on day 6 are centrifuged at 1200 rpm for 5 min and then resuspend with SF-RBC medium containing EPO (final: 2 IU/mL) only. Change the medium every 3–4 days if the medium shows yellow color.
2. In liquid culture, more and more erythroid cells are expanded. After 20 days, the purity of erythrocytes reaches up to 95 %.

Notes:

The erythrocytes in this stage are more and more fragile over time. Try best not to centrifuge or at a low centrifugation speed to protect cells when change the medium.

2.3.7 *Characterization of hPSC-Derived Erythrocytes by Flow cytometry Assay*

Erythroid cells are originated from hPSCs coculture with mFLSCs/mAGMS-3, and then, erythrocytes are expanded and matured in suspension culture. It is known that mature human erythrocytes from hCB-CD34<sup>+</sup> cells are GPA<sup>+</sup>/CD34<sup>neg</sup>/CD45<sup>neg</sup>/CD71<sup>medium</sup>/CD47<sup>neg</sup>/EPO-R<sup>neg</sup> [12, 45]. To demonstrate the phenotypic expression pattern of erythrocytes, erythroid cells at different inducing stages are detected by flow cytometry assay. A representative flow cytometry data of erythroid cells derived from hESC (H1)/mAGMS-3 coculture are shown in Fig. 2.3. Based on our data, the erythrocytes generated in our coculture system are typically GPA<sup>+</sup>CD45<sup>neg</sup>, and the phenotypic pattern of the mature erythrocytes (by day 12 + 24 H1/mAGMS-3) is similar to that of the adult-type mature erythrocytes.



**Fig. 2.3** Representative flow cytometry profiles of erythroid cells derived from hESC (H1)/mAGMS-3 coculture. **a** CD45 and GPA co-expression overtime of cells derived from H1/mAGM-S3 coculture. **b** CD45 and GPA co-expression overtime of erythroid cells of suspension culture stage derived from day 12 H1/mAGM-S3 coculture. Abundant mature erythrocytes (GPA<sup>+</sup>CD45<sup>neg</sup>) are generated. **c** The c-kit, CD34, CD47, CD81, and CD71 expression pattern of erythrocytes derived from day 12 + 24 H1/mAGMS-3 coculture

1. The total harvested coculture cells are incubated with normal rabbit serum. Add 10  $\mu$ L rabbit serum to  $1 \times 10^6$  cells.
2. The total harvested coculture cells are distributed into  $0.2\text{--}0.5 \times 10^6$  per sample in 0.1 mL volume.
3. Add monoclonal antibodies that conjugated with FITC, PE, or APC. 7-AAD is used to exclude dead cells. Cells are stained on ice in dark for 30 min.
4. Wash the incubated cells twice with SM, and resuspend them with 0.1–0.2 mL SM solution. Then, filter the cell suspension through a 38- $\mu$ m nylon membrane to remove cell masses.
5. The stained samples are analyzed on FACS Canto<sup>TM</sup>II (Becton Dickinson Company). The cells are gated by SSC and FSC first, and then, the living cells are gated by 7-AAD negative fraction. Record of the data can be further analyzed by BDFACSDiva software or FlowJo software (Version 7.2.5).

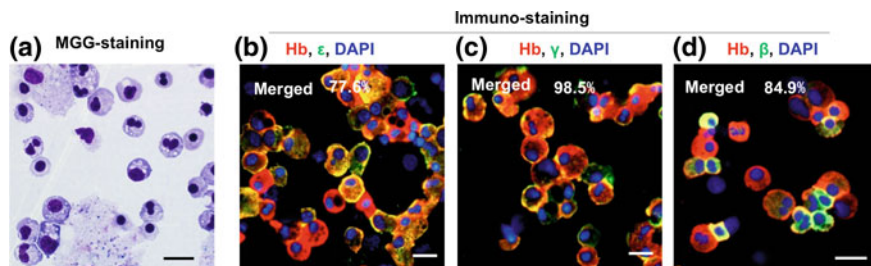
#### Notes:

1. The GPA antibody (Clone, JC159) from DAKO company is more sensitive for detecting embryonic and fetal erythroid cells than others.
2. The CD45 antibody (Clone, 2D1) from DAKO company is more sensitive to embryonic hematopoietic cells than others.

### 2.3.8 *Hb Components of hPSC-Derived Erythrocytes Detected by Immuno-Staining Assay*

The various globins are encoded by two gene clusters, the  $\alpha$  cluster on chromosome 16 encodes including the embryonic  $\zeta$ -globin and adult  $\alpha$ -globin, and the  $\beta$  cluster on chromosome 11 encodes containing the embryonic  $\varepsilon$ -globin, the fetal  $\gamma$ -globin, and adult  $\delta$ - and  $\beta$ -globins [46]. There are two accompanying switches, a switch from embryonic-to-fetal globins early in gestation and then from fetal-to-adult globins around the time of birth. The Hb content is a good criterion to evaluate the maturity of erythrocytes. We detect the Hb components of hPSC-derived erythrocytes by immuno-staining assay as reported [27]. Erythrocytes derived from hPSCs by our coculture system are able to mature progressively and overwhelmingly express high rate of adult-type  $\beta$ -globin (Figs. 2.2d and 2.4d).

1. Circle the cells cytopspined on the glass slides using the liquid blocker pen.
2. Fix the cells with 4 % PFA solution at 4 °C for 30 min and then wash the cells with D-PBS (-) for three times.
3. Treat the membrane of cells with 5 % SM/0.1 % Triton-100/D-PBS (-) at 4 °C for 30 min to degrade the lips on the membrane, block the unspecific binding sites on/in cells, and then wash with 5 % SM/D-PBS (-) for three times.
4. Incubate the cells with the primary Abs Hb [1: 100 diluted with 5 % SM/D-PBS (-)] at 4 °C over night.



**Fig. 2.4** Characteristics of erythroid cells generated from hESC (H1)/mAGMS-3 coculture on day 12 + 24. **a** Photographs of MGG staining. (60× magnification; bar = 20 μm). **b–d** The Hb components of erythrocytes derived from day 12 + 24 H1/mAGMS-3 (red Hb; green  $\epsilon$ -,  $\gamma$ -,  $\beta$ -globin; blue DAPI; 60× magnification; bar = 20 μm)

5. After wash with 5 % SM/D-PBS (-) for three times, stain the cells with Cy3-conjugated secondary Abs [1: 100 diluted with 5 % SM/D-PBS (-)] at room temperature (RT) for 30 min and then wash with 5 % SM/D-PBS (-) and 0.1 % Tween-20/D-PBS (-) for 3 times, respectively.
6. Stain the nucleus with DAPI (0.1 μg/mL) at RT for 5 min and then wash with D-PBS (-) for 3 times.
7. Dispense one drop of mounting medium on to the section to mount cells. Cover slips can be permanently sealed around the perimeter with nail polish.
8. Observe with a fluorescence microscope (Olympus, BX53), count the percentage of Hb and  $\epsilon$ -,  $\gamma$ -,  $\beta$ -globin positive cells, and then take photographs by using a CellSens XV image processing software (Olympus).

#### Notes:

1. Optimal working dilutions of primary and secondary antibodies should be determined experimentally.
2. Both of negative and positive controls should be set in every test. They are the standard for judging negative or positive expressing cells.
3. Washing technique after incubation with primary and secondary is very important. Excessive and insufficient wash will lead to false negative and false positive results, respectively.
4. Dispense one drop of mounting medium on each cover slip and then cover the slides onto the cytopspined area one by one.
5. Store mounted slides at 4 °C and protect from light.

## 2.4 Future Perspectives

Because of their distinctive properties for erythrocytes in the progressive maturation from embryonic stage to fully mature adult-type RBCs, the Hb-switching pattern has long been served as classical standard to characterize the maturity of

erythrocytes derived from embryonic hematopoiesis. Based on the *in vitro* developmental model of hPSC-derived erythrocytes, clues for human early hematopoiesis are uncovered partly. Recent reports show that expression of GPA (CD253a), previously known as a typical lineage-specific marker for erythrocytes, defines an initiation of a novel earliest population enriched with definitive multipotential hematopoietic progenitors [47–50].

However, there is still a long way to challenge toward clinical translation. First, a more efficient large-scale manufacturing method should be optimized to significantly save the prohibitively expensive cost of culturing cells *in vitro*. Many efforts have been made for this purpose, such as optimizing the formula of differentiation medium [51], using semipermeable membranes to save complementary molecules in medium [5], replacing cytokines with cheaper mimetics [52], adding specific small molecules to increase expansion folds of erythroid precursors [53], establishing immortalized erythroid progenitor cell lines [54–57], and using genetic manipulation to upregulate erythroid commitment and amplification [55, 58]. Secondly, there are still some obfuscations in the pivotal regulatory axis of erythropoiesis. Especially, it requires practical solution to the puzzles of enucleation [59] and Hb switching [60] in erythrocytes developed from hPSCs. The synthetic three-dimensional (3D) system to mimic hematopoietic niches [61–64] might be a promising means. New-type polymer materials and nanomaterials should also be explored in this field. Thirdly, in order to fulfill successful therapeutic application, an *in vivo* model of efficient transplantation and functional assay for hPSC-derived erythrocyte progenitors in immuno-deficiency mice should be established. In addition, although the membrane structure molecules of adult erythrocytes has been studied systematically by Mohandas N and Xiuli An group [45, 65, 66], little is known about the characteristics of embryonic and fetal erythrocyte membrane. Both humanized culture system [67] and animal evaluating models [68] must be developed before clinical translation of the hPSC-derived RBCs.

Since the erythrocytes derived from EBs are of primitive properties, mimicking the erythropoiesis in yolk sac, it is essential to establish an efficient culture system to produce large-scale definitive erythrocytes from hPSCs. To our experiences, large quantity of functionally mature erythrocytes can be reproducibly generated from hPSCs by coculture with murine mFLSCs/mAGMS-3 stromal cell lines derived from normal fetal mice [27]. The culture models established in our laboratory provide an ideal platform to further promote the study on uncovering the mechanisms controlling human early erythropoiesis and erythropoiesis-related diseases and the clinical application of hPSC-derived cRBCs.

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