

# The Time-Dependent Morphological Alteration and Enucleation Process during the Differentiation of Mammalian Erythroid Cells

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## Abstract

One characteristic of the erythroid terminal differentiation is hemoglobin expression and enucleation. In the present study, by using real-time cultured Friend Virus Anemia-inducing (FVA) cells, scanning electron microscopy (SEM), immunofluorescence combined with laser scanning confocal microscopy (LSCM), the FVA cells induced by Erythropoietin (EPO) for were used for: 1) to study the shape and proportion of cells at different differentiating stages, enucleation process, the formation of blood islands, and engulfment of nuclei by macrophages in real time, 2) to quantitatively analyze erythroid cell surface markers including CD71 and Ter119, and cytoskeletal-associated proteins (stathmin, septin8 and RBBP4). The results of real-time monitoring of enucleation indicated that it took about 7 to 8 hours to extrude the nuclei from karyopyknosis (polychromatic erythroblasts). It further showed that the macrophages engulfed the expelled erythroid nuclei. SEM showed a variety of shapes of the nascent reticulocytes. In the process of erythroid differentiation, expressions of both the transferring receptors CD71 and Ter119 were higher than that of adult blood cells, whereas, cytoskeletal-associated proteins (stathmin, septin8 and RBBP4) decreased gradually. Therefore, systematic observation of the process of differentiation and enucleation will provide a deeper understanding of cellular and molecular mechanisms for erythroid differentiation and carcinogenesis.

**Keywords:** Erythroid differentiation; Enucleation; Real-time cultured cell monitoring; Morphology; LSCM; Occurrence of Leukemia

## Introduction

Erythroid differentiation is a complex process, and the terminally differentiated cells are characterized by shrinkage of the cells, karyopyknosis, enucleation, and ultimately differentiating into mature red blood cells. Mice were injected with FVA, and the erythroid cells from their spleen were synchronized as proerythroblast and retained the differentiation characteristics of Colony Forming Unit-Erythroid (CFU-E) cells [1-3]. Therefore, the medium was supplemented with EPO in the culture, the cells mainly generated basophilic erythroblasts at 12 h to 24 h, polychromatic erythroblasts at 36 h, dominantly orthochromatic erythroblasts and reticulocytes at 48 h to 60 h [4]. There were many changes in the cell morphology, haemoglobin accumulation, the expelled nuclei were wrapped by small amount of cytoplasm and plasma membrane, and then formed reticulocytes [5,6] in the process of differentiation.

In the early stages of erythroid differentiation, the macrophage functions as a “nurse” cell for forming blood islands [7-9], the direct contact of macrophages and erythroid cells improves the proliferation of erythroid cells [10-13], and provides conditions for erythrocyte formation [14], the nuclei are engulfed by macrophages through related molecules on the nucleus that interact with macrophages [15].

Recently, there are a few reports regarding to the erythroid differentiation and enucleation [14,16]. However, there is still a lack of systematic study about cell morphology at various differentiation stages, surface markers, changes in cell number, the time needed for enucleation and polymorphism of the nascent reticulocytes.

This research has systematically studied the morphology and enucleation at various differentiation stages of the FVA cells. It took about 7 to 8 hours from polychromatic erythroblasts to the exclusion of nuclei and showed a variety of shapes of the nascent reticulocytes.

## Materials and Methods

Animal and virus Six to eight-weeks old mature BALB/c female mice (20 -25 g) were purchased from Hangzhou Normal University

(Hangzhou, China). Friend virus anemia-inducing (FVA) was purchased from ATCC (American Tissue Culture Collection, Rockville, USA).

## Reagents

IMDM medium (Gibco, Grand Island, USA), fetal bovine serum (HyClone, Logan, USA), 4% paraformaldehyde (Sigma-Aldrich, Steinheim, Germany), antibodies for stathmin, Ter-119, CD68, and CD71 (Abcam, Cambridge, UK), antibodies for septin8 (Santa, Dallas, USA), antibodies for RBBP4 (Epitomics, Burlingame, USA), EPO, FITC-conjugate goat anti-mouse IgG and Cy3-conjugate goat anti-rabbit IgG (Invitrogen, Eugene, USA).

## FVA cells isolation and culture

Mice were injected with FVA via tail vein. Spleens from these mice were harvested two weeks later. Single cell suspension of the spleens was prepared, and cultured in IMDM medium containing 30% FBS, 0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, USA),  $10^{-4}$  mol/L 1-Thioglycerol (Sigma-Aldrich, St. Louis, USA), and 2 mmol/L L-glutamine (Gibco, Grand Island, USA).  $1 \times 10^6$ /mL FVA cells were seeded in the culture flask with  $2 \times 10^4$  IU/L EPO, and were grown in the incubator in 5% CO<sub>2</sub> at 37°C for 2 h, 12 h, 24 h, 36 h, 48 h, 60 h, 72 h.

## Benzidine and Wright's staining for the morphology of erythroid cells

**Benzidine staining:** FVA cells were collected and then centrifuged at 1000 rpm for 3 min, stained with 100  $\mu$ L of 0.02% benzidine dye

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Received February 21, 2015; Accepted April 02, 2015; Published April 12, 2015

**Citation:** Yang Z, Zheng Y, Hu F, Zhang Z, Zhao F, et al. (2015) The Time-Dependent Morphological Alteration and Enucleation Process during the Differentiation of Mammalian Erythroid Cells. J Leuk 3: 188. doi:10.4172/2329-6917.1000188

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(Yuanhang, Shanghai, China) premixed with 30% H<sub>2</sub>O<sub>2</sub>, and then examined. Wright's stain: FVA cells were fixed with 4% paraformaldehyde for 20 min., and stained according to the established procedure [17] described elsewhere, and then examined with Nikon ECLIPSE TE2000-U microscope.

### The sizes of FVA cells

Count Star Automated Cell Counter was used to study the statistics of the size of FVA cells after cultured with EPO for 0 h, 2 h, 12 h, 24 h, 36 h, 48 h, 60 h and 72 h.

### Scanning electron microscopy for morphology of FVA cells

FVA cells were washed twice with PBS (GN, Hangzhou, China), fixed with 1 mL 3% glutaraldehyde (SH, Shanghai, China) solution at 4°C for 2 h and washed 3 times with PBS. They were then fixed with 1 mL of 1% osmium tetroxide at 4°C for 2 h, washed 3 times with PBS for 10 min each time. The cells were then dehydrated in graded ethanol (50%, 70%, 80%, 90%, 100%; Changzheng, Hangzhou, China) every 5 min, replaced with acetonitrile (50%, 70%, 80%, 90%, 100%; Tedia, USA) every 15 min until dry. Then the slice was plated platinum and detected with Hitachi s-4800 scanning electron microscopy.

### Morphology of macrophages during erythroid differentiation

Murine ErythroLeukemia (MEL) cells, mouse peritoneal macrophages, and FVA cells were fixed with 4% paraformaldehyde for 20 min, washed 3 times with PBS, and stained with 500 µL DAPI (1 mg/L, KeyGEN, Nanjing, China) working solution at 37°C for 15 min. The cells were then rinsed with methanol (Changzheng, Hangzhou, China), washed 3 times with PBS, and blocked with 1% BSA for 30 min. Furthermore, the cells were incubated with anti-CD68 primary antibody (1:200) at 4°C overnight. After incubation, they were washed 3 times with PBST (Phosphate buffer solution with Tween 20), incubated with Cy3-conjugate goat anti-rabbit IgG (1:10,000) for 1 h, rinsed with PBST. Anti-fade mounting medium was then added and observed with Nikon C1SI LSCM.

### Real-time cultured cell monitoring for enucleation

FVA cells were seeded at a concentration of  $1 \times 10^6$  /mL, EPO ( $2 \times 10^{-4}$  IU/L) was added and the cells were grown in 5% CO<sub>2</sub> at 37°C in miniature incubator for real-time cultured cell monitoring. These cells in the same position were imaged one frame per minute for 48 h.

### Quantitative analysis of Ter119 and CD71 in erythroid cells and adult blood cells

FVA cells, mouse bone marrow cells, mouse blood cells, MEL cells and NIH3T3 cells were fixed with 4% paraformaldehyde for 20 min, washed 3 times with PBS, and blocked with 1% BSA for 30 min. The samples were then incubated with CD71 (1:3,000) or Ter119 (1:6,000) antibodies at 4°C overnight and then washed 3 times with PBST, incubated with Cy3-conjugate secondary antibody (goat anti-rabbit IgG (1:10,000) and FITC-conjugate secondary antibody (goat anti-mouse) IgG (1:10,000) for 1 h. They were then rinsed with PBST and anti-fading agent was added, and then observed with LSCM. Results were quantified using the EZ-C1 3.20 software for CD71 and TER119. All data were expressed as means  $\pm$  SD of three independent experiments.

### The expression of stathmin, RBBP4, and septin8 in erythroid cells

Immunofluorescence was combined with LSCM to detect the expression of the three cytoskeletal-associated proteins, i.e. stathmin, RBBP4, and septin8.

### Statistical analysis

Quantitative experimental data were expressed as mean  $\pm$  SD and analyzed using IBM SPSS Statistics 21.0 software with t-test. The difference with  $P < 0.05$  was regarded as statistically significant.

### Results

As shown in Figure 1, the weight of the normal adult mouse spleen was about 0.1 g, that increased to 1~1.5 g due to the cells being synchronized as proerythroblasts after transfection with FVA that increased the blood volume of spleen.

### Change in the sizes of FVA Cells

Benzidine (Figure 2a-e) was used to detect the differentiation process of cells, while Wright's staining (Figure 2f-j) could observed the cell morphology, the cells could be divided into: mostly proerythroblast with diameter of 12 µm at 2h, basophilic erythroblast with diameter of 10 µm after 12h, mainly polychromatic and orthochromatic erythroblasts with diameter from 10 µm to 8 µm at 36 h. Most of the cells were differentiated into reticulocytes with diameter of 8 µm at 48 h, and almost all of the erythroblast was differentiated into reticulocytes after 60 h. And 14 µm represented small cell clusters (Table 1). The results showed that the ratio of cells with 12 µm in diameter was decreased from about 43% at 2 h to below 10% at 72 h, while the number of cells with a diameter of 8 µm gradually increased from 15% at 2 h to 85% at 72 h (Figure 3).

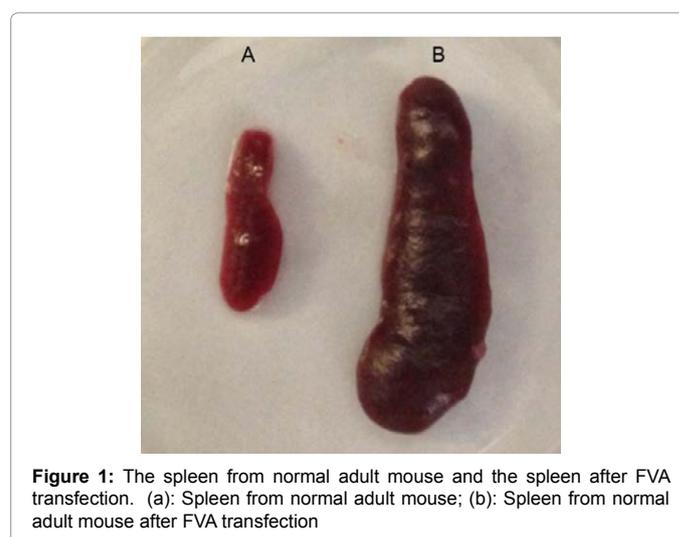
The result of SEM showed that (Figure 4), the cellular volume gradually decreased. The volume of minimal cell was orthochromatic Erythroblasts (Figure 4e). Further, the cellular volume was slightly increased and the reticulocytes formed biconcave discoid structures.

### Enucleation process of Erythroblast

The SEM results of the whole process of enucleation are shown in Figure 5. Before enucleation, the nucleus deviated and then the cytoplasm extended into a gourd shape. During the process, the nuclear was extruded from the cell and the entire nuclear and cytoplasm was separated. The nascent reticulocytes formed irregular shapes after enucleation (Figure 6).

### Real-Time cultured cell monitoring of enucleation

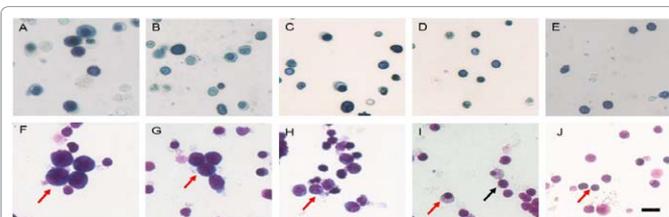
Figure 7a-f show that it spent about 7-8 hours from the karyopyknosis to enucleation, and ultimately the formation of reticulocytes. During



differentiation, the cellular volumes were gradually decreased, and the cells eventually changed from the original round nucleated cells to enucleated reticulocytes.

### Morphologic changes of the macrophages during erythroid differentiation

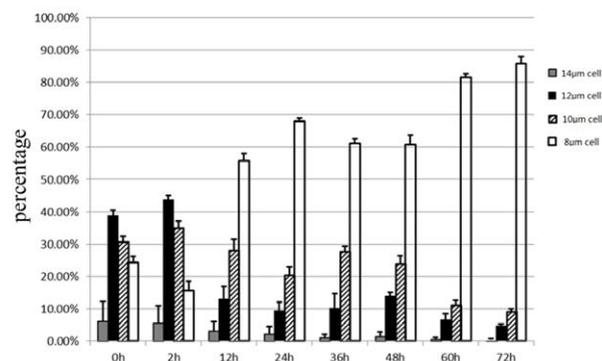
As shown in Figure 8a-b, erythroid cells gathered around macrophages and the number was gradually increased, then the macrophages became large gradually due to engulfment of nuclei (Figure 8c-d). Fetal liver cells from E14.5 d fetal mouse cultured *in vitro* had similar results (Figure 9). Furthermore, the macrophage-specific cell surface marker CD68 confirmed that it was macrophage. The volumes of the macrophages increased tremendously due to the endocytosis of naked nuclei. After 72 h, macrophages expanded into oval shapes, and their volumes were decreased with the intracellular naked nuclei being gradually digested (Figure 10).



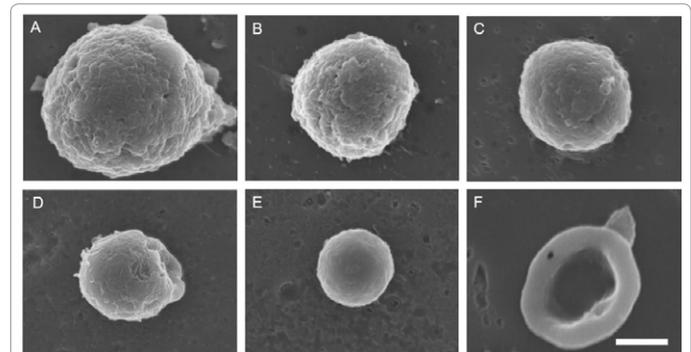
**Figure 2:** Benizidine staining of FVA cells cultured after 2 h-60 h (a-e): 2 h; (b) 24 h; (c) 36 h; (d) 48 h; (e) 60 h; Wright's staining of FVA cells cultured after 2 h-60 h (f-j): (f) 2 h, Proerythroblasts, Red arrow; (g) 24 h, Basophilic Erythroblast, Red arrow; (h) 36 h, polychromatic Erythroblast, Red arrow; (i) 48 h, orthochromatic erythroblast, red arrow; reticulocytes, black arrow; (j) 60 h, nucleus, red arrow. Bar =10  $\mu$ m.

FVA cells	8 $\mu$ m cells	10 $\mu$ m cells	12 $\mu$ m cells	14 $\mu$ m cells
0h	3183 $\pm$ 176	4018 $\pm$ 207	5067 $\pm$ 265	799 $\pm$ 59
2h	277 $\pm$ 23	621 $\pm$ 39	775 $\pm$ 31	98 $\pm$ 14
12h	633 $\pm$ 41	319 $\pm$ 32	148 $\pm$ 17	35 $\pm$ 12
24h	887 $\pm$ 23	264 $\pm$ 22	123 $\pm$ 10	30 $\pm$ 15
36h	534 $\pm$ 24	242 $\pm$ 13	89 $\pm$ 12	9 $\pm$ 5
48h	2426 $\pm$ 213	954 $\pm$ 70	556 $\pm$ 19	58 $\pm$ 20
60h	4004 $\pm$ 124	548 $\pm$ 25	330 $\pm$ 17	27 $\pm$ 7
72h	3055 $\pm$ 190	321 $\pm$ 10	168 $\pm$ 3	14 $\pm$ 6

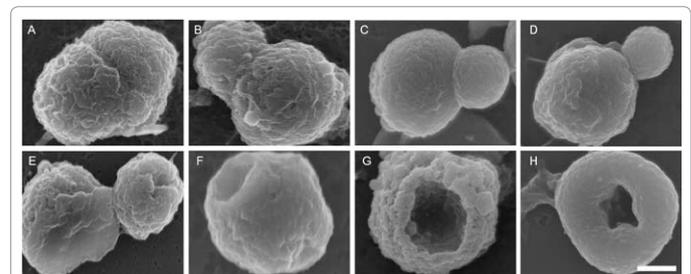
**Table 1:** The sizes of cells at induced different times the number of 8  $\mu$ m, 10  $\mu$ m, 12  $\mu$ m, and 14  $\mu$ m diameters cells.



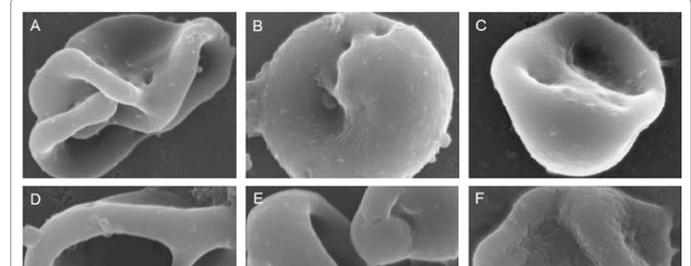
**Figure 3:** Trends of cells with diameters of 8  $\mu$ m, 10  $\mu$ m, 12  $\mu$ m, 14  $\mu$ m. 14  $\mu$ m cells represented small cell clusters, 12  $\mu$ m cells were Proerythroblasts, 10  $\mu$ m cells represented basophilic and polychromatic erythroblasts, and 8  $\mu$ m cells were orthochromatic Erythroblasts and reticulocytes.



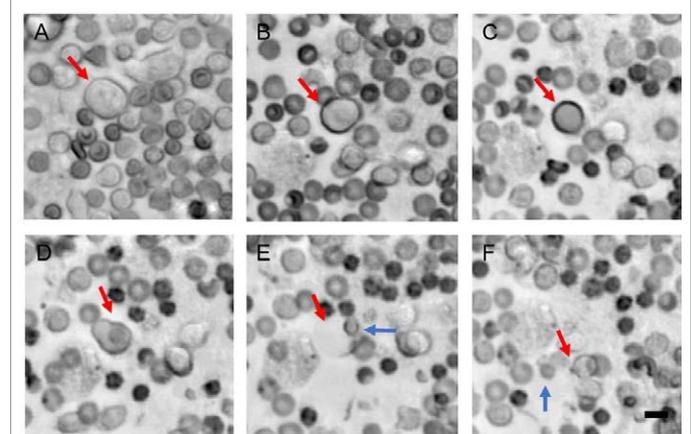
**Figure 4:** The SEM results of FVA cells cultured after 2h-48h. (a-c) nucleated cells, (d-e) Cellular volume gradually reduced; (f) Nascent reticulocytes after enucleation. Bar =2  $\mu$ m.



**Figure 5:** The SEM results of FVA cells cultured after 2 h-48 h. (a-e) Enucleating cell; (f-h) Cell after enucleation. Bar =1  $\mu$ m.



**Figure 6:** Distinct shapes of nascent reticulocytes after enucleation. Bar =2  $\mu$ m.



**Figure 7:** Real-time cultured of cells at Bright field. The red arrows indicate continuous change of the same cell from nuclear karyopyknosis to enucleation, and the blue arrows indicate extruded nucleus. Bar =5  $\mu$ m.

### Expression of erythroblast specific surface markers

Transferrin receptor (CD71) was involved in the absorption of iron and essential proteins that regulate the cell growth [18]. Presence of Ter119 was a sign of nucleated red blood cells. The expression of both markers could be used to determine the differentiation capability of erythroid cells. FVA cells cultured for 12 h, mouse bone marrow cells, mouse peripheral blood cells, MEL cells and NIH3T3 cells were tested. The results showed a decrease in CD71 and Ter119 expression from erythroid cells to NIH3T3 cells (Figure 11). The expression of both the proteins (CD71 and Ter119) in FVA cells cultured for 12 h were significantly higher than that of bone marrow cells and peripheral blood cells (Table 2).

### Expression of the three cytoskeletal-associated proteins in erythroblast

The expression of three cytoskeletal-associated proteins (stathmin, RBBP4, and septin8) was decreased in the order of proerythroblasts, basophilic, polychromatic, and orthochromatic erythroblasts (Figure 12).

### Discussion

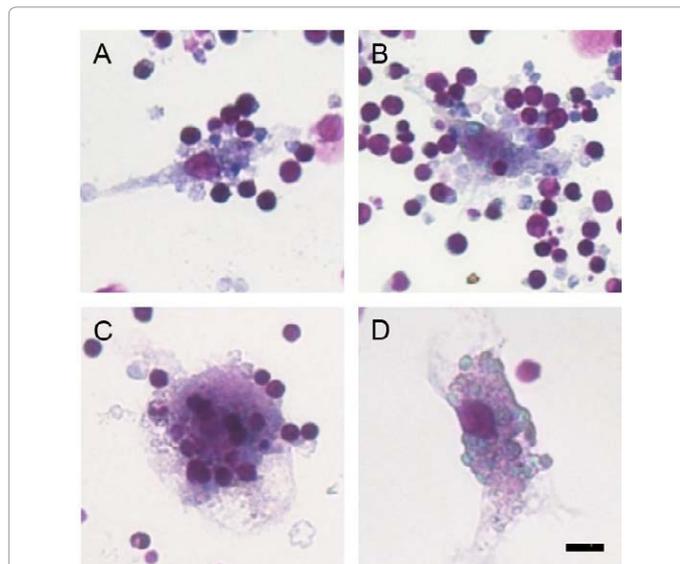
Enucleation in the terminal differentiation of erythroblast is a multi-step complex process in which the nucleus, membrane, and cytoskeletal system are all involved. In this study, FVA cells were used for

systematically studying the processes of differentiation and enucleation. By the stimulation of EPO, FVA cells proliferated and synergetically differentiated into basophilic, polychromatic, and orthochromatic erythroblasts. SEM results showed karyopyknosis, nuclear deviation, enucleation, and then generation of reticulocytes.

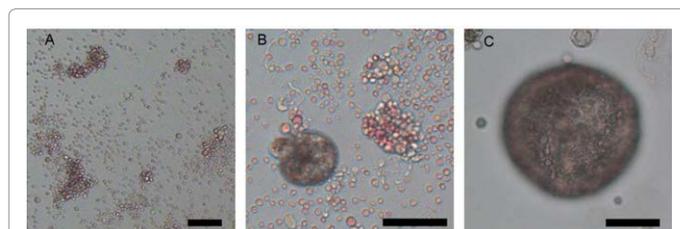
Real-time monitoring results showed it took about 7~8 hours from karyopyknosis to enucleation. During differentiation, immature nucleated cells gradually gathered around macrophages. Macrophages then engulfed the extruded nuclei, resulting in increased volumes at first and narrowed volumes later because the nuclei were digested in the latter phase.

Quantitative analysis of erythroid-specific marker proteins (CD71 and Ter119) showed that the expressions of both were higher than adult blood cells. All the results indicate that the differentiation of erythroid cells requires the synthesis of hemoglobin in abundance, so the transferrin receptor CD71 is highly expressed. When erythroid differentiation was induced in the constant presence of EPO in an *in vitro* differentiation system, CD71 expression was maintained during all stages of erythroid differentiation [19]. When EPO was removed from the culture medium, erythroid differentiation was better coupled with down-regulation of CD71 expression [20].

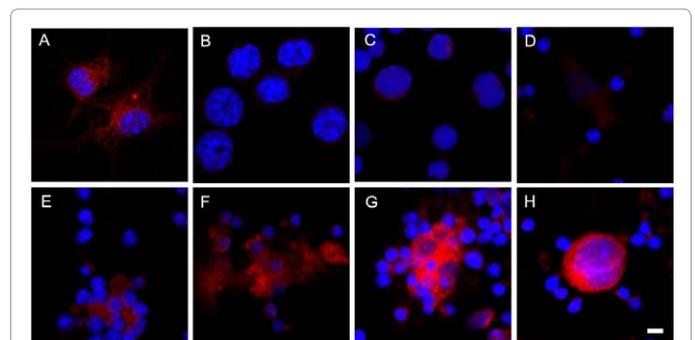
The microtubule depolymerization protein stathmin is a member of the protein family, which regulates the microtubule cytoskeleton. It also regulates microtubule polymerization via promoting microtubule depolymerization and preventing tubulin heterodimers [21]. Septin8 is a member of class conserved cytoskeletal GTP-binding proteins. It plays an important role in cell division, cell polarization, vesicle trafficking, membrane remodeling and many other important physiological processes [22]. Histone-binding protein RBBP4 is a component of



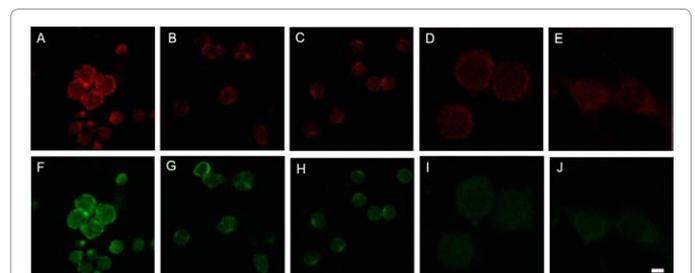
**Figure 8:** Wright's staining results of blood islands during Erythroid differentiation. (a-c) Engulfing nuclei by macrophage, (d) The shrunken cells due to the digestion of the nuclei. Bar = 10 µm.



**Figure 9:** Bright field results of Fetal liver cells from E14.5 d fetal mouse after being induced by EPO for 24 h, 48 h, and 60 h in vitro. (a) Formation of blood islands at 24 h; (b) Increase in blood islands at 48 h (c) engulfment of nuclei at 60 h. Bar = 20 µm.



**Figure 10:** LSCM results of macrophage specific cell surface marker CD68 (red) and DAPI (blue). (a) Macrophages, CD68 positive control; (b) MEL cells, CD68 negative control; (c-h) CD68 and DAPI co-staining results of FVA cells in vitro after 12 h, 24 h, 36 h, 48 h, 60 h, 72 h. Bar = 5 µm.

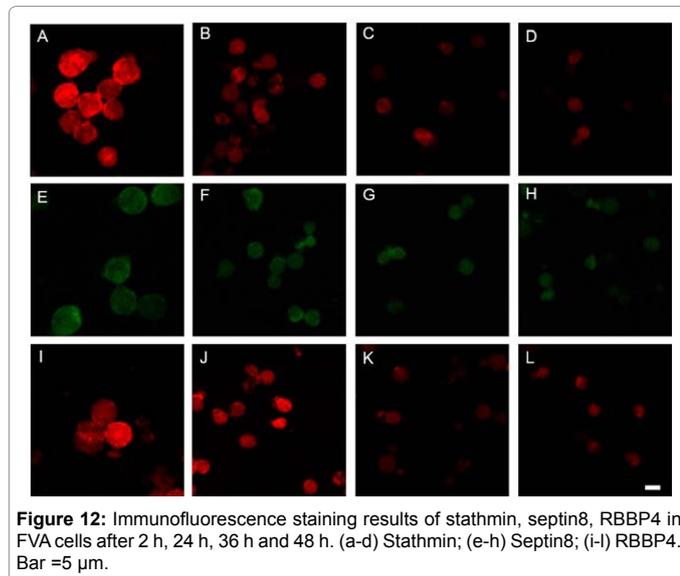


**Figure 11:** (a-e) The CD71 immunofluorescence staining results (red) of FVA 12 h cells, bone marrow cells, peripheral blood cells, MEL cells, and NIH3T3 cells, respectively. (f-j): The Ter119 (green) of FVA 12 h cells, bone marrow cells, peripheral blood cells, MEL cells, and NIH3T3 cells, respectively. Bar = 5 µm.

Surface markers	FVA 12h cells	Bone marrow cells	Peripheral blood cells	MEL cells	NIH3T3 cells
CD71	1204.01 ± 25.02	374.56 ± 32.00*	425.48 ± 17.25*	258.16 ± 15.54*	222.43 ± 6.56*
Ter119	694.85 ± 23.43	230.46 ± 15.03*	214.48 ± 4.29*	100.31 ± 5.38*	67.76 ± 5.31*

P<0.05

**Table 2:** Fluorescence intensity of CD71 and Ter119 in five kinds of cells The fluorescence intensities of CD71 and Ter119 in FVA cells were significantly different from those of the other four cells (P <0.05)



**Figure 12:** Immunofluorescence staining results of stathmin, septin8, RBBP4 in FVA cells after 2 h, 24 h, 36 h and 48 h. (a-d) Stathmin; (e-h) Septin8; (i-l) RBBP4. Bar =5 µm.

ATP-dependent chromosomal rearrangements complex, such as the nuclear chromatin remodeling and histone deacetylase complexes Sin3, and regulates the functions of the Sin3 [23]. The contents of the three cytoskeletal-associated proteins (stathmin, septin8, and RBBP4) decrease gradually during differentiation.

During the development and differentiation of mammalian erythroid cells, the changes in cell morphology and gene expression are showed regularly [24]. Enucleation is regulated by microtubules and PI3K signaling in a manner mechanistically similar to directed cell locomotion [25]. However, most molecular events in enucleation remain unclear, which gene regulates this process is not clear. Functions of cytoskeletal proteins, gene regulation and the mechanism of Erythroid differentiation need to be clarified by further studies.

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