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A Simple Embryonic Stem Cell-Based *in vitro* Differentiation System That Recapitulates Early Erythropoietic Events in the Mouse Embryo

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An embryonic stem (ES) cell-based *in vitro* model system was examined to determine whether a simple differentiation of embryoid bodies (EBs) in the suspension medium is useful to dissect early erythropoiesis. Characteristics of the differentiating EBs were monitored for their differentiation potential to generate hematopoietic cell types by general morphology, benzidine staining and two-step colony assays, and expressivity of several erythroid marker genes by the RT-PCR analysis for total cellular RNA prepared from the differentiating EBs. Every hematopoietic lineage cells were generated from the differentiating EBs with reproducible frequencies, similar to the other sophisticated differentiation protocols. Furthermore, the globin gene switching in differentiating ES cells paralleled the sequence of events found in the mouse embryo, and such that their expression was activated by at least 12 hrs later than those of erythroid-specific transcription factors, GATA-1 and Tal-1. The erythropoietic differentiation program initiated reproducibly and efficiently in this simple differentiation system in a suspension culture, such that this system may be useful for dissection of the molecular events of early erythropoiesis.

KEY WORDS: Gene Expression, Globin Gene, Transcription Factor, Embryonic Stem Cells, *in vitro* Differentiation

Erythropoiesis is a multi-step process which starts from the pluripotent hematopoietic stem cells and in which other blood cell types are generated by multiple commitment and determination steps [see Metcalf (1989) for review]. This event in vertebrates is characterized by sequential changes in erythropoietic site, cell morphology, and type of globin. In mammals, erythropoiesis first proceeds in the yolk sac during the embryonic stage, later shifts to the liver for the fetal stage, and finally shifts to the bone marrow around the time of birth. Regulation of the rate of erythropoiesis is achieved by controls at a number of critical steps (Marks and Rifkind, 1978).

Recently, several cytokines and transcription factors have been identified and characterized at the molecular level, which may play important roles in regulating genes related to the terminal differentiation of the red blood cell [see Kim (1993) and Orkin (1995) for review]. Very little is known about the molecular network of gene expression for the induction and the maintenance of early erythropoiesis, partly due to inaccessibility of the early embryos and the limited amount of tissue available at this stage of development. It is possible to purify the normal progenitor cells, but it is hard to study the molecular characteristics of the undifferentiated progenitor cells, due to the limited amounts of purified cells available for direct study, and the spontaneous differentiation during

the culture of purified stem cells. Study of progenitor cells has attempted through the production of targeted mutations in the genes encoding these factors and the analyses of their developmental consequences in transgenic animals (Capecchi, 1989). But this system is also limited by early embryonic lethal mutations in a homozygous state.

The early events at the embryonic stages have been studied by using *in vitro* differentiation of the mouse embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981). Previous studies have demonstrated that ES cells differentiate in culture and generate multiple hematopoietic lineages (Whitelaw *et al.*, 1990; Lindenbaum and Grosveld, 1990; Schmitt *et al.*, 1991; Wiles and Keller, 1991; Keller *et al.*, 1993). One of the most successful systems is of Keller *et al.* (1993), in which they could enumerate hematopoietic precursors (colony forming cells, CFC) and show proper globin gene switching. This system depends on the two-step colony assay, in which differentiating embryoid bodies (EBs) cultured in semisolid methylcellulose medium are disaggregated and subsequently replated into methylcellulose culture containing several cytokines, such that complicated and sophisticated steps of manipulation are needed, and quality of the reagents, like serum and cytokines, should be well controlled. Thus, a simpler differentiation system is required to dissect early erythropoiesis. Here, as an effort to establish a simple ES-based *in vitro* model system, ES cells were differentiated by a simple suspension culture of EBs, and differentiating EBs were monitored for their differentiation potential to generate hematopoietic cell types and regulated gene expression.

Materials and Methods

Embryonic stem cell culture

The mouse ES cell lines used in these experiments were 129/sv-derived CCE (a gift from M. Groudine) and E14-TGa (a gift from Y. Zhuang). These cells were adapted to grow in the presence of leukemia-inhibitory factor (LIF) without feeder cells (Wiles and Keller, 1991). Undifferentiated ES cells were maintained on gelatinated petri dishes (Costar) in Dulbecco's modified Eagle medium (DMEM, Gibco/BRL) supplemented with LIF, 15% bovine calf serum (Hyclone), and 1.5×10^{-4}M monothioglycerol (Sigma). LIF was obtained as a conditioned medium from a LIF-transformed CHO cell line (a gift from M. Groudine).

In vitro differentiation of Embryonic stem cells in suspension culture

Confluent ES cell cultures were harvested by trypsinization and ES cells were then resuspended at a concentration of 1×10^5 cells per ml in differentiation medium (DMEM supplemented with 10% fetal bovine serum). Ten ml aliquots were dispensed into 100-mm bacterial petri dishes (Baxter) to allow EBs to form. The suspension was diluted 1:3 on day 3 and maintained in a humidified 5% CO_2 atmosphere as long as the experiment required; the medium was changed every 3 days.

To see the erythropoiesis, benzidine staining was done by adding an equal volume of staining solution (0.2% benzidine hydrochloride and 0.3% hydrogen peroxide in 3% acetic acid) directly into the suspension culture; the dark blue staining can be visualized within a few minutes (Garrett and Kredich, 1981).

In vitro colony assay within the embryonic bodies

Differentiating EBs in suspension culture were harvested after 5 to 7 days by centrifugation. The cells within the embryonic bodies were dissociated by incubation (1 hr at 37°C) in 0.25% collagenase solution dissolved in phosphate buffered saline supplemented with 20% serum. Following this incubation, the cells were gently broken up by passing through a syringe with a 20-gauge needle. About 5×10^4 cells were plated in 1.0% methylcellulose in Iscove's modified Dulbecco's medium (IMDM, Gibco/BRL) with 10% fetal bovine serum and a source of hematopoietic growth factors (Epo, 2 U/ml; IL-1α, 10 ng/ml; IL-3, 10 ng/ml; G-CSF, 100 ng/ml; SLF, 150 ng/ml) (Keller *et al.*, 1993). Colonies at 7 to 8 days of incubation were collected and stained by

Giemsa to score the differentiating blood cell types.

RNA preparation and reverse transcriptase-PCR

Total RNA was isolated from differentiating EBs using RNAzol (Chromczynsky and Sacchi, 1987). The quality of prepared total RNA samples was checked by electrophoresis on TBE-based native agarose gels using normal DNA loading buffer in the increased electric field (10 V/cm) (Park *et al.*, 1996).

The reverse transcriptase-PCR (RT-PCR) assays were performed essentially as described (Zhuang *et al.*, 1992). Briefly, 100 ng of each RNA sample was used for the RT reaction with random hexamers; the resulting cDNAs were amplified with exon-specific primers (see Table 1) for 28 cycles. A series of dilutions of cDNA synthesized from known amounts of total cellular RNA was used in PCR analysis to find the linear amplification range; 1/25th and 1/10th of cDNA were optimal for globins and 12S rRNA genes, and for other transcription factors, respectively. Because a small fraction of radiolabeled nucleotide (α^{32}P-dCTP) was included in the PCR reaction, the PCR products were directly analyzed on a 5% polyacrylamide gel. Using a 12S rRNA standard, the relative level of each resulting autoradiogram band was measured by densitometry. It should be noted that as the genes of interest in this study are probably expressed in only a subset of cells, the amount of expression may not be quantitative.

Results and Discussion

Embryoid bodies in suspension culture

To examine the process of embryonal erythropoiesis, the observation that mouse ES cells can be made to undergo differentiation *in vitro*, giving rise to various lineages in the embryoid bodies (EBs), was made. For *in vitro* differentiation, single-celled suspension of ES cells was cultured on bacterial dishes in the absence of LIF (Zhuang *et al.*, 1992). The best differentiation, as judged by morphological observation, benzidine staining, and RT-PCR assay (see below), was at a density of $1{\sim}2 \times 10^6$ cells per plate. One day after plating the trypsinized cells, 50 to 100 suspended cells started to clump together to form EBs. The EBs were too big to differentiate appropriately when plated at a higher concentration, whereas the EBs were too small to survive during the next several days when plated at a lower concentration.

Some examples of differentiating EBs are shown in Fig. 1. Three- to four-day-old EBs showed good oval to spherical shape (Fig. 1A). EBs started to expand to a hollow ball (cystic EBs, Fig. 1B) at days 10 to 14, and then burst off. After 2 weeks, the majority of the EBs attached to the plate and the variety of cell types was generated, including some beating myocardia. One or two red blood islands per plate could be seen at this stage (Fig. 1C). However, judged by benzidine-staining, more than 50% of EBs at as early as 7 to 8 days of culture showed positive signals (Fig. 1D). These results suggest that an early stage of erythropoiesis autonomously proceeds well, but only small proportions of cells in EBs differentiate further to

Table 1. Oligonucleotide primers for studying gene expression

Gene	5′ sequence	3′ sequence	size (bp)
12S rRNA (RLP7)	GAAGCTCATCTATGAGAAGGC	AAGACGAAGGAGCTGCAGAAC	202
c-kit ligand (SLF)	CGCTGCCTTTCCTTATGAAGAAGA	CGGGACCTAATGTTGAAGAGAGCA	528
β-globin (EY)	AACCCTCATCAATGGCCTGTGG	TCAGTGGTACTTGTGGGACAGC	415
β-globin (βH1)	GGAAACCCCCGGATTAGAGC	CAGCCTGCACCTCTGGGGTG	219
β-globin (β-maj)	ATGGTGCACCTGACTGATGCTG	GGTTTAGTGGTACTTGTGAGCC	444
GATA-1	AGGGGCCCTGGGGACCTCAG	GAGGCAGGGTAGAGTGCCGT	303
TAL-1	GGGGAACCGGATGCCTTCCC	GTCCGGGCTGGCTGCCCCAT	450
ID-1	GCCATCTCGCGCTGCGCTGGG	GCCTCCGGTGGTCCCGCTTC	231

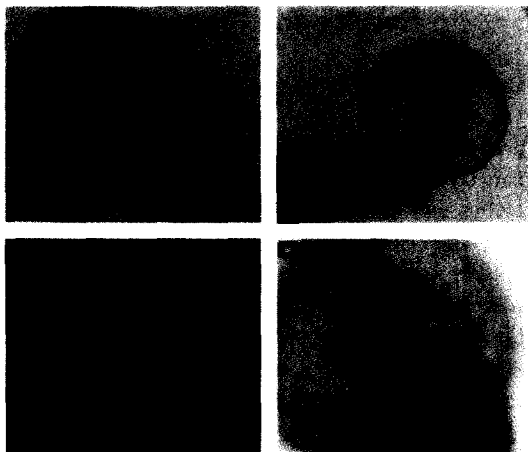

Fig. 1. Typical examples of differentiating embryoid bodies. A, four-day-old EBs; B, cystic (expanded hollow ball) EBs at day 8; C, red blood islands at 2 weeks culture; D, benzidine-staining positive EBs at day 7. Magnifications: A, × 100; B and C, × 50; D, × 200.

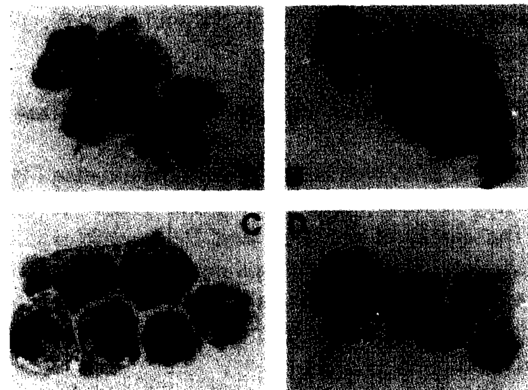

Fig. 2. Hematopoietic cell types generated within the developing embryoid bodies cultured in suspension medium. *In vitro* colony assay of the cells dissociated from 5-day-old EBs in suspension culture was performed as described in Materials and Methods. Each panel (A through D) shows different phases of the Giemsa-stained 7-day-differentiated cells that were cultured in methylcellulose medium supplemented with several kinds of cytokines. All kinds of hematopoietic cell types are seen. Arrows indicate maturing erythrocytes and proerythroblasts. Magnification (A through D), × 1,000.

the blood island-forming stage when developing EBs are in suspension culture. The decline of the progression of later erythropoiesis in EBs is probably due to the lack of appropriate growth factors that should be supplied exogenously in developing embryos, and/or the defect of structural integrity to form an appropriate microenvironment.

Hematopoietic lineage cell types in the developing embryoid bodies

To determine the frequency and types of hematopoietic cells developed in differentiating ES cells *in vitro*, EBs differentiated for various periods of time in suspension were harvested, dissociated, and then replated in secondary methylcellulose cultures containing Epo, IL-1, IL-3, and G-CSF (see Materials and Methods for detail). These cytokines are known to be regulators of erythroid and macrophage development, which are the first two lineages to be detected within the embryonic yolk sac. Keller *et al.* (1993) have already demonstrated that addition of these growth factors could create a better environment for the late stage of hematopoietic differentiation. Hematopoietic colony numbers originating from 5- to 7-day-old EBs averaged about 1,000 per 35mm dish, while the numbers from 3- and 4-day-old EBs were approximately 100 and 500, respectively (data not shown). Colonies from the EBs cultured longer than 9 days formed far fewer. These data suggest that, within the early stage EBs in suspension culture, hematopoietic precursors (colony-forming cells, CFC) are efficiently produced and generate every kinds of hematopoietic cells, although the proper erythropoietic environment is gradually lost after day 9, probably due to the lack of proper cytokine or growth factor influxes. Indeed, when the colonies derived from 5- to 7-day-old EBs were tested, all kinds of hematopoietic lineage cells were easily observed (Fig. 2) in more than 90% of colonies with the frequencies of 34.9%, red cell types: 47.6%, white cell types: 17.5%, megakaryocytes (Table 2).

Expression profiles of β-like globin genes and several marker genes

These reproducible and predictable formation of EBs in suspension culture suggested an analysis of the expression pattern of a number of genes known to be involved in erythropoiesis. Expression

Table 2. Distribution of blood cell types generated in colony assay. Differentiating EBs in suspension culture were removed after 7 days by centrifugation. The cells within the embryonic bodies were dissociated by collagenase and then gently broken up by passing through a syringe (see Materials and Methods). About 5 × 10^4 cells were plated in methylcellulose medium with 10% fetal bovine serum and a source of hematopoietic growth factors. Colonies at 7 days of incubation were collected and stained by Giemsa to score the differentiating blood cell types.

No. of Colony Scored (%)			
Erythroids	Myeloids	Megakaryocytes	Total
88 (34.9)	120 (47.6)	44 (17.5)	252 (100)

studies were accomplished by using RT-PCR (see Materials and Methods for detail). RNAs isolated from the pools of EBs every 24 hours for up to 2 weeks of the differentiation process were analyzed, and their results are shown in Fig. 3. In this analysis, steady state RNA levels of β-type globins (βh1, εy and β^{major}), transcription factors (GATA-1, Tal-1, and Id-1), and cytokine (SLF) were analyzed.

The transcripts for the embryonic βh1-globin were detected from 4th day EBs with the maximal expression at days 8 through 10, while another embryonic εy-globin started its expression at day 7 with the peak activity at days 8 through 11 (Fig. 3). On the other hand, adult type β-major globin RNA started its expression at day 6 but lasted longer than embryonic transcripts. Among the β-type globins in mouse, primitive erythrocytes arising early from the yolk sac at day 8 through 11 contain largely the embryonic type of globin (βh1 and εy) and a small quantity of adult type (β^{major} and β^{minor}) (Fantoni *et al.*, 1967). βh1- and εy-globins are expressed asynchronously, such that βh1-globin mRNA levels peak 2 days earlier than those of εy-globin in both yolk sac and peripheral blood (Whitelaw *et al.*, 1990). Definitive erythrocytes formed later in the fetal liver, spleen, and bone marrow were reported to contain only the adult type. These definitive cells first appear at day 11.5 in blood and at day 12.5 in fetal liver (Wong *et al.*, 1983). On the other hand, Whitelaw *et al.* (1990) reported that large amounts of εy-globin mRNA were also detected in fetal liver, reaching a peak at day 13.5, such that εy-globin mRNA was produced both in the yolk sac and in the fetal liver during mid-gestation. Thus, it appeared that the entire complement of mouse β-type globin genes is expressed in ES-derived EBs and that this ES cell differentiation system is capable of carrying out the switch from the embryonic to the fetal expression pattern. It should be noted again that the β-major globin RNA, in accordance with the efficiency of erythropoietic differentiation potential as described before, is not maintained at high levels at later stages (see below).

GATA-1 was expressed at low levels in undifferentiated ES cells and then underwent a rapid down-regulation to nearly undetectable levels at day 3 of differentiation. At days 4 to 6 of differentiation (which preceded slightly, but overlapped the peak of globin synthesis in these EBs), GATA-1 transcripts rose dramatically but were not maintained afterwards (Fig. 3). As one of the prominent GATA transcription factor family, GATA-1 has been known to be expressed at high levels in erythroid cells, mast cells, megakaryocytes (Barnhart *et al.*, 1989; Martin *et al.*, 1990; Romeo *et al.*, 1990) and Sertoli cell lineages in testis (Ito *et al.*, 1993), and are essential for normal primitive and definitive erythropoiesis (Pevny *et al.*, 1991, 1995; Weiss *et al.*, 1994). The relative expression profile of GATA-1 to the globins, in general, is quite well correlated to the other previous differentiation systems (Wiles and Keller, 1991; Keller *et al.*, 1993) and even to that of the developing mouse embryos (Whitelaw *et al.*, 1990). However, a finding that GATA-1 message is detectable in undifferentiated ES cells is consistent with the report of Lindenbaum and Grosveld (1990) but contrary to others (Simon *et al.*, 1992; Keller *et al.*, 1993). This controversy is a matter of debate, although the noted difference may be caused by the sensitivity of the assay and/or the culture condition; the pros come from EBs cultured in suspension medium while the cons from cultures in semisolid medium supplemented with cytokines. However, GATA-1 expressivity in undifferentiated ES cells may not be critical for the

normal development, because GATA-1 deficient ES cells could go through the onset of globin gene expression without difficulty (Kim, 1996). The disappearance of GATA-1 message at later stages of differentiation in suspension culture is also correlated to the findings described previously. Thus, CFC formation and globin expression seem to follow the timing and relative levels of GATA-1 expression, such that GATA-1 expression is required for the maintenance of the erythroid lineage.

The expression profiles of two helix-loop-helix (HLH) transcription factors, Tal-1 and Id-1, were also examined (Fig. 3). Both genes showed quite similar patterns of expression to that of GATA-1, with an exception of no expression of Tal-1 in undifferentiated ES cells. Tal-1 has been known to be expressed specifically in erythroid cells (Begley *et al.*, 1989) and megakaryocytes as well as basophilic granulocytes (Mouthon *et al.*, 1993), and it appears to be essential for embryonic blood formation *in vivo* (Shivdasani *et al.*, 1995). The homozygous Tal-1 knock-out mice showed complete embryonic lethality at embryonic day

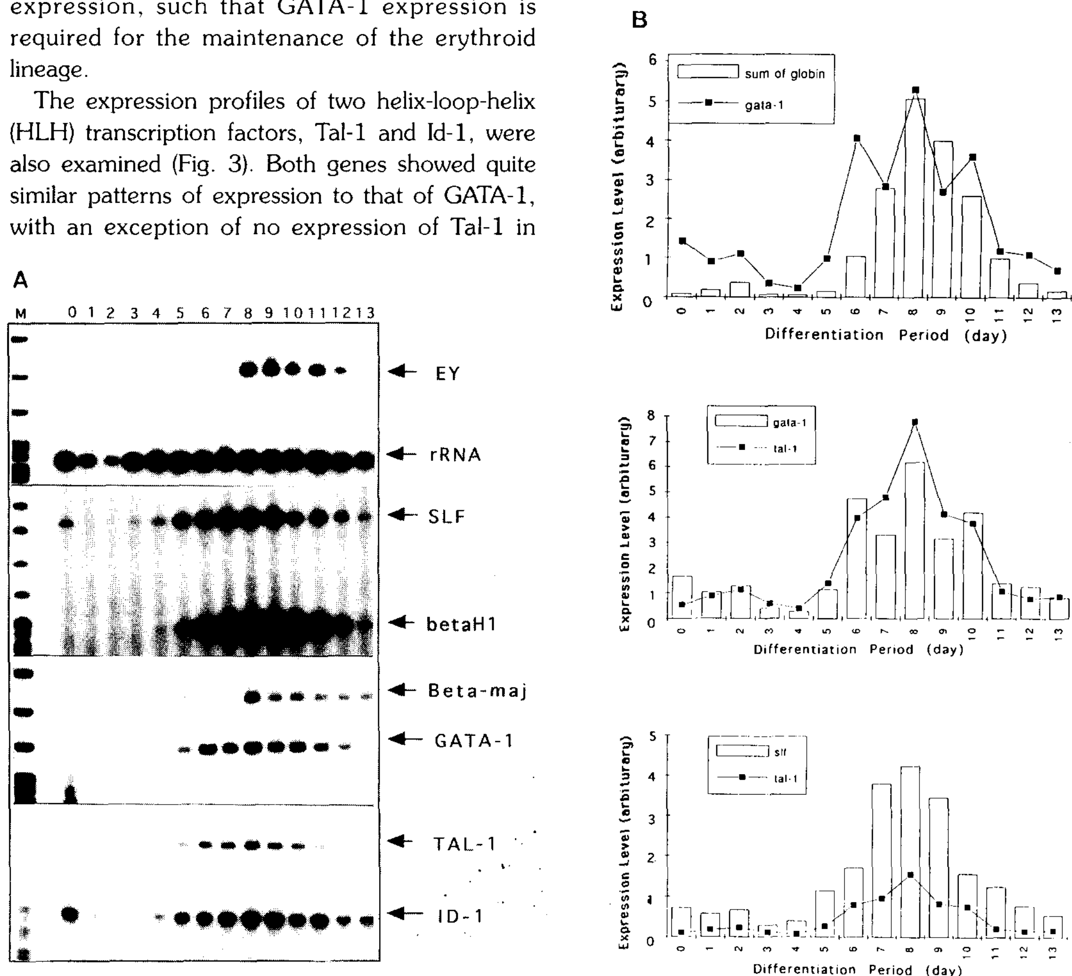

Fig. 3. Expression profiles of several erythroid marker genes in the developing Embryoid bodies. Total RNAs were isolated everyday for 2 weeks from pools of about 50 EBs in the culture. RT-PCR was performed as described in Materials and Methods. A. M and 0 to 13 at the top of the figure denote DNA molecular weight size marker (pBR322 plasmid MspI cut) and days of differentiation of ES cells *in vitro*, respectively. Letters at the righthand of the figure are genes that are tested for expression: EY, fetal β-type globin εy; rRNA, RLP7 gene coding for mitochondrial 12S rRNA; SLF, steel factor; betaH1, the earliest expressing fetal β-type globin βH1; Beta-maj, adult-type β globin; GATA-1, erythroid-specific transcription factor GATA-1; TAL-1, erythroid-specific transcription factor Tal-1; ID-1, helix-loop-helix transcription repressor Id-1. B. Each autoradiographic band in panel A was scanned by densitometer and then the values were normalized with those of rRNA at each corresponding day. Top panel, comparison between the levels of GATA-1 and the sum of globins; Middle panel, between GATA-1 and Tal-1; Bottom panel, between SLF and Tal-1.

10.5. Id-1 is known to be expressed in a variety of cell types (Benezra *et al.*, 1990), and it's expression was reported to increase slightly during day 9.5 to 15.5 of gestation (Riechmann *et al.*, 1994).

SLF (*steel* factor), a recently identified growth factor which is the gene product of the murine *steel* locus and a ligand for the *c-kit* tyrosine kinase receptor, started to express at day 3, increased over time and reached a comparatively high level till day 6 of differentiation (Fig. 3). A SLF-specific RNA signal was detected in 6.5- and 7.5-day egg cylinders, in the 6.5 day decidua, and in the 8.5 day yolk sac region and the developing embryo (Keller *et al.*, 1993). Thus, the SLF expression profile is also well matched to that of the normal mouse embryo. Furthermore, in results similar to those of Schmitt *et al.* (1991), low levels of SLF RNA in undifferentiated ES cells were detected and the amount of SLF-specific RNA fell to undetectable levels during the next 48 hrs of differentiation.

These data indicate that a reproducible erythropoietic differentiation program ensues, which is similar to that observed in the sophisticated two-step colony assay (Keller *et al.*, 1993), and even in early mouse embryos, demonstrating that this simple differentiation system is useful to dissect the molecular events of early erythropoiesis. Compared to the sophisticated and well defined 2 step differentiation system (Keller *et al.*, 1993), this system has a merit in its simplicity and ease of manipulation, while preserving reproducibility and efficiency.

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생쥐 배아에서의 초기 적혈구 분화를 재현할 수 있는 배아주 세포에 기초한 간단한 시험관내 분화체계

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현탁 배양액에서 생쥐 배아주 세포를 배아체(embryoid body, EB)로 분화시키는 간단한 시험관내 모델 체계가 초기 적혈구 분화 분석에 유용한 지의 여부를 조사하였다. 분화중인 배아체로부터 각 혈구계열 세포 유형이 만들어지는 지(분화능)의 여부는 혈도형성, benzidine 염색법 및 2단계 콜로니 분석법으로 조사하였고, 발생과 분화시기에 맞추어 적혈구 표지 유전자들이 발현되는 지(발현능)의 여부는 각 분화시기별 배아체로 부터 추출한 RNA를 RT-PCR 방법으로 조사하였다. 분석 결과, 다른 기존의 복잡한 분화 방법에 의한 것과 마찬가지로 모든 혈구계열 세포 유형이 반복성있게 유도되었다. 더구다나, 분화중인 배아주 세포에서의 글로빈 유전자 발현 전환은 생쥐 배아에서와 유사하게 진행되었으며, 글로빈 유전자의 발현은 적혈구-특이 전사인자인 GATA-1과 Tal-1 보다 적어도 12시간 늦게 활성화되었다. 이와같이 간단한 분화 체계에서도 적혈구 분화과정이 효율적으로 반복성 있게 나타나는 것으로 보아, 간단한 현탁배양에서의 배아체 분화는 초기 적혈구 분화과정의 분자적 기작을 분석하는데 유용하게 이용될 수 있으리라 본다.