stringent statistical conditions. Grey columns indicate no statistical differences.

Fig. 7. FACS analysis to check the content of CD19(+) cells

A. FACS analysis of PBMNC stained with anti-CD3 and anti-CD19 antibodies. B and C, FACS analysis of CD19(+)-depleted cell population (B) and CD19(+) cell population (C) stained with anti-CD19 antibody after MACS beads separation, respectively. Lower column indicates statistical analysis for each Region shown in figures.

Fig. 8. V(D)J recombination

PCR products were separated on a gel and the amplified V(D)J recombination products are shown. Fibroblasts were used as a recombination-negative control. Mononuclear cells derived from peripheral blood cells (PBMNCs) and $CD19^{+}$ cells (B lineage cells) were used as multiclonal recombination-positive controls. If the cell line is monoclonal, then a single band is generated by PCR.

Table 1 Mongoloid minority populations included in this study

Country	Location	Tribe	Other name	Latitude (degrees N)	Longitude (degree W)	B-LCL Established M	F	Total
Venezuela	1	Sanema (Sanumá)	Sanumá, Chirichano, Guaika, Samatali, Samatari, Sanima, Tsanuma, Xamatari	3.5	64.5	13	9	22
	2	Ye'Kuana	Maquiritari, Cunuana, Defcuana, Maiongong, Maquiritai, Maquiritare, Pawana, Soto, Ye fcuana	3.5	64.5	6	14	20
Colombia/Venezuela	3	Piaroa	Adole, Ature, Guagua, Kuakua, Quaqua	5	67	2	2	4
Colombia	4	Amorua;Guahibo	-	6	67.5	0	2	2
	5	Etnia Siquani;Guahibo	-	6	67.5	2	1	3
	6	Siquani;Guahibo	-	6	67.5	4	1	5
	7	Guahibo	Goahibo, Goahiva, Guaigua, Guajibo, Guayba, Sicuani, Sikuani, Wahibo	6	67.5	3	2	5
	8	Ticuna	Tikuna, Tucuna, Tukúna	4	69.5	6	14	20
	9	Wayu	Wayuu, Guajiro, Goajiro, Guajira, Uáira, Waiu, Wayúu, Wayuunaiki	11.5	72	36	62	98
	10	Wayuu Epiayu	-	11.5	72	0	2	2
	11	Wayuu Puchaina	-	11.5	72	1	1	2
	12	Wayuu Uriana	-	11.5	72	2	2	4
	13	Matapi	Yucuna, Yukuna	-1	69.5	1	0	1
	14	Miraña	Bora, Boro, Meamuyna	-1	69.5	0	1	1
	15	Cumbal	-	1	77	4	11	15
	16	Inga	Highland Inga	1	77	3	13	16
	17	Kamsa	Camsá, Camëntsëá, Coche, Kamemtxa, Kamse, Sibundoy	1	77	3	17	20
Ecuador	18	Cañar	Cañar Highland Quichua	-2.5	79	7	20	27
	19	Saraguro	Loja Quichua, Saraguro Quichua	-3.5	79.5	7	15	22
Bolivia	20	Aymara	-	(-16) - (-18.5)	68.5 - 69	15	13	28
	21	Chipaya	Puquina	-18.5	67.5	5	3	8
	22	Quechua	North La Paz Quechua	(-21.5) - (-21)	66 - 67.5	13	12	25
	23	Mestizo		(-20) - (-21)	65.5 - 66	2	1	3
Paraguay	24	Chaco (Lengua)	Enxet	-22.5	59.5	18	17	35
	25	Chaco (Nivaclé)	Ashlushlay, Axluslay, Chulupe, Chulupí, Chulupie, Churupí, Nivaklé	-22.5	59.5	9	2	11
	26	Chaco (Sanapaná)	Lanapsua, Quiativis, Quilyacmoc, Saapa, Sanam	-22.5	59.5	1	0	1
Chile	27	Atacama	Kunza, Atacameño, Likanantaí, Lipe, Ulipe	(-23) - (-23.5)	68	14	13	27
	28	Mapuche	Mapudungun, Araucano, Mapudungu	-39	71.5 - 72	26	33	59
	29	Huilliche	Huiliche, Veliche	-43	73	5	4	9
Argentine	30	Puna	-	-22.5	65.5	7	17	24
			Total			215	304	519

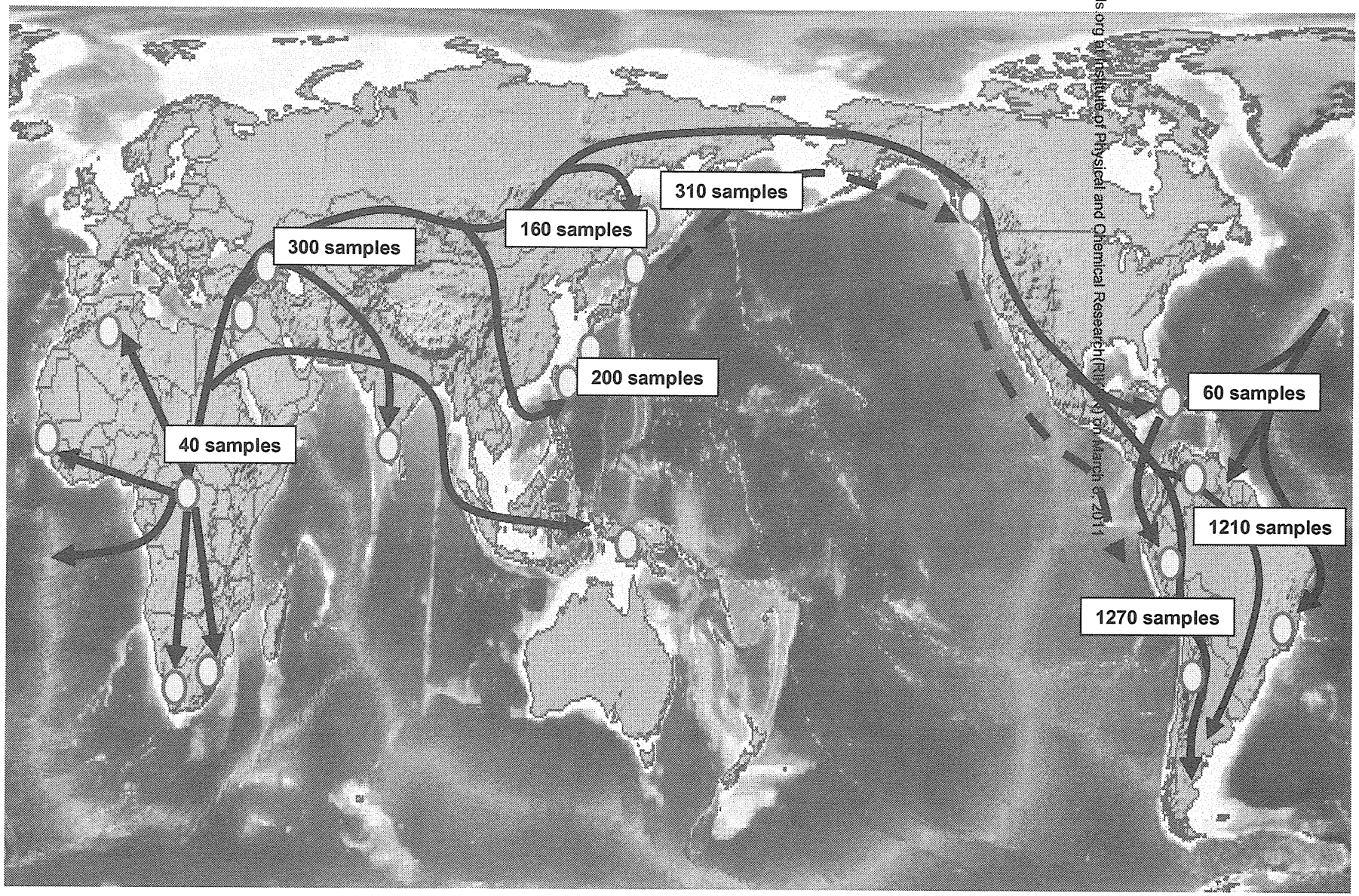
Other names were referred by Ethnologue country index (http://www.ethnologue.com/country_index.asp)
"-, not entried in Linguist group"

Table 2 Genomic loci where aberrations were detected

Chromosome	Cytoband	Start	Stop	pattern	Gene name
detected in all samples					
2	p11.2	88916534	89743016	deletion	immunoglobulin kappa J, V clusters
7	p14.1	38262501	38349233	amplification	T cell receptor gamma C, J, V clusters
14	q11.2	21485151	22050050	amplification	T cell receptor alpha V, J clusters
14	q32.33	105314054	106286079	deletion	immunoglobulin heavy chain V, D, J clusters
detected in over 5 samples					
7	q34	141988254	142202474	amplification	T cell receptor beta V, J clusters
16#$	q22.1	66945730	66967713	amplification/deletion	LOC100129324, Sphingomyelin Phosphodiesterase 3
22	q11.22	20717615	21576029	deletion	immunoglobulin lambda V, J clusters
detected in 2-4 samples					
2#	q35	218971508	218979039	amplification	MIRN26B, CTDSP1
3	p21.31	48577843	48600744	amplification	UCN2, COL7A1
detected only in Dann and PBMC					
5	q35.3	179147890	179177396	amplification	LTC4S, MGAT4B

#, aberration was not detected in B lymphocyte population
$, aberration was not detected in B-LCL established from adult Japanese

Figure 1



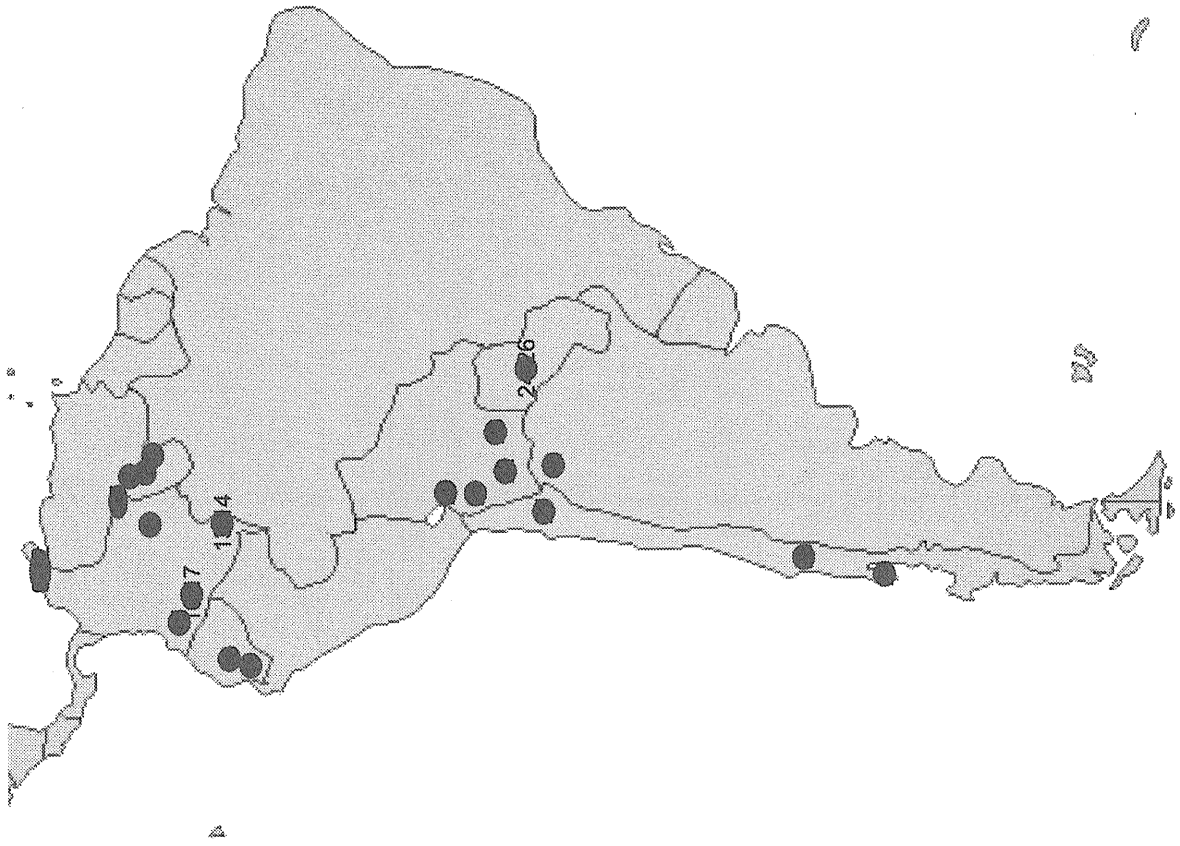
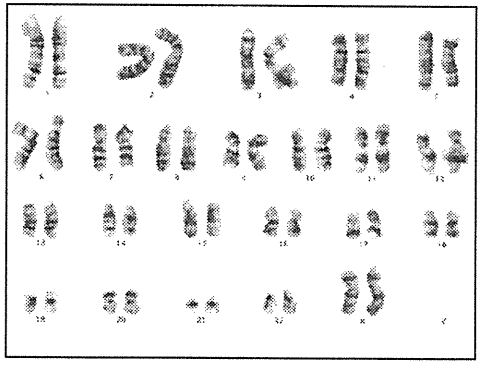


Figure 2

Figure 3

A



YAN3268

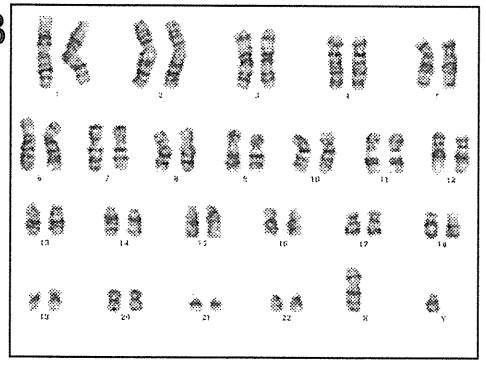
Mode of chromosome number

number of chromosomes	46			
number of analyzed cells	50			

Karyotype

46,XX [20]

B



WY084

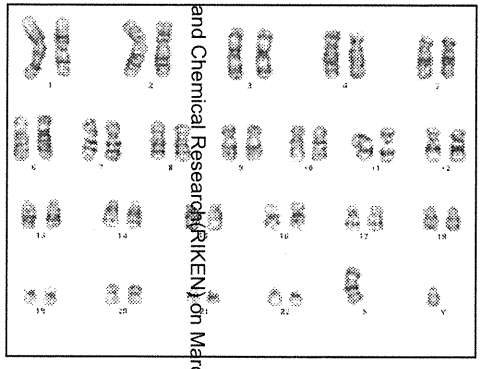
Mode of chromosome number

number of chromosomes	46	90	91	
number of analyzed cells	48	1	1	

Karyotype

46,XY [18]
90,XXYY,-6,-10 [1]
91,XXYY,-22 [1]

C



YAN3191

Mode of chromosome number

number of chromosomes	46	92		
number of analyzed cells	46	4		

Karyotype

46,XY,1qh+(presumable) [20]

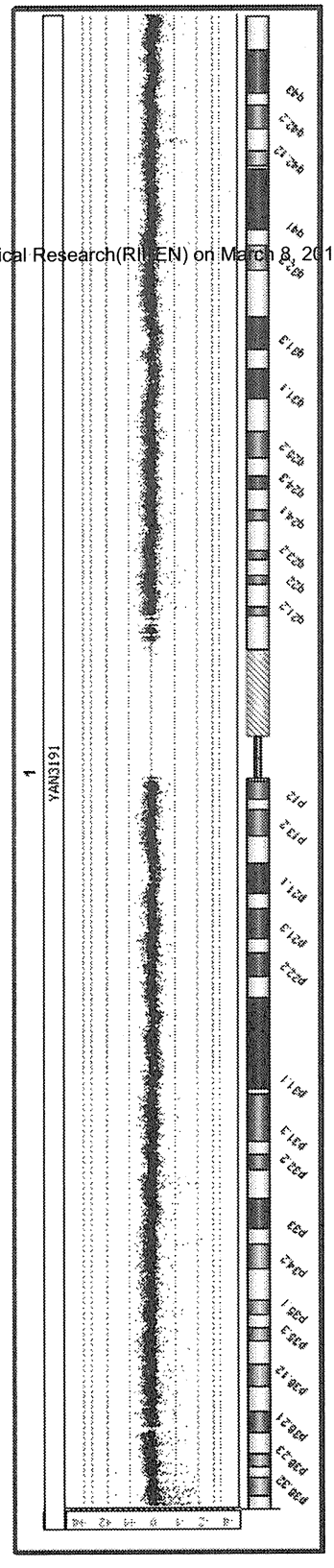


Figure 4

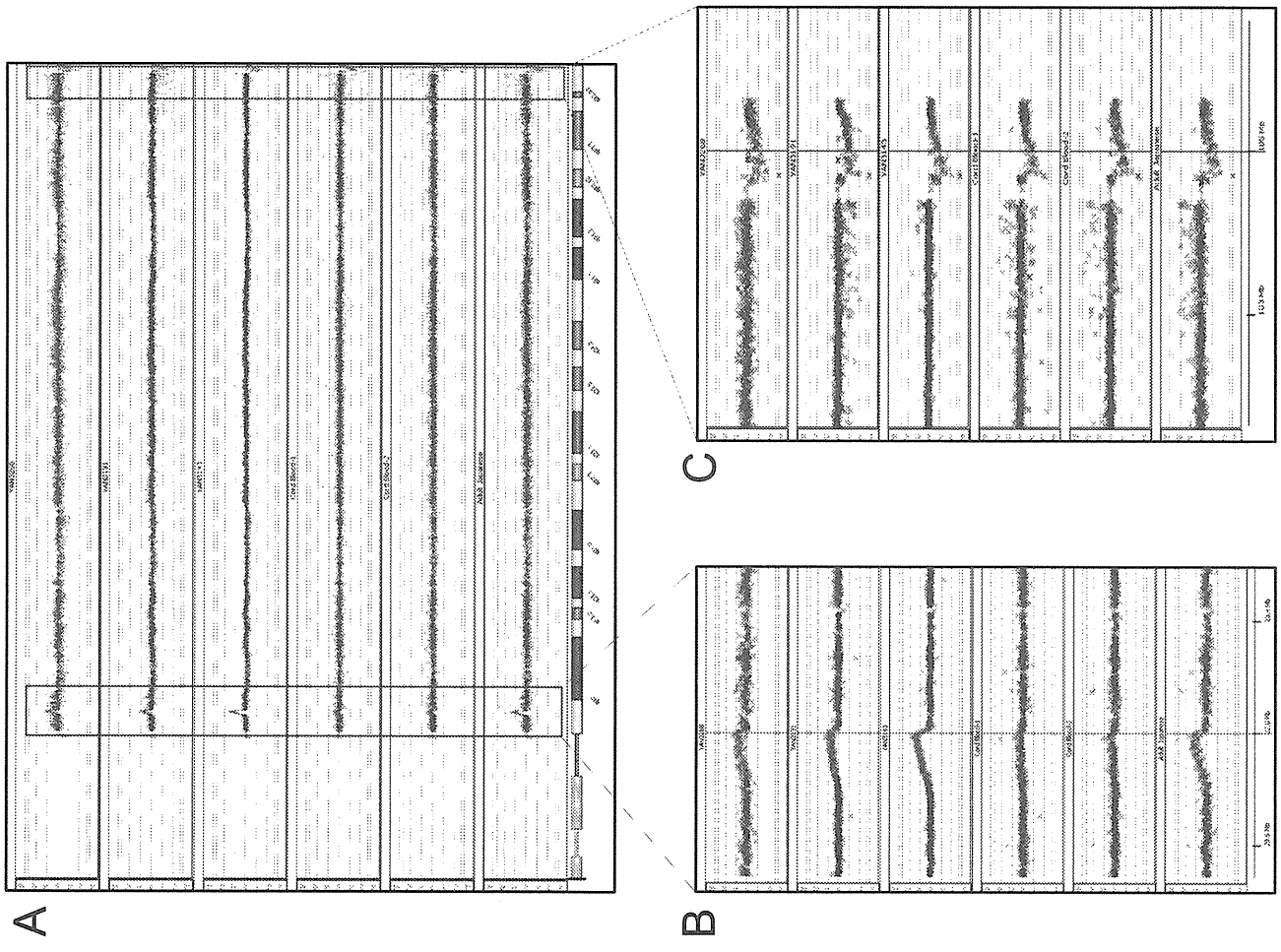


Figure 5

Figure 6

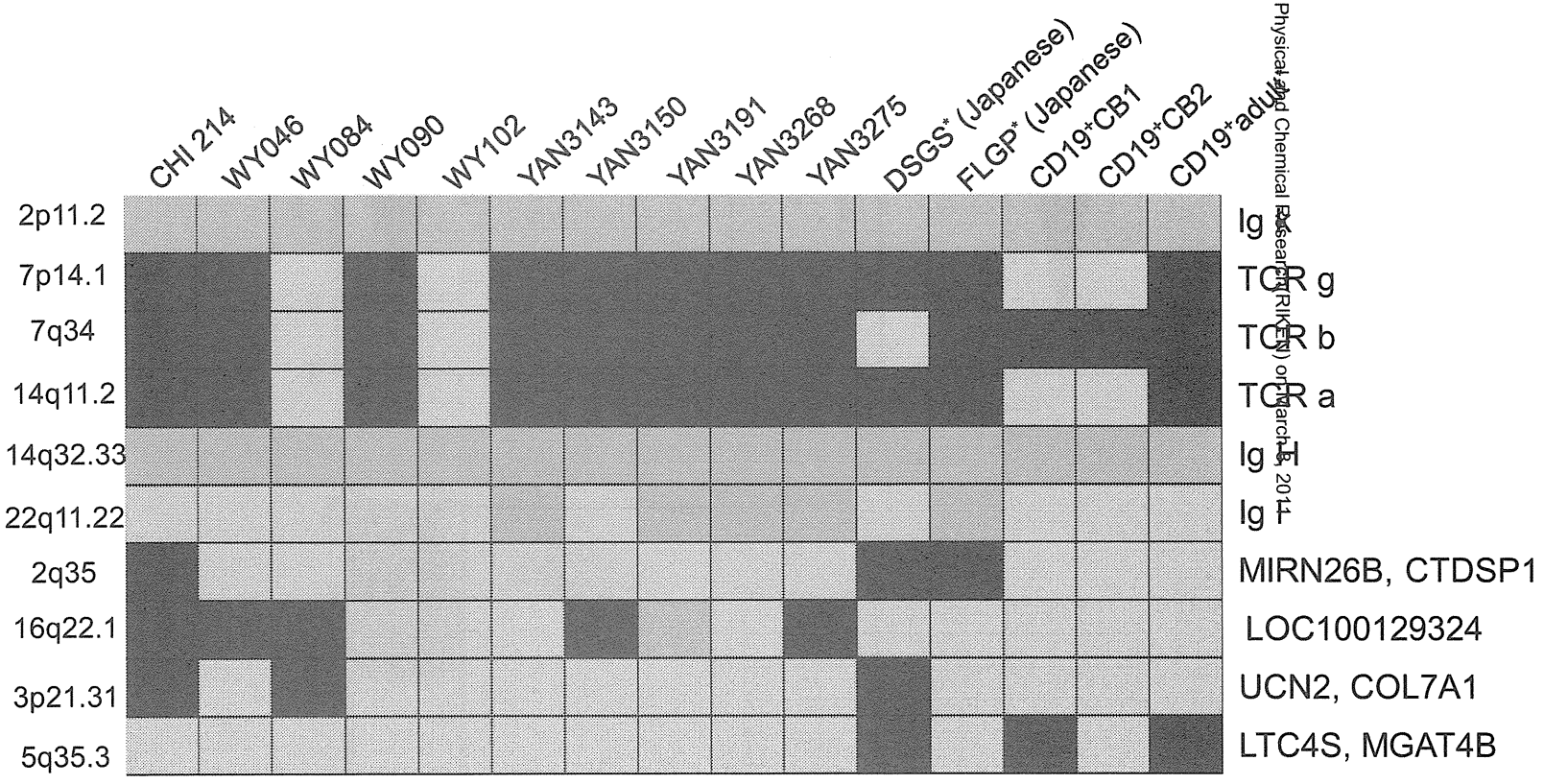
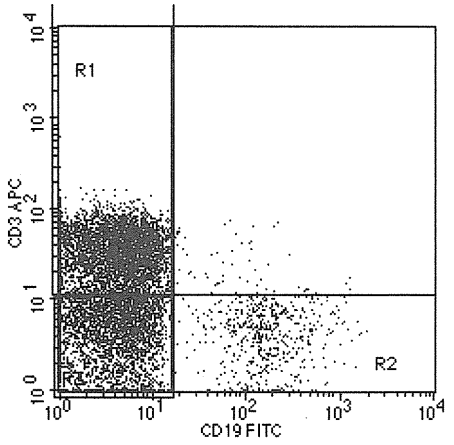


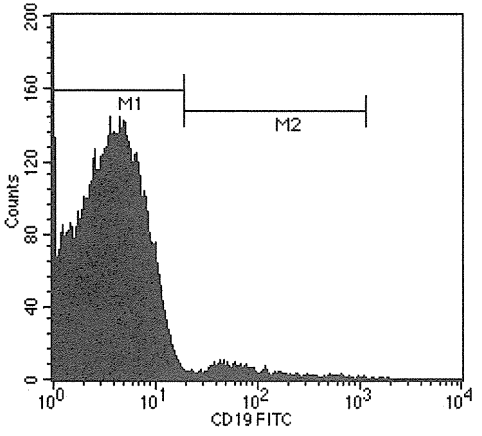
Figure 7

A



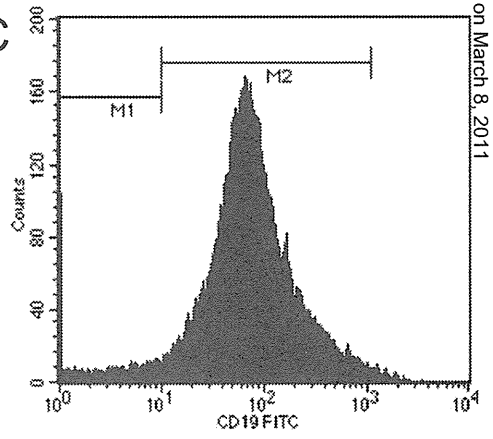
Label	Events	% Total
All	30000	100
R1	16019	53.4
R2	2024	6.75
R3	11468	38.23

B



Label	Events	% Total
All	30000	100
M1	29095	96.98
M2	897	2.99

C



Label	Events	% Total
All	30000	100
M1	2362	7.87
M2	27515	91.72

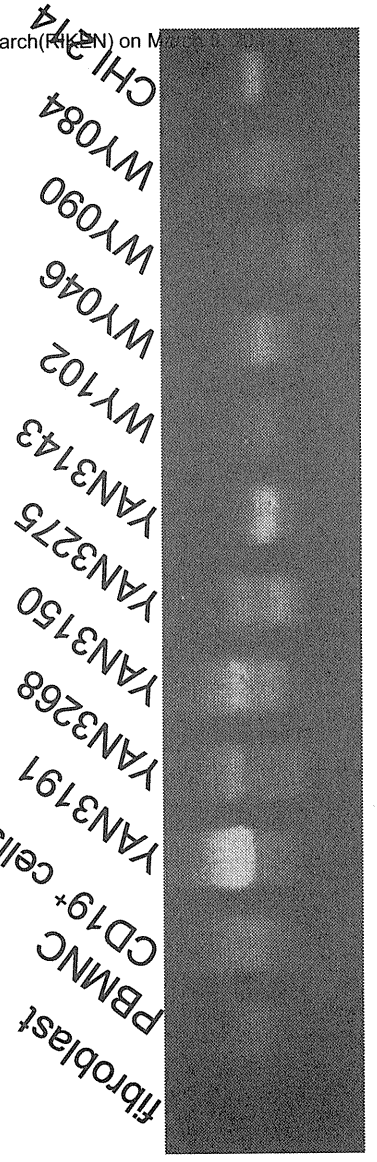


Figure 8

15

ES Cell-derived Erythroid Cell Lines Able to Produce Mature Red Blood Cells

Yukio Nakamura
Cell Engineering Division, RIKEN BioResource Center
Japan

1. Introduction

Transfusion therapies involving red blood cells (RBCs), platelets, and neutrophils depend on the donation of these cells from healthy volunteers. However, unpredictable adverse results can ensue from transfusion therapies because of the donation of cells from a very large number of anonymous volunteers. For example, transfusion of blood products that include hazardous viruses or prions is difficult to prevent completely because, occasionally, tests to detect them yield pseudo-negative results. This comment is, of course, not intended as a criticism of the current system that is dependent on volunteers who act as blood donors from their own good will. However, there is little doubt that RBCs, platelets, and neutrophils produced in vitro might be a preferable means of producing such cells, thereby reducing, or even eliminating, the need for a large pool of anonymous donors. To date, however, the use of hematopoietic cells produced in vitro has not proved practical for routine therapeutic applications.

RBC transfusion was the first transplantation procedure to be established and is now routine and indispensable for many clinical purposes. However, in many countries the supply of transfusable materials is not always sufficient. In Japan, for example, the supply of RBCs with an AB/RhD(-) phenotype is always lacking because individuals with this RBC phenotype are rare. This problem of inequalities in the supply and demand for RBCs has stimulated interest in the development of in vitro procedures for the generation of transfusable and functional RBCs from hematopoietic stem cells or progenitor cells present in bone marrow or umbilical cord blood (Figure 1) (Neildez-Nguyen et al., 2002; Giarratana et al., 2005; Miharada et al., 2006; Douay and Andreu, 2007).

In addition, it is important to realize that clinical risk factors associated with RBC transfusions have not been entirely excluded. One notable and very severe complication of the procedure can be transfusion-related acute lung injury (TRALI), which has only recently been recognized and has not yet been eliminated (Silliman et al., 2009; Looney et al., 2010). One of the possible causes of TRALI may be a factor in the transfused materials, such as antibodies in the transfused materials against antigens on the leukocytes. This type of adverse outcome also results from the dependence of blood transfusion on the supply of blood from a large number of anonymous individuals. Problems may arise if donated blood is utilized without sufficient preliminary trials being carried out on each sample. The use of RBCs derived from selected human resources may help to alleviate these problems, since they can be intensively tested for pathogens before clinical use. Trial transfusions of a minimal amount of material into each

recipient could also be performed to determine if there are unexpected complications. Therefore, the establishment of resources for in vitro production of RBCs (Figure 1) will provide a means to alleviate many problems associated with RBC transfusion.

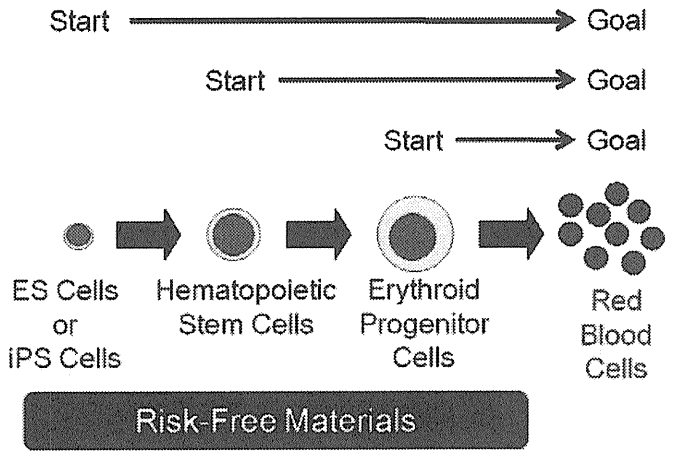


Fig. 1. A summary of some of the ways in which RBCs could be produced in vitro.

2. RBC production from hematopoietic stem cells

The hematopoietic stem cells present in bone marrow and umbilical cord blood are promising materials for in vitro production of RBCs and this has stimulated interest in the development of in vitro procedures for the generation of functional RBCs from these tissues (Neildez-Nguyen et al., 2002; Giarratana et al., 2005; Miharada et al., 2006). Umbilical cord blood cells are of particular interest as they are readily available but are usually discarded. Provided the mother of a neonate consents to use of the umbilical cord blood, this material can provide a useful resource without any further complicating critical or ethical concerns.

It was reported that human erythroid cells (nucleated cells) produced on a large scale ex vivo could differentiate in vivo into enucleated RBCs (Neildez-Nguyen et al., 2002). This study demonstrated that erythroid progenitor cells produced in vitro from hematopoietic stem and progenitor cells could have a clinical application as an alternative method for transfusing terminally differentiated RBCs. More recently, the same group described an ex vivo methodology for producing fully mature human RBCs from hematopoietic stem cells (Giarratana et al., 2005). The enucleated RBCs produced by this approach are potentially even more valuable as they should be functional immediately after transfusion without requiring the long latency period for enucleation normally necessary for erythroid cells.

The mechanism of erythroblast enucleation, a critical step in RBC production, has not yet been fully elucidated (Lee et al., 2004; Kingsley et al., 2004). The role of interactions between erythroblasts and other cells, such as macrophages, in this process is a controversial topic (Ohneda and Bautch, 1997; Yanai et al., 1997; Hanspal et al., 1998; Iavarone et al., 2004; Spike et al., 2004). Macrophages in retinoblastoma gene (Rb)-deficient embryos are unable to physically interact with erythroblasts and RBC production is impaired in these embryos (Iavarone et al., 2004). In addition, in vitro production of enucleated RBCs from immature

hematopoietic stem/progenitor cells proceeds efficiently in the presence (Giarratana et al., 2005) but not in the absence (Neildez-Nguyen et al., 2002) of feeder cells.
Of note, however, enucleation can apparently be initiated in vitro in erythroblasts that have been induced to differentiate in vivo to a developmental stage that is competent for nuclear self-extrusion (Spike et al., 2004; Yoshida et al., 2005). Moreover, we have developed a method to produce enucleated RBCs efficiently in vitro without use of feeder cells (Figure 2) (Miharada et al., 2006). The culture system has allowed erythroid cells to differentiate to a developmental stage competent for nuclear self-extrusion (Miharada et al., 2006). Taken together, although it has generally been thought that efficient enucleation of erythroblasts is largely dependent on signals mediated by cells in their local environment (Ohneda and Bautch, 1997; Yanai et al., 1997; Hanspal et al., 1998; Iavarone et al., 2004), the interaction of erythroblasts with other cells is not necessary for efficient erythroblast enucleation (Miharada et al., 2006). Signals mediated by humoral factors appear to be sufficient for the efficient autonomous completion of erythroblast enucleation.

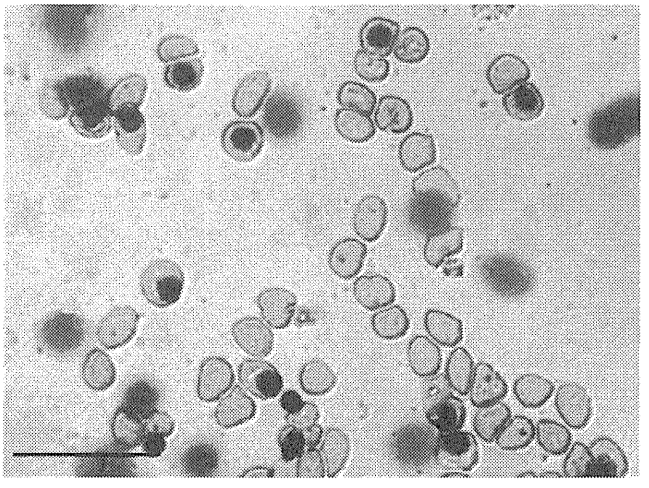


Fig. 2. Enucleated RBCs produced in vitro from hematopoietic stem cells. Scale bar indicates 50 μm.

Since culture without the use of feeder cells is technically easier and less expensive, the method we established (Miharada et al., 2006) has the potential to be a cost-effective means of producing transfusable RBCs on a large scale from immature hematopoietic stem/progenitor cells. Currently, however, cost factors means that it is not yet realistic to produce RBCs on a large scale, approximately 200 ml or more, using our in vitro culture system. In particular, patents on the growth factors used in the culture system are a major obstacle, because these growth factors are very expensive, at least at the moment. After the relevant patents expire, our in vitro culture system will become a more realistic scenario.

3. RBC production from ES cells

ES cells possess the potential to produce various differentiated cells able to function in vivo and thus represent another promising resource for RBC production. Furthermore, since ES

cell lines are immortalized, they can be used repeatedly and have potential to produce abundant differentiated cells in the quantities required for clinical use. However, it will be important to carry out routing screening of the ES cell lines for de novo chromosomal aberrations and/or genetic mutations that may arise in vitro, before these long term cell cultures are applied in the clinic. Unsurprisingly, there is now a widespread and enthusiastic debate on standardization of the characteristics of ES cells for regenerative medicine protocols that exploit these cell lines. In my opinion, since chromosomal aberrations and genetic mutations are inevitable in long term cell cultures, only ES cell lines that have been cultured for a limited period, e.g., less than 30 passages, should be selected for clinical use.

Hematopoietic cells, including those in the erythroid lineage, have been generated from mouse ES cells (Keller et al., 1993: Nakano et al., 1994; Nakano et al., 1996; Carotta et al., 2004), non-human primate ES cells (Li et al., 2001; Umeda et al., 2004; Kurita et al., 2006), and human ES cells (Kaufman et al., 2001; Chadwick et al., 2003; Cerdan et al., 2004; Vodyanik et al., 2005; Wang et al., 2005; Olivier et al., 2006). We have also established a long-term in vitro method for culturing hematopoietic cells derived from ES cells of the non-human primate, the common marmoset (Hiroyama et al., 2006). Recently, abundant production of enucleated RBCs from human ES cells was reported (Lu et al., 2008).

Taken together, we can now produce mature RBCs by in vitro culture of ES cells or the hematopoietic stem/progenitor cells present in umbilical cord blood. In practice, however, the efficiency of RBC generation varies with the quality of the ES cell line or the umbilical cord blood sample. Since ES cell lines can be utilized repeatedly, derivation of RBCs from ES cells appears to be more practical. However, even with optimal experimental procedures and the most appropriate ES cell line the generation of abundant RBCs directly from primate ES cells is a costly and time-consuming process (Hiroyama et al., 2006; Lu et al., 2008). If human erythroid progenitor cell lines can be established that have efficient production of mature RBCs, they would provide a much more useful resource than ES cell lines.

4. Establishment of mouse RBC progenitor cell lines

Several mouse and human erythroid cell lines have been established. However, to the best of our knowledge, there is no cell line that can efficiently differentiate into enucleated RBCs. For example, the human erythroid cell line K562, derived from chronic myelogenous leukemia cells, can differentiate to mature erythroid cells and produce haemoglobin but cannot produce enucleated RBCs.

It is generally difficult to establish hematopoietic cell lines from adult hematopoietic stem or progenitor cells, since these somatic cells are quite sensitive to DNA damage and are unable to maintain the lengths of their telomere repeats on serial passage (Lansdorp, 2005). In contrast, ES cells are relatively resistant to DNA damage and maintain telomere length on serial passage (Lansdorp, 2005). Therefore, we speculated that these characteristics of ES cells might be advantageous for the establishment of cell lines, since differentiated cells derived from ES cells should retain these beneficial characteristics. In addition, mouse cells tend to immortalize more readily than human cells. Hence, we attempted to evaluate the feasibility of establishing hematopoietic cell lines, erythroid cell lines in particular, from mouse ES cells.

4.1 Establishment of RBC progenitor cell lines from mouse ES cells

To induce differentiation of hematopoietic cells from mouse ES cells, we cultured ES cells using OP9 feeder cells (Nakano et al., 1994; Nakano et al., 1996; Kodama et al., 1994) in the presence of specific factors (Hiroyama et al., 2008). OP9 cells were used not only for induction of hematopoietic differentiation but also for establishment of cell lines in the early phase of long term culture of the induced hematopoietic cells (Hiroyama et al., 2008). In most cases, the induced cells stopped proliferating within two months of the initial induction of differentiation from ES cells (Hiroyama et al., 2008). This phenomenon is similar to that observed in primary culture of human cells such as fibroblasts, the so-called "crisis" of primary cells. In general, normal human cells cannot bypass this crisis stage and thus it is impossible to obtain immortalized cells from normal cells by continuous culture alone.

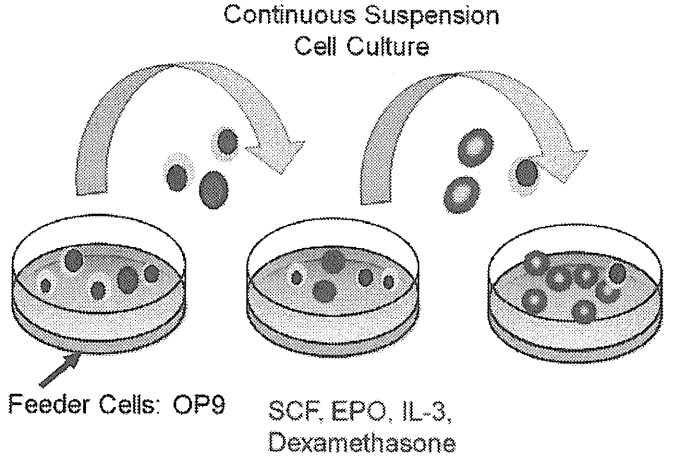


Fig. 3. A diagrammatic outline of the method to establish erythroid cell lines from ES cells.

Induced cells that could proliferate continuously for approximately two months (60 days) were cultured in the absence of OP9 cells and in the presence of hematopoietic humoral factors (Figure 3). Cells that continued to proliferate in the absence of OP9 cells were selected to establish cell lines. These cell lines acquired independency from OP9 cells within three months of the initial induction of differentiation from ES cells (Hiroyama et al., 2008). At approximately four months after initial induction, we sought to determine the factor(s) that were essential for the proliferation of each cell line. After this evaluation, each cell line was cultured in the presence of these essential factor(s) alone with changes of the medium every two or three days.

In addition to the method described in Figure 3 (Method A), we developed a second protocol (Method B) identical to Method A but omitting IL-3 at all stages. We attempted long term cultures of 63 lines, 51 with Method A and 12 with Method B. Five independent immortalized cell lines were successfully established, 4 with Method A and 1 with Method B (Hiroyama et al., 2008). These five cell lines continued to proliferate for more than a year. Morphological and flow cytometric analyses suggested that three of the lines were erythroid in nature, while the other two were mast cell-like (Hiroyama et al., 2008). We designated the erythroid cell lines MEDEP (mouse ES cell-derived erythroid progenitor line) and the mast

cell-like cell lines MEDMC (mouse ES cell-derived mast cell line). MEDEP-E14, MEDEP-BRC4, and MEDEP-BRC5 were derived from E14TG2a (129 strain), BRC4 (C57BL/6N strain), and BRC5 (C57BL/6N strain) mouse ES cell lines, respectively. The presence of IL-3 in the culture medium (Method A) may not be necessary for the establishment of erythroid cell lines, as we were able to establish one erythroid line, MEDEP-BRC4, following culture of the cells in the absence of IL-3 (Method B) (Hiroyama et al., 2008). MEDEP cells could proliferate from single cells following sorting by flow cytometry, i.e., cloning was possible.
All three MEDEP cell lines retained the morphological characteristics of erythroid cells and also showed cytokine dependency after cloning (Hiroyama et al., 2008). MEDEP-E14 and MEDEP-BRC5 were dependent on erythropoietin (EPO) and stem cell factor (SCF), respectively (Hiroyama et al., 2008). Although MEDEP-BRC5 appeared to respond to EPO, it could not proliferate long term in the presence of EPO alone. MEDEP-BRC4 proliferated most efficiently in the presence of SCF, EPO and dexamethasone (Hiroyama et al., 2008). The cytokine dependency of these MEDEP cell lines has not changed since they were induced to differentiate from ES cells more than a year ago.
RT-PCR analyses demonstrated that all MEDEP lines expressed genes specific for erythroid cells: GATA-1, EKLF (Erythroid Krüppel-like factor) and EPOR (erythropoietin receptor) (Hiroyama et al., 2008). In addition, all MEDEP lines expressed α- and β-globin, but not γ-, ε-, or ζ-globin (Hiroyama et al., 2008), indicating that they were adult and not primitive erythroid progenitor cells. Since it has been reported that definitive erythropoiesis can be induced in mouse ES cells, i.e., the induction of adult type erythroid cells (Nakano et al., 1996), all MEDEP lines appear to be derived from adult type erythroid progenitor cells.

4.2 In vitro differentiation of MEDEP

Next, we evaluated the potential of MEDEP cells to differentiate into more mature erythroid cells and found that the various lines could be induced to differentiate by the following treatments: MEDEP-E14 by deprivation of EPO; MEDEP-BRC5 by deprivation of SCF and addition of EPO; and, MEDEP-BRC4 by deprivation of SCF and dexamethasone and addition of EPO (Hiroyama et al., 2008). EPO appeared to be necessary for MEDEP-BRC5 and MEDEP-BRC4 cells to maintain cell viability during the differentiation process.
The three MEDEP lines exhibited differential expression of TER119 (a cell surface antigen specific for mature erythroid cells) and CD71 (transferrin receptor). For example, expression of CD71 was slightly higher in MEDEP-E14 cells than in MEDEP-BRC5 cells (Hiroyama et al., 2008). $TER119^{-}CD71^{-}$ cells differentiate first to $TER119^{-}CD71^{+}$ cells, subsequently to $TER119^{++}CD71^{+}$ cells, and then finally to $TER119^{+}CD71^{-}$ cells (Miharada et al., 2005). Consistent with the differences in their cytokine dependency, the three MEDEP cell lines appeared to represent different stages of erythroid differentiation. Nevertheless, after induction of differentiation in vitro by the methods described above, expression of TER119 and CD71 in each of the MEDEP lines exhibited a pattern consistent with a more mature lineage (Hiroyama et al., 2008). This expression pattern suggests that each of the three lines was able to differentiate into a more mature lineage. At present, the cause of the variability between MEDEP cell lines remains uncertain. However, these results clearly demonstrated that erythroid progenitor cells could be immortalized at different stages of their differentiation.
Of note, the vast majority of cells in each MEDEP line could differentiate into more mature cells, although each MEDEP line included cells possessing abnormal karyotypes (Hiroyama et al., 2008). This result strongly suggested that cells with abnormal karyotypes still retained

the potential to differentiate into more mature erythroid cells. In general, most immortalized cell lines are not necessarily homogenous in many aspects such as karyotype, genotype, phenotype etc., even after cloning. The emergence of cells possessing different characteristics is often observed following long term utilization of immortalized cell lines. Hence, periodic recloning and selection of cell lines is recommended to maintain cell cultures with desirable characteristics for clinical application.

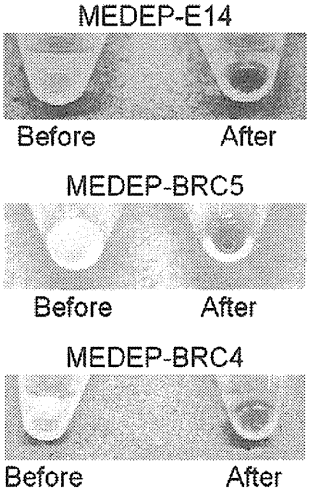


Fig. 4. MEDEP cell pellets show different coloration before and after differentiation.

Following induction of differentiation of MEDEP cells in vitro, the cell pellets collected after centrifugation appeared red while those before differentiation were white (Figure 4). In addition, following differentiation, enucleated cells could be identified by flow cytometric analysis using SYTO85 staining (Hiroyama et al., 2008). Morphological analysis confirmed that enucleated RBCs were present in addition to very mature erythroblasts (Figure 5).

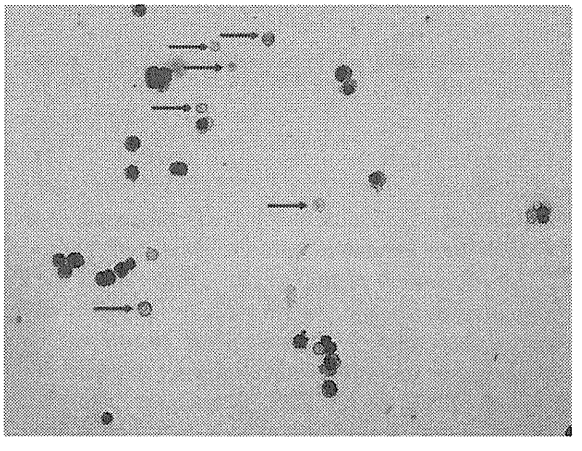


Fig. 5. Enucleated RBCs produced from MEDEP cells. Arrows indicate enucleated cells.

4.3 In vivo proliferation and differentiation of MEDEP

To evaluate the functional potential of MEDEP cells in vivo, we established a subline of MEDEP-E14 expressing the GFP marker Venus (Nagai et al., 2002). Although the expression of TER119 was slightly higher in MEDEP-E14-Venus cells than in parent MEDEP-E14 cells, the MEDEP-E14-Venus cells retained the ability to proliferate and differentiate into more mature erythroid cells in vitro (Hiroyama et al., 2008).

In general, the ablation of endogenous hematopoietic cells in mice is required to allow efficient detection of transplanted hematopoietic cells. Acute anemia induced by phlebotomy or hemolysis is commonly used in the study of urgent erythropoiesis (Alter et al., 1982; Miharada et al., 2005). We induced acute anemia in mice by intraperitoneal injection of phenylhydrazine, an inducer of hemolysis, and transplanted MEDEP-E14-Venus cells ($2x10^7$ cells/mouse) 24 hours later. Three days after transplantation, Venus-positive cells were present in the bone marrow and spleen (Hiroyama et al., 2008). Since the spleen is the major organ supporting urgent erythropoiesis in mice (Miharada et al., 2005), the transplanted cells were more abundant in the spleen than the bone marrow (Hiroyama et al., 2008). Venus-positive cells (the transplanted cells) demonstrated a phenotype consistent with differentiation into more mature erythroid cells compared to their phenotype just before transplantation (Hiroyama et al., 2008). Of note, MEDEP-E14-Venus cells differentiated into much more mature lineages in vivo than they did in vitro (Hiroyama et al., 2008).

To investigate whether transplanted cells could proliferate in vivo, we determined the absolute number and proportion (%) of Venus-positive cells in the spleen in a cell transplantation experiment. The absolute number of Venus-positive cells was elevated approximately two fold at three days compared to one day after cell transplantation (Hiroyama et al., 2008). This result indicates that transplanted cells can proliferate in vivo.

The expression of Venus in the transplanted cells decreased following their differentiation, i.e., the expression of Venus was lower in $TER119^{++}$ cells than in $TER119^{+}$ cells (Hiroyama et al., 2008). Thus, although we could not detect Venus-positive cells in peripheral blood, this was likely the result of disappearance of Venus following terminal differentiation. We therefore sought to confirm that MEDEP cells could differentiate into terminally-differentiated RBCs in vivo.

4.4 Increase of RBC number in mice suffering from acute anemia following transplantation of MEDEP

MEDEP cells ($2x10^7$ cells/mouse) were transplanted 24 hours after induction of acute anemia. As a control experiment, MEDMC cells ($2x10^7$ cells/mouse) were transplanted into control mice. Since $2x10^7$ transplanted RBCs correspond to a mere 2 μl of transfused cells, the number of RBCs in the transplanted mice will only increase if these transplanted MEDEP cells proliferate to some degree and differentiate into terminally-differentiated RBCs in vivo. Five days after transplantation, blood cell counts were performed in peripheral blood. The transplantation of MEDEP-E14 significantly ameliorated anemia compared to the control (Figure 6). The data obtained from the mice transplanted with control cells did not differ significantly from the data obtained from anemic mice that were not transplanted with any cells.

Since the RBC count in peripheral blood reflects the number of enucleated cells, whereas erythroblasts (nucleated cells) are included in the count of white blood cells (WBC), the

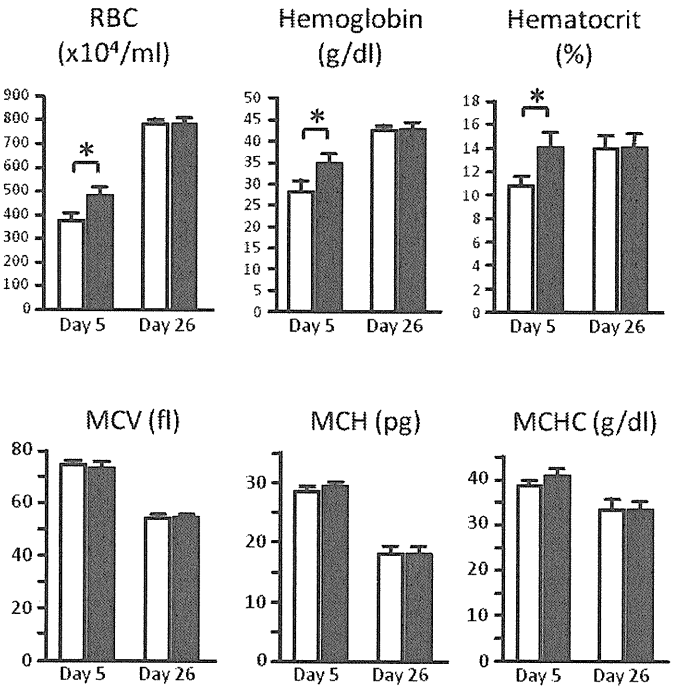


Fig. 6. Blood counts in control and MEDEP transplanted mice. White bars and red bars indicate mice transplanted with control cells and MEDEP cells, respectively. Asterisks indicate statistically significant differences ($P<0.05$).

increased number of RBC observed in mice transplanted with MEDEP cells indicated that the transplanted MEDEP cells could efficiently differentiate into enucleated cells (Hiroyama et al., 2008). The life span of RBCs is approximately 50 days in the mouse; therefore, it is highly likely that the RBCs produced from the transplanted MEDEP cells accumulated in the transplanted mouse.

Increases in mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and MCH concentration (MCHC) (Figure 6) are commonly observed in the recovery phase of acute anemia (Miharada et al., 2005). In addition, an increase in the number of WBC is observed in the recovery phase of acute anemia (Hiroyama et al., 2008) and is due to the presence of erythroblasts in the peripheral blood, since erythroblasts are counted as WBC by the automatic counter (Miharada et al., 2005). Given that there was no difference in MCV, MCH and MCHC levels between the transplanted and control mice in the recovery phase of acute anemia (Figure 6), RBCs derived from MEDEP cells in vivo appeared to possess characteristics similar to those derived from erythroid progenitor cells in the host mice. Twenty-six days after transplantation (27 days after the induction of acute anemia), all mice had recovered from the anemia and there were no differences in the blood counts of the two groups (Figure 6).

The transplantation of MEDEP-E14-Venus and MEDEP-BRC5 cells also ameliorated anemia compared to the control (Hiroyama et al., 2008). However, the transplantation of MEDEP-