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In vitro Production of Transfusable Red Blood Cells

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Abstract

Unpredictable adverse results can ensue from blood transfusion therapies because of the donation of cells from a very large number of anonymous volunteers. There is little doubt that transfusable blood cells produced on a large scale *in vitro* would be candidate materials to replace cells donated from such a large group of anonymous individuals. The recent progress of technology for *in vitro* production of transfusable red blood cells is reviewed.

Introduction

Hematopoietic cells, including hematopoietic stem and progenitor cells and terminally differentiated cells, are utilized in a range of clinical therapies. As the supply of these cell types is limited, the *in vitro* expansion of hematopoietic cell numbers would be very beneficial. One of the potential clinical applications for *in vitro* expanded cells is bone marrow reconstitution therapy following chemotherapy and/or radiotherapy of hematopoietic malignancies and other malignancies of solid organs, in a similar fashion to ordinary bone marrow transplantation and umbilical cord blood transplanta-

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Abbreviations: BSA, bovine serum albumin; EPO, erythropoietin; ES, embryonic stem; FCS, fetal calf serum; Flt3-L, Flt3 ligand; IGF-I, insulin-like growth factor-I; IGF-II, insulin-like growth factor-II; IL-3, interleukin-3; iPS, induced pluripotent stem; MAP, mixture of mannitol, adenine and phosphate; MEDEP, mouse ES cell-derived erythroid progenitor; MHC, major histocompatibility; NOD/SCID mice, non-obese diabetic/severe combined immunodeficient mice; MSC, mesenchymal stromal cells; RBC, red blood cell; SCF, stem cell factor; TNF-α, Tumor necrosis factor-α; TPO, thrombopoietin; TRALI, transfusion-related acute lung injury; VEGF, vascular endothelial growth factor

tion. In light of the potential benefits, many studies have been undertaken to identify *in vitro* culture systems that can be used to successfully expand hematopoietic stem and progenitor cells (Aglietta *et al.*, 1998; Ziegler and Kanz, 1998; Pei, 2002; Takagi, 2005; Hofmeister, 2007).

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 volunteers who donate the hematopoietic cells. However, there is little doubt that RBCs, platelets, and neutrophils produced *in vitro* would be candidate materials to replace cells donated from such a large group of anonymous individuals. To date, the use of hematopoietic cells produced *in vitro* has not proved practical for routine therapeutic applications.

RBC transfusion

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 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)
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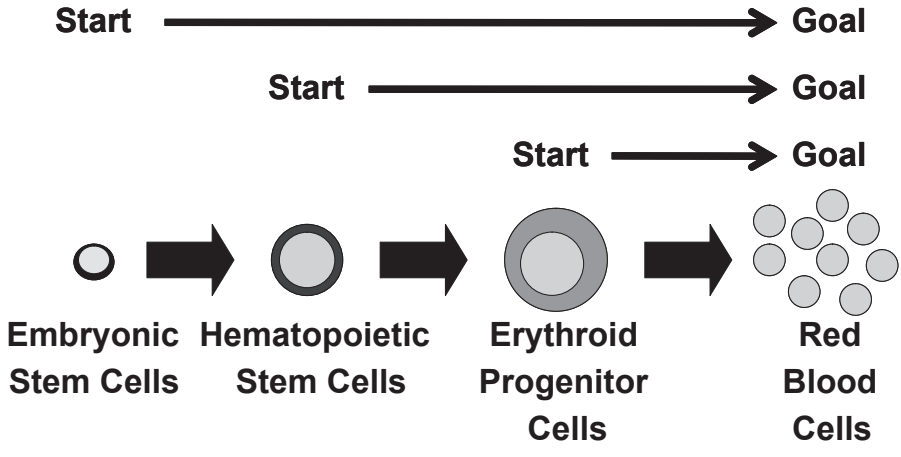

Figure 1. *In vitro* production of red blood cells from embryonic stem cells, hematopoietic stem cells, and erythroid progenitor cells.

Risk of RBC transfusion

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 transfusion is transfusion-related acute lung injury (TRALI), which has only recently been recognized and has not yet been eliminated. One of the possible causes of TRALI may be a factor in the transfused materials, such as antibodies against antigens on the leukocytes of the recipient. As mentioned above, this type of adverse outcome results from the dependence of blood transfusion on the supply of blood from many individuals. Problems may arise if this 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.

Erythropoiesis *in vivo*

The numbers of RBCs and progenitor cells are tightly regulated *in vivo* by homeostatic mechanisms. Although these mechanisms have not been elucidated, they are likely to involve factors with the ability either to induce or suppress proliferation of the RBC compartment. One factor that is known to play a role in the regulation of RBC numbers is the cytokine, erythropoietin (EPO) (Zhu and D'Andrea, 1994; Constantinescu *et al*., 1999). EPO prevents apoptosis and induces mitosis of erythroid progenitor cells, thereby accelerating their proliferation and increasing RBC numbers. Interestingly, physiological levels of EPO are not sufficient to prevent apoptosis of cultured erythroid progenitors (Koury and Bondurant, 1990). Intracellular factors, such as the tyrosine phosphatase SHP-1 (Klingmuller *et al*., 1995), members of the CIS/SOCS protein family (Naka *et al*., 1999; Yasukawa *et al*., 2000; Chen *et al*., 2000; Krebs and Hilton, 2001), and a truncated EPO receptor (Nakamura *et al*., 1992; Nakamura *et al*., 1994; Nakamura *et al*., 1998) have been shown to suppress EPO-mediated signals. However, an excess of EPO appears to prevent apoptosis of erythroid progenitor cells *in vitro* (Koury and Bondurant, 1990).

Tumor necrosis factor-α (TNF-α) is reported to be an important humoral factor that suppresses RBC numbers in rheumatoid arthritis (Papadaki *et al.*, 2000). Since inhibition of TNF-α function is insufficient to reverse anemia in rheumatoid arthritis (Papadaki *et al.*, 2000), it seems likely that another, as yet unidentified, suppressive modulator(s) is involved in the control of RBC numbers under normal and/or pathological conditions. Lipocalin 2 (LCN2) is one such suppressive modulator and can inhibit proliferation and/or differentiation of erythroid progenitor cells (Miharada *et al.*, 2005 and 2008).

RBC production from hematopoietic stem cells

The hematopoietic stem cells that are present in bone marrow and umbilical cord blood

are promising materials for the *in vitro* production of RBCs. In particular, umbilical cord blood cells are readily available as they are usually discarded. Provided the mother of a neonate consents to use of the umbilical cord, this material can provide a useful resource without any further complicating critical or ethical concerns.

Neildez-Nguyen *et al.* (2002) have reported that human erythroid cells (nucleated cells) produced on a large scale *ex vivo* could differentiate *in vivo* into enucleated RBCs. This study demonstrates 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 time for enucleation as is necessary with the erythroid cells.

In vitro expansion of erythroid progenitor cells

Many factors that act on hematopoietic stem and progenitor cells have been identified and analyzed (Metcalf, 1998, 1999), and can be utilized for attempts to expand the numbers of these cells *in vitro*.

Neildez-Nguyen *et al*. developed a culture protocol to expand $CD34^+$ erythroid progenitor cells based on a 3-step expansion of cells by sequential supply of specific combinations of cytokines to the culture medium (Kobari *et al*., 2000). Serum-free medium was supplemented with 2% deionized bovine serum albumin (BSA), 150 µg/ml iron-saturated human transferrin, 900 µg/ml ferrous sulfate, 90 µg/ml ferric nitrate, 100 µg/ml insulin, lipids (30 µg/ml soybean lecithin and 7.5 µg/ml cholesterol) and 10^{-6} M hydrocortisone. In the first step (days 0−7), 10^4/ml $CD34^+$ cells were cultured in the presence of 50 ng/ml Flt3 ligand (Flt3-L), 100 ng/ml thrombopoietin (TPO), and 100 ng/ml stem cell factor (SCF). In the second step (days 8−14), the cells obtained on day 7 were resuspended at $5x10^4$/ml in the same medium containing 50 ng/ml SCF, 3 units/ml EPO, and 50 ng/ml insulin-like growth factor-I (IGF-I). In the third step, the cells collected on day 14 were resuspended at $2x10^5$/ml and cultured for a further two to seven days in the presence of the same cytokine mixture as in the previous step but without SCF. The cultures were incubated at 37°C in 5% CO_2 in air and the medium was changed every three days to ensure good cell proliferation. This procedure allowed the *ex vivo* expansion of $CD34^+$ hematopoietic stem and progenitor cells into a pure erythroid precursor population. When these erythroid precursor cells were injected into non-obese diabetic, severe combined immunodeficient (NOD/SCID) mice, the erythroid cells proliferated and underwent terminal differentiation into mature enucleated RBCs.

Enucleation of erythroid progenitor 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 the interaction of erythroblasts with other cells, such as macrophages, is a controversial

topic in this process (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 progenitor cells proceeds efficiently in the presence (Giarratana *et al.*, 2005) but not in the absence (Neildez-Nguyen *et al.*, 2002) of feeder cells.

Giarratana *et al*., (2005) also used a 3-step approach to expand erythroid progenitor cells and to induce efficient enucleation. The cells were first cultured in a modified serum-free medium (Kobari *et al.*, 2000; Giarratana *et al*., 2000) supplemented with 1% deionized BSA, 120 μg/ml iron-saturated human transferrin, 900 ng/ml ferrous sulfate, 90 ng/ml ferric nitrate and 10 μg/ml insulin. In the first expansion step (days 0−8), 10^4/ml $CD34^+$ cells were cultured in the presence of 10^{-6} M hydrocortisone, 100 ng/ml SCF, 5 ng/ml interleukin-3 (IL-3) and 3 IU/ml EPO. On day 4, one volume of cell culture was diluted with four volumes of fresh medium containing hydrocortisone, SCF, IL-3 and EPO. In the second step (3 d), the cells were resuspended at $5x10^4$, 10^5, $2x10^5$ or $3x10^5$/ml and co-cultured on an adherent stromal layer in fresh medium supplemented with EPO. In the third step (up to 10 d), the cells were cultured on an adherent stromal layer in fresh medium without cytokines. The cultures were maintained at 37°C in 5% CO_2 in air. The adherent cell layer consisted of either the MS-5 stromal cell line (Suzuki *et al*., 1992) or mesenchymal stromal cells (MSCs) (Prockop, 1997) established from whole normal adult bone marrow in RPMI supplemented with 10% fetal calf serum (FCS). Adherent MSCs were expanded and purified through at least two successive passages. Since MSCs appear to be obtainable from various human tissues (Sudo *et al*., 2007), this method should be useful for producing transfusable RBCs *in vitro*.

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). Miharada *et al.* (2006) described the development of a method, with two slightly different protocols, to produce enucleated RBCs efficiently *in vitro* without use of feeder cells. Their system for expanding erythroid progenitor cells and inducing efficient enucleation of those progenitor cells is shown in *Figure 2*. $CD34^+$ cells were isolated from human umbilical cord blood by magnetic microbead selection and cultured using a procedure involving four passages (*Figure 2*). In the first three passages, cells were cultured in the presence of humoral factors (see below) and erythroid differentiation medium (EDM): StemSpan® H3000 supplemented with 5% Plasmanate® cutter, α-tocopherol (20 ng/ml), linoleic acid (4 ng/ml), cholesterol (200 ng/ml), sodium selenite (2 ng/ml), iron-saturated human transferrin (200 μg/ml), human insulin (10 μg/ml), ethanolamine (10 μM), and 2-mercaptoethanol (0.1 mM). In passage I, $1x10^5$ $CD34^+$ cells were cultured in 10 ml of EDM ($1x10^4$ cells/ml) either in the presence of human SCF (50 ng/ml), human EPO (6 IU/ml), human IL-3 (10 ng/ml) (Protocol A) or in the presence of SCF (50 ng/ml), EPO (6 IU/ml), IL-3 (10 ng/ml), human vascular endothelial growth factor (VEGF, 10 ng/ml), and human insulin-like growth factor-II (IGF-II, 250 ng/ml) (Protocol B) for six days. In passage II, $3x10^5$ cells, approximately 1/30 of cells expanded in passage I, were cultured in 10 ml of EDM ($3x10^4$ cells/ml) in the presence of SCF (50 ng/ml) and EPO (6 IU/ml) for four days. In passage III, $5x10^5$ cells,

approximately 1/50 of cells expanded in passage II, were cultured in 10 ml of EDM ($5x10^4$ cells/ml) in the presence of SCF (50 ng/ml) and EPO (2 IU/ml) for six days. In passage IV (enucleation step), $5x10^6$ cells, approximately 1/10 of cells expanded in passage III, were cultured for four days in 10 ml of enucleation medium ($5x10^5$ cells/ml): IMDM supplemented with 0.5% Plasmanate® cutter, D-mannitol (14.57 mg/ml), adenine (0.14 mg/ml), disodium hydrogen phosphate dodecahydrate (0.94 mg/ml), and mifepristone (an antagonist of glucocorticoid receptor, 1 μM). The cultures were incubated at 37°C in 5% CO_2 under humidified conditions.

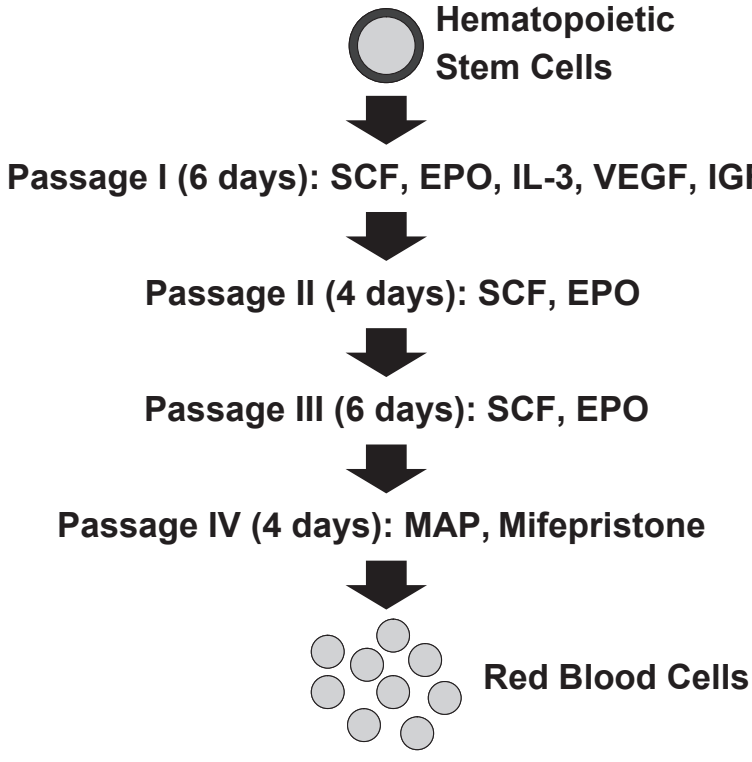

Figure 2. Culture protocol developed by Miharada *et al.* (2006) for the efficient production of enucleated red blood cells without feeder cells from hematopoietic stem cells. SCF, stem cell factor. EPO, erythropoietin, IL-3, interleukin-3. VEGF, vascular endothelial growth factor. IGF-II, insulin-like growth factor-II. MAP, mixture of D-mannitol, adenine, and disodium hydrogen phosphate dodecahydrate.

The increase in cell numbers following passage III of protocol B described above was similar that obtained by Neildez-Nguyen *et al.* (2002) with a method that avoided the use of feeder cells. Glucocorticoids promote proliferation and inhibit differentiation of erythroid progenitors (Kolbus *et al.*, 2003) and are usually present at some level in serum. This suggests that the use of serum-free medium may be preferable for inducing terminal differentiation in cultured cells. However, the removal of serum from the medium could also have a negative effect upon cell viability. In the procedure outlined in *Figure 2*, it was found that serum was necessary in passage IV to maintain cell viability. Miharada *et al.* (2006) also found that fetal bovine serum could be substituted by human Plasmanate. Mifepristone, an antagonist of glucocorticoid function, was

added to the medium in passage IV to accelerate the process of enucleation. They found that Mifepristone was more effective at inducing enucleation in the presence of a lower concentration of Plasmanate (0.5%) than at a higher concentration (10%), strongly suggesting that glucocorticoids were present in the Plasmanate (Miharada *et al.*, 2006). In both protocols, the vast majority of cells produced following passage IV expressed Rh-D antigen, a specific marker of erythroid progenitors and terminally differentiated RBCs (Miharada *et al.*, 2006). Notably, a higher proportion of the cells produced by protocol B (nearly 80%) was enucleated compared to protocol A (Miharada *et al.*, 2006).

The method developed by Miharada *et al.* (2006) included VEGF and IGF-II in the culture medium. These two factors have been reported to promote the survival, proliferation, and/or differentiation of hematopoietic progenitors (Gerber and Ferrara, 2003; Zhang and Lodish, 2004; Hiroyama *et al.*, 2006). Consistent with these findings, these factors promoted the expansion of erythroid progenitors (Miharada *et al.*, 2006). However, a much more important feature of their culture system is that it allowed erythroid cells to differentiate to a developmental stage competent for nuclear self-extrusion (Miharada *et al.*, 2006). 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). However, the data reported by Miharada *et al.* (2006) demonstrate that the interaction of erythroblasts with other cells is not necessary for efficient erythroblast enucleation. Signals mediated by humoral factors appear to be sufficient for the efficient autonomous completion of erythroblast enucleation. In addition, since culture without the use of feeder cells is technically easier and less expensive, the method of Miharada *et al.* (2006) has the potential to be a cost-effective means of producing transfusable RBCs on a large scale from immature hematopoietic progenitor cells.

RBC production from embryonic stem (ES) cells

The induction of hematopoietic cells from mouse ES cells is well established (Weiss, 1997; Orkin, 1998; Lazner *et al.*, 2001; Kyba and Daley, 2003; Chen *et al.*, 2003; Nakano, 2003; Olsen *et al.*, 2006). The stromal cell line, OP9 (Kodama *et al.*, 1994), has proved to be a useful feeder cell line for hematopoietic cell induction from mouse ES cells (Nakano *et al.*, 1994). OP9 cells enable induction of both primitive and definitive erythropoiesis from mouse ES cells (Nakano *et al.*, 1996). However, mass culture of mouse ES cells to produce pure erythroid progenitor cells and embryoid bodies can also be achieved through the addition of exogenous growth factors (Carotta *et al.*, 2004). Similarly, the induction of hematopoietic cells from non-human primate ES cells (Li *et al.*, 2001; Umeda *et al.*, 2004) and human ES cells (Kaufman, 2001; Chadwick, 2003; Cerdan, 2004; Wang, 2004; Zhan, 2004; Vodyanik, 2005; Wang, 2005a, 2005b, 2005c; Zambidis, 2005) has been reported recently.

Before any cells derived from human ES cells can be used in the clinic, it will be necessary to carry out preclinical studies, possibly in experimental primates (Hematti *et al.*, 2005). Attempts have been made to induce hematopoietic cells from primate ES cells (Umeda *et al.*, 2004; Hiroyama *et al.*, 2006) using the CMK-6 line that was

derived from the cynomolgus monkey (Suemori *et al.*, 2001). Hiromaya *et al.* (2004) modified a previously described method (Umeda *et al.*, 2004) to induce differentiation in CMK-6 cells on OP9 stromal cells without embryoid body formation, and observed that hematopoietic cells could be induced from CMK-6 cells. However, OP9 cells are very sensitive to variations in maintenance conditions, including medium source and serum lot, and this sensitivity can influence the ability of OP9 cells to support hematopoiesis (Vodyanik *et al.*, 2005). To avoid any possible difficulties posed by this characteristic, Hiroyama *et al.* (2006) sought to identify an alternative feeder cell line and also searched for specific humoral factors that improved the efficacy of the method. As a result, they found that use of the well-known cell line, C3H10T1/2 along with insulin-like growth factor-II (IGF-II) had considerable and beneficial effects on induction of hematopoietic cells from primate ES cells. Furthermore, the C3H10T1/2 cells were capable of long-lasting *in vitro* production of terminally differentiated blood cells including RBCs (Hiroyama *et al.*, 2006).

Given that the ultimate goal of developing RBCs from human ES cells is for clinical application, then the use of feeder cells derived from non-human species should be avoided. It has been reported that human ES cells express an immunogenic non-human sialic acid when they are cultured with non-human-derived materials (Martin *et al.*, 2005). This observation is very critical and merits further intensive investigation. Before clinical applications of blood cells derived from human ES cells are undertaken, pre-clinical *in vivo* studies using experimental primates are likely to provide essential safety information. With regard to RBCs, the method developed by Hiroyama *et al.* (2006) may enable a preclinical study in primates as it is able to produce abundant RBCs from ES cells. If *in vivo* immunological reactions originating from non-self antigens peak at negligible levels in experimental primates, then RBCs obtained from human ES cells using a method involving non-human feeder cells may still be suitable for clinical applications.

Establishment of erythroid progenitor cell lines able to produce transfusable RBCs

It is notable that the efficiency of generation of erythroid progenitor cells and RBCs varies depending on the culture methods employed and the ES cell lines used. 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 time-consuming process (Hiroyama *et al.*, 2006). If human erythroid progenitor cell lines that efficiently produce transfusable and functional RBCs can be established, they would represent a much more valuable resource for producing RBCs than ES cell lines (*Figure 1*).

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. It is generally difficult to establish hematopoietic cell lines from adult hematopoietic stem and progenitor cells as both are sensitive to DNA damage and are unable to maintain the lengths of telomere repeats on serial passage (Lansdorp, 2005). In contrast, ES cells are relatively resistant to DNA damage and maintain telomere lengths on serial passage (Lansdorp, 2005). Therefore, these characteristics of ES cells may be advantageous for the establishment of cell lines since differentiated cells derived from ES cells may retain them.

Recently, Hiroyama *et al.* (2008) developed a robust method to obtain differentiated cell lines following the induction of hematopoietic differentiation of mouse ES cells (*Figure 3*), and established five independent hematopoietic cell lines using this method. Three of these lines exhibited characteristics of erythroid cells, and they were designated mouse ES cell-derived erythroid progenitor (MEDEP) cell lines. Although their precise characteristics varied, each of the MEDEP lines could differentiate *in vitro* into more mature erythroid cells, including enucleated RBCs. Following transplantation into mice suffering from acute anemia, MEDEP cells proliferated transiently and subsequently differentiated into functional RBCs. Treated mice showed a significant amelioration of acute anemia. In addition, MEDEP cells did not form tumors following transplantation into mice. This report was the first to demonstrate the feasibility of establishing erythroid cell lines able to produce mature RBCs.

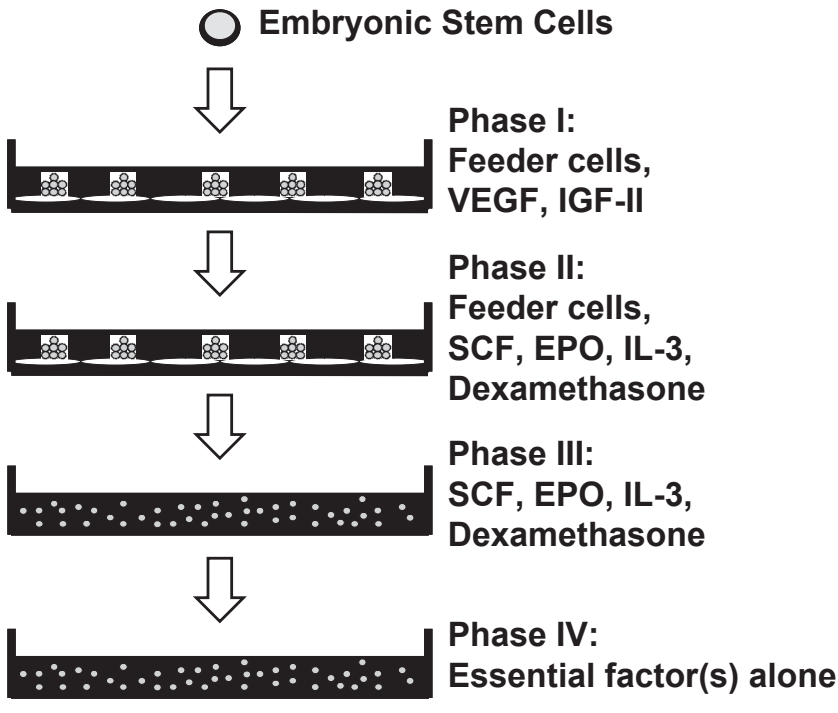

Figure 3. Culture protocol developed by Hiroyama *et al.* (2008) to establish an erythroid progenitor cell line from embryonic stem cells. VEGF, vascular endothelial growth factor. IGF-II, insulin-like growth factor-II. SCF, stem cell factor. EPO, erythropoietin, IL-3, interleukin-3.

At present, the mechanism underlying the establishment of differentiated cell lines from ES cells has not been elucidated. Nevertheless, published data clearly indicate that useful erythroid cell lines can be reproducibly obtained from mouse ES cells. Given that differentiation strategies developed for mouse ES cells often differ from those applied to human ES cells (Reubinoff *et al.*, 2000), it is likely that the method developed by Hiroyama *et al.* (2008) will not be directly applicable to human ES

cells and will require some modification. However, given the number of human ES cell lines established to date, it is possible that intensive testing of these lines for their erythroid potential may allow establishment of human erythroid cell lines similar to those of the mouse.

Induced pluripotent stem (iPS) cells as a source for establishing erythroid progenitor cell lines

To establish the MEDEP cell lines, Hiroyama *et al.* (2008) screened eight types of mouse ES cell line and succeeded in establishing MEDEP cell lines from three of these. By extrapolation from this result, it may be that many more human ES cell lines than currently available worldwide will be necessary to establish usable erythroid cell lines. In this context, the establishment of human induced pluripotent stem (iPS) cell lines (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Nakagawa *et al.*, 2008; Park *et al.*, 2008; Lowry *et al.*, 2008) should help to solve the problem of a potential shortfall, since human iPS cells have very similar characteristics as human ES cells.

Clinical application of erythroid progenitor cell lines

Hiroyama *et al.* (2008) reported that MEDEP cells did not exhibit tumorigenicity *in vivo*. Nevertheless, the tumorigenic potential of any human erythroid cell line will need to be thoroughly analyzed prior to clinical use (Vogel, 2005; Hentze *et al.*, 2007). In addition, it may be advisable to engineer these cells in such a way that they are eliminated if a malignant phenotype arises for any reason (Schuldiner *et al.*, 2003). Alternatively, the use of terminally differentiated cells that no longer have the capability of proliferating should allow clinical applications of ES cell derivatives without the associated risk of tumorigenicity. Thus, for example, RBCs lack nuclei following terminal differentiation, and are highly unlikely to exhibit tumorigenicity *in vivo*. As such, even if the original ES cells and/or their derivatives possessed abnormal karyotypes and/or genetic mutations, they might nonetheless be useful for clinical applications, provided that they can produce functional RBCs. Indeed, the MEDEP lines included many cells possessing abnormal karyotypes, however, the vast majority of the cells in each cell line nevertheless differentiated into mature erythroid cells and transplantation of these cells significantly ameliorated anemia (Hiroyama *et al.*, 2008). In general, immortalized cell lines are not necessarily homogenous in karyotype, even after cloning. The emergence of cells possessing abnormal karyotypes is often observed following continuous culture of immortalized cell lines. Hence, periodical recloning and selection of cell lines is recommended to maintain their genotype.

As described above, various methods have been developed that enable the production of enucleated RBCs from human hematopoietic stem and progenitor cells (Neildez-Nguyen *et al.*, 2002; Giarratana *et al.*, 2005; Miharada *et al.*, 2006). Therefore, once appropriate erythroid cell lines have been established, it should be possible to use these methods to produce enucleated RBCs *in vitro*. Since RBCs are much smaller than normal nucleated cells, RBCs produced *in vitro* could be selected by size prior to use in the clinic so as to exclude nucleated cells, for example, by filtration. In addition, X-ray irradiation might be useful for eradicating any contaminating nucleated cells without affecting the RBCs.

Another potential obstacle to the clinical use of ES cell derivatives is that of immunogenicity (Drukker and Benvenisty, 2004; Boyd *et al.*, 2005). Transplanted MEDEP cells could not ameliorate acute anemia in mouse strains other than those from which each individual cell line was derived or in immunodeficient mice (Hiroyama *et al.*, 2008), suggesting immunological rejection in heterologous strains. Hence, the clinical application of erythroid cell lines will require use of many cell lines that express different major histocompatibility (MHC) antigens. However, *in vitro*-generated RBCs need to be compatible with ABO and RhD antigens alone (8 types in total), meaning that 8 types of erythroid cell line would suffice to generate RBCs of all the different blood types required for clinical application. Recently, a technique was developed for the removal of the A and B antigens from RBCs (Liu *et al.*, 2007). Thus, removal of the antigens from RBCs that initially expressed A or B antigens, may allow transfusion of these RBCs into individuals that possess antibodies against A or B antigens. Such a technique might also be useful for transfusion of RBCs generated from erythroid cell lines *in vitro*. Of note, the establishment of a human erythroid cell line lacking the genes to produce A, B and RhD antigens would be a very useful resource for clinical application, since such a cell line would produce O/RhD(-) RBCs, which would, in theory, be transfusable into all individuals.

References

Aglietta, M., Bertolini, F., Carlo-Stella, C., *et al.* (1998) Ex vivo expansion of hematopoietic cells and their clinical use. *Haematologica* **83**, 824-48.

Boyd, A.S., Higashi, Y. and Wood, K.J. (2005) Transplanting stem cells: potential targets for immune attack. Modulating the immune response against embryonic stem cell transplantation. *Advanced Drug Delivery Reviews* **57**, 1944-1969.

Carotta, S., Pilat, S., Mairhofer, A., *et al.* (2004) Directed differentiation and mass cultivation of pure erythroid progenitors from mouse embryonic stem cells. *Blood* **104**, 1873-80.

Cerdan, C., Rouleau, A. and Bhatia, M. (2004) VEGF-A165 augments erythropoietic development from human embryonic stem cells. *Blood* **103**, 2504-12.

Chadwick, K., Wang, L., Li, L., *et al.* (2003) Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood* **102**, 906-15.

Chen, D., Lewis, R.L. and Kaufman, D.S. (2003) Mouse and human embryonic stem cell models of hematopoiesis: past, present, and future. *Biotechniques* **35**, 1253-61.

Chen, X.P., Losman, J.A. and Rothman, P. (2000) SOCS proteins, regulators of intracellular signaling. *Immunity* **13**, 287-90.

Constantinescu, S. N., Ghaffari, S. and Lodish, H. F. (1999) The erythropoietin receptor: Structure, activation and intracellular signal transduction. *Trends in Endocrinology and Metabolism* **10**, 18-23.

Douay, L. and Andreu, G. (2007) Ex vivo production of human red blood cells from hematopoietic stem cells: what is the future in transfusion? *Transfusion Medicine Reviews* **21**, 91-100.

Drukker, M. and Benvenisty, N. (2004) The immunogenicity of human embryonic stem-derived cells. *Trends in Biotechnology* **22**: 136-41.

Gerber, H.P. and Ferrara, N. (2003) The role of VEGF in normal and neoplastic hematopoiesis. *Journal of Molecular Medicine* **81**, 20-31.

Giarratana, M.C., Kobari, L., Lapillonen, H., et al. (2005) Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. *Nature Biotechnology* **23**, 69-74.

Giarratana, M.C. Vergé, V., Schmitt, C., et al. (2000) Presence of primitive lymphoid progenitors with NK or B potential in *ex vivo* expanded bone marrow cell cultures. *Experimental Hematology* **28**, 46−54.

Hanspal, M., Smockova, Y. and Uong, Q. (1998) Molecular identification and functional characterization of a novel protein that mediates the attachment of erythroblasts to macrophages. *Blood* **92**, 2940−50.

Hematti, P., Obrtlikova, P. and Kaufman, D.S. (2005) Nonhuman primate embryonic stem cells as a preclinical model for hematopoietic and vascular repair. *Experimental Hematology* **33**, 980-986.

Hentze, H., Graichen, R. and Colman, A. (2007) Cell therapy and the safety of embryonic stem cell-derived grafts. *Trends in Biotechnology* **25**, 24-32.

Hiroyama, T., Miharada, K., Aoki, N., et al. (2006) Long lasting *in vitro* hematopoiesis derived from primate embryonic stem cells. *Experimental Hematology* **34**, 760-9.

Hiroyama, T., Miharada, K., Sudo, K., et al. (2008) Establishment of mouse embryonic stem cell-derived erythroid progenitor cell lines able to produce functional red blood cells. *PLoS ONE* **3**, e1544.

Hofmeister, C.C., Zhang, J., Knight, K.L., et al. (2007) Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. *Bone Marrow Transplantation* **39**, 11-23.

Iavarone, A., King, E.R., Dai, X.M., et al. (2004) Retinoblastoma promotes definitive erythropoiesis by repressing Id2 in fetal liver macrophages. *Nature* **432**, 1040-45.

Kaufman, D.S., Hanson, E.T., Lewis, R.L., et al. (2001) Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proceedings of the National Academy of Sciences U.S.A.* **98**, 10716-21.

Kingsley, P.D., Malik, J., Fantauzzo, K.A., et al. (2004) Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis. *Blood* **104**, 19-25.

Klingmuller, U., Lorenz, U., Cantley, L.C., et al. (1995) Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell* **80**, 729-38.

Kobari, L., Pflumio, F., Giarratana, M., et al. (2000) In vitro and *in vivo* evidence for the long-term multilineage (myeloid, B, NK, and T) reconstitution capacity of *ex vivo* expanded human CD34(+) cord blood cells. *Experimental Hematology* **28**, 1470-80.

Kodama, H., Nose, M., Niida, S., et al. (1994) Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. *Experimental Hematology* **22**, 979-84.

Kolbus, A., Blázquez-Domingo, M., Carotta, S., et al. (2003) Cooperative signaling between cytokine receptors and the glucocorticoid receptor in the expansion of erythroid progenitors: molecular analysis by expression profiling. *Blood* **102**, 3136-46.

Koury, M.J. and Bondurant, M.C. (1990) Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science* **248**, 378-81.

Krebs, D.L. and Hilton, D.J. (2001) SOCS proteins: negative regulators of cytokine signaling. *Stem Cells* **19**, 378-87.

Kyba, M. and Daley, G.Q. (2003) Hematopoiesis from embryonic stem cells: lessons from and for ontogeny. *Experimental Hematology* **31**, 994-1006.

Lansdorp, P.M. (2005) Role of telomerase in hematopoietic stem cells. *Annals of the New York Academy of Sciences* **1044**, 220-7.

Lazner, F., Kola, I., Stadler, E., et al. (2001) Embryonic stem cells in the study of hematopoiesis. *Methods in Molecular Biology* **158**, 287-300.

Lee, J.C., Gimm. J.A., Lo, A.J. et al. (2004) Mechanism of protein sorting during erythroblast enucleation: role of cytoskeletal connectivity. *Blood* **103**, 1912-19.

Li, F., Lu, S., Vida, L., et al. (2001) Bone morphogenetic protein 4 induces efficient hematopoietic differentiation of rhesus monkey embryonic stem cells *in vitro*. *Blood* **98**, 335-42.

Liu, Q.P., Sulzenbacher, G., Yuan, H. et al. (2007) Bacterial glycosidases for the production of universal red blood cells. *Nature Biotechnology* **25**, 454-64.

Lowry, W.E., Richter, L., Yachechko, R., et al. (2008) Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proceedings of the National Academy of Sciences U.S.A.* **105**, 2883-8.

Martin, M.J., Muotri, A., Gage, F., et al. (2005) Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nature Medicine* **11**, 228-32.

Metcalf, D. (1998) Regulatory mechanisms controlling hematopoiesis: principles and problems. *Stem Cells* **16** (Supplement 1), 3-11.

Metcalf, D. (1999) Cellular hematopoiesis in the twentieth century. *Seminars in Hematology* **36** (4 Supplement 7), 5-12.

Miharada, K., Hiroyama, T., Sudo, K., et al. (2005) Lipocalin 2 functions as a negative regulator of red blood cell production in an autocrine fashion. *FASEB Journal* **19**, 1881-3.

Miharada, K., Hiroyama, T., Sudo, K., et al. (2006) Efficient enucleation of erythroblasts differentiated *in vitro* from hematopoietic stem and progenitor cells. *Nature Biotechnology* **24**, 1255-6.

Miharada, K., Hiroyama, T., Sudo, K., et al. (2008) Lipocalin 2-mediated growth suppression is evident in human erythroid and monocyte/macrophage lineage cells. *Journal of Cellular Physiology* **215**, 526-37.

Naka, T., Fujimoto, M. and Kishimoto, T. (1999) Negative regulation of cytokine signaling: STAT-induced STAT inhibitor. *Trends in Biochemical Sciences* **24**, 394-8.

Nakagawa, M., Koyanagi, M., Tanabe, K., et al. (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nature Biotechnology* **26**, 101-6.

Nakamura, Y., Komatsu, N. and Nakauchi, H. (1992) A truncated erythropoietin receptor that fails to prevent programmed cell death of erythroid cells. *Science* **257**, 1138-41.

Nakamura, Y. and Nakauchi, H. (1994) A truncated erythropoietin receptor and cell death; A reanalysis. *Science* **264**, 588-9.

Nakamura, Y., Takano, H., Osawa, M., et al. (1998) Impaired erythropoiesis in transgenic mice overexpressing a truncated erythropoietin receptor. *Experimental Hematology* **26**, 1105-10.

Nakano, T. (2003) Hematopoietic stem cells: generation and manipulation. *Trends in Immunology* **24**, 589-94

Nakano, T., Kodama, H. and Honjo, T. (1994) Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science* **265**, 1098-101.

Nakano, T., Kodama, H. and Honjo, T. (1996) In vitro development of primitive and definitive erythrocytes from different precursors. *Science* **272**, 722-4.

Neildez-Nguyen, T.M., Wajcman, H., Marden, M.C., et al. (2002) Human erythroid cells produced *ex vivo* at large scale differentiate into red blood cells *in vivo*. *Nature Biotechnology* **20**, 467-72.

Ohneda, O. and Bautch, V.L. (1997) Murine endothelial cells support fetal liver erythropoiesis and myelopoiesis via distinct interactions. *British Journal of Haematology* **98**, 798−808.

Olsen, A.L., Stachura, D.L. and Weiss, M.J. (2006) Designer blood: creating hematopoietic lineages from embryonic stem cells. *Blood* **107**, 1265-75.

Orkin, S.H. (1998) Embryonic stem cells and transgenic mice in the study of hematopoiesis. *International Journal of Developmental Biology* **42**, 927-34.

Papadaki, H.A., Kritikos, H.D., Valatas, V., et al. (2000) Anemia of chronic disease in rheumatoid arthritis is associated with increased apoptosis of bone marrow erythroid cells: improvement following anti-tumor necrosis factor-α antibody therapy. *Blood* **100**, 474-82

Park, I.H., Zhao, R., Weat, J.A. et al. (2008) Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451**, 141-6.

Pei, X. (2002) Stem cell engineering: the new generation of cellular therapeutics. *International Journal of Hematology* **76**, 155-6.

Prockop, D.J. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**, 71−4.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., *et al.* (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nature Biotechnology* **18**, 399-404.

Schuldiner, M., Itskovitz-Eldor, J. and Benvenisty, N. (2003) Selective ablation of human embryonic stem cells expressing a "suicide" gene. *Stem cells* **21**, 257-65.

Spike, B.T., Dirlam, A., Dibling, B.C., *et al.* (2004) The Rb tumor suppressor is required for stress erythropoiesis. *EMBO Journal* **23**, 4319-29.

Sudo, K., Kanno, M., Miharada, K., *et al.* (2007) Mesenchymal progenitors able to differentiate into osteogenic, chondrogenic, and/or adipogenic cells *in vitro* are present in most primary fibroblast-like cell populations. *Stem Cells* **25**, 1610-7.

Suemori, H., Tada, T., Torii, R., *et al.* (2001) Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. *Developmental Dynamics* **222**, 273-9.

Suzuki, J., Fujita, J., Taniguchi, S., *et al.* (1992) Characterization of murine hemopoietic-supportive (MS-1 and MS-5) and non-supportive (MS-K) cell lines. *Leukemia* **6**, 452-8.

Takagi, M. (2005) Cell processing engineering for ex-vivo expansion of hematopoietic cells. *Journal of Bioscience and Bioengineering* **99**, 189-96.

Takahashi, K., Tanabe, K., Ohnuki, M., *et al.* (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-72.

Umeda, K., Heike, T., Yoshimoto, M., *et al.* (2004) Development of primitive and definitive hematopoiesis from nonhuman primate embryonic stem cells *in vitro*. *Development* **131**, 1869-79.

Vodyanik, M.A., Bork, J.A., Thomson, J.A., *et al.* (2005) Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood* **105**, 617-26.

Vogel, G. (2005) Ready or not? Human ES cells head toward the clinic. *Science* **308**, 1534-8.

Wang, L., Li, L., Menendez, P., *et al.* (2005) Human embryonic stem cells maintained in the absence of mouse embryonic fibroblasts or conditioned media are capable of hematopoietic development. *Blood* **105**, 4598-603.

Wang, L., Li, L., Shojaei, F., *et al.* (2004) Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. *Immunity* **21**, 31-41.

Wang, L., Menendez, P., Cerdan, C., *et al.* (2005) Hematopoietic development from human embryonic stem cell lines. *Experimental Hematology* **33**, 987-96.

Wang, L., Menendez, P., Shojaei, F., *et al.* (2005) Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression. *Journal of Experimental Medicine* **201**, 1603-14.

Weiss, M.J. (1997) Embryonic stem cells and hematopoietic stem cell biology. *Hematology/Oncology Clinics of North America* **11**, 1185-98.

Yanai, N., Sato, Y. and Obinata, M. (1997) A new type-II membrane protein in erythropoietic organs enhances erythropoiesis. *Leukemia* (Supplement 3) **11**, 484−5.

Yasukawa, H., Sasaki, A. and Yoshimura, A. (2000) Negative regulation of cytokine signaling pathways. *Annual Review of Immunology* **18**, 143-64.

Yoshida, H., Kawane, K., Koike, M., *et al.* (2005) Phosphatidylserine-dependent engulfment by macrophages of nuclei from erythroid precursor cells. *Nature* **437**, 754-8.

Yu, J., Vodyanik, M.A., Smuga-Otto, K. *et al.* (2007) Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-20.

Zambidis, E.T., Peault, B., Park, T.S., *et al.* (2005) Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. *Blood* **106**, 860-70.

Zhang, C.C. and Lodish, H.F. (2004) Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells. *Blood* **103**, 2513-21.

Zhan, X., Dravid, G., Ye, Z., *et al.* (2004) Functional antigen-presenting leucocytes derived from human embryonic stem cells *in vitro*. *Lancet* **364**, 163-71.

Zhu, Y. and D'Andrea, A. D. (1994) The molecular physiology of erythropoietin and the erythropoietin receptor. *Current Opinion in Hematology* **1**, 113-8.

Ziegler, B.L. and Kanz, L. (1998) Expansion of stem and progenitor cells. *Current Opinion in Hematology* **5**, 434-40.