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Standardization of progenitor cell assay for cord blood banking

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Summary. - Cord blood has proved itself, if correctly stored with rational criteria, an excellent source of stem cells for related and unrelated transplants. It has been recently proven that the factor which predicts the best the speed of engraftment in cord blood transplants is the dose of progenitor cells injected per kg of body weight of the recipient. This result has been obtained thanks to a careful standardization of the neonatal progenitor cell assay. This manuscript describes such a standardization realized as a joined effort by the Istituto Superiore di Sanità, Rome, and the pivotal cord blood bank founded as a feasibility study by the National Institutes of Health, Bethesda at the New York Blood Center.

Key words: cord blood, cord blood banking, progenitor cell assay, transplantation, ontogenesis.

Riassunto *(Standardizzazione del saggio dei progenitori emopoietici nell'ambito delle banche di sangue di cordone)*. - Il sangue di cordone rappresenta, se conservato correttamente e con criteri razionali, una eccellente sorgente di cellule staminali per trapianto non solo da donatori consanguinei ma anche da donatori estranei. Recentemente è stato dimostrato che il parametro che predice meglio la velocità di recupero delle funzioni ematologiche (numero di cellule bianche e di piastrine circolanti) dopo trapianto con sangue di cordone è la dose dei progenitori trapiantati per kg di peso corporeo del ricevente. Questa correlazione è stata possibile grazie all'accurata standardizzazione del saggio clonogenetico per i progenitori emopoietici presenti nel sangue di cordone realizzata nell'ambito del controllo di qualità della prima banca di sangue cordonale, la banca del New York Blood Center. Questo lavoro descrive la standardizzazione di questo saggio frutto di un progetto di collaborazione tra l'Istituto Superiore di Sanità ed il New York Blood Center.

Parole chiave: banche di sangue di cordone, saggi clonogenetici di progenitori emopoietici, trapianti, ontogenesi.

Introduction

Transplantation of hematopoietic stem and progenitor cells from neonatal cord blood can restore the bone marrow function and sustain hematopoietic recovery in both related and unrelated recipients [1-3]. For patients for whom no suitable related marrow donor is available, this source of hematopoietic stem cells offers substantial advantages (relative ease of procurement; absence of risk to the donor, low risk of transmitting to the recipient cytomegalovirus and Epstein-Barr virus, lower incidence of graft-versus-host disease -GVHD-, etc.) [4].

The Placental Blood Program at the New York Blood Center was founded in 1992 as a special feasibility study by the National Institutes of Health (NIH) Bethesda, MD, USA to collect placental blood units and to make them available for transplantation [5]. As of today January 2001, the program has collected more than 11 000 units and provided grafts for 1027 transplants.

When first establishing criteria for cord blood banking, the Blood Center discussed the necessity to include, as a part of the characterization of the units, an estimate of the number and quality of the stem cells present in the sample. This estimate could serve as an internal quality control of the units being stored over time and as a mean to follow the "health" of the stem cells during banking manipulations. Furthermore, at the end of the study, it could prove itself as an important predictive factor for the match of a particular unit with a specific recipient [5].

Definitive hemopoietic stem cells (HSC) are formally defined as cells capable to engraft syngeneic recipients giving rise to all the blood elements for all the life of the host [6]. They cannot be systematically studied in human samples unless species other than man are used as recipients (immunodeficient xenotransplant) [7, 8]. However, xenotransplant assays are so cumbersome to be unusable in a cord blood bank setting. Alternatively, estimate of the number of hematopoietic stem cells present in a given human sample is provided by the number of cells expressing CD34, an antigen present on the surface of both stem and progenitor cells [9, 10]. Clinical studies have shown that, in the case of autologous mobilized peripheral blood stem cells transplantation, the speed of engraftment is directly correlated with the number of $CD34^+$ cells injected per kg of body weight of the recipient [11, 12]. However, the number of cells expressing the CD34 antigen present

in cord blood is too low (0.5-2%) to be determined directly with commercial kits. In preliminary experiments, the feasibility to standardize $CD34^+$ cell determination for cord blood banking was explored. These experiments indicated that, with the techniques available in 1992, at least 2 ml (ie ≅ 3% of a unit) of cord blood had to be dedicated to this testing (results not shown). Furthermore, $CD34^+$ cell determination is a phenotypic analysis which does not provide any indication on the quality of the cells being stored. As such, it cannot be used as a toxicity test in the optimization of the cord blood storing process itself [13].

During the process of hematopoietic differentiation, stem cells generate a series of progenitor cell compartments that are progressively more restricted in their proliferative potential [9]. The number of each type of progenitor cell can be quantified in specific clonogenic assays performed in well established culture conditions. Extensive investigations in the mouse have shown that the number of progenitor cells in a tissue is genetically determined [14] and in constant ratio (specific for every inbred strain, sex and ontogenetic stage) with the number of stem cells as determined by *in vivo* reconstitution assay [15]. In humans, the dose of marrow progenitor cells transplanted is predictive of the speed of hematopoietic recovery after autologous bone marrow transplantation [16]. However, all the attempts to standardize colony assay for adult bone marrow undertaken so far have failed. These failures have discouraged the use of progenitor cell determination in the human marrow setting. The progenitor cells which are in cord blood are metabolically very active. Furthemore, in contrast with clinical bone marrow samples the majority of which are from patients treated with toxic drugs, cord blood is a normal "healthy" tissue. In the case of murine bone marrow (a normal tissue normalized for age and health status of the donor), the progenitor cell assays had been standardized with less than 5-10% variability among replicates. Because of the inherent genetic heterogeneity of the human samples, the human assays cannot reach the precision of the murine determination. However, it should be possible to standardize progenitor assay also with normal human tissues (such as cord blood). Once the problem of standardization has been solved, progenitor cell assays would have three advantages over CD34 determination as surrogate stem cell assay in cord blood banking:

a) the small volume necessary for the assay. In fact, cord blood is a very rich source of progenitor cells. As shown in Fig. 1, only 1-2 μl of blood are sufficient to generate many hemopoietic colonies. Therefore, only 50-100 μl of material must be devoted to this test;

b) it is a direct determination (no purification step required). This fact reduces the contribution of sample and data manipulation to the error of the measure;

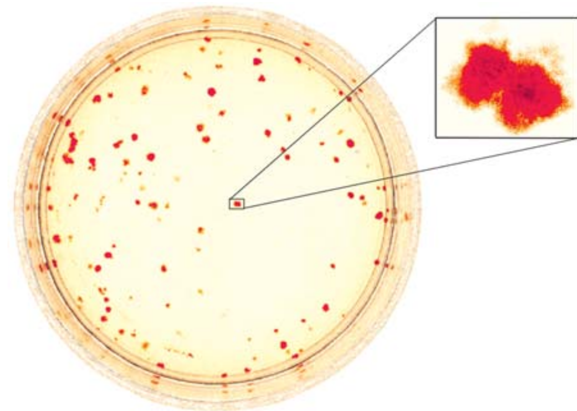

Fig. 1. - Photography of a representative 1 ml culture dish containing 1ml of cord blood. The picture was taken 12 days after the beginning of the growth. The insert shows a higher magnification of a representative colony. The colony is made red by the presence of the maturing erythroblasts.

c) it is a functional assay: Cells must proliferate and differentiate for several days in culture to generate a colony. The quality of proliferation and differentiation of each colony is evident during the scoring (Fig. 1) and provides an idea on the "health" status of cells very close to the stem cell compartment.

On the basis of all these considerations, it was decided to use progenitor cell assays, as an estimate of the quality and quantity of stem cells present in the cord blood units being stored at the Blood Center [5]. Since commercial kits for colony assay were not available in 1992, Dr. Rubinstein established a collaboration with the Istituto Superiore di Sanità with the specific aim of standardizing progenitor cell assay for cord blood banking.

Standardization assay

The main criteria used in the standardization was to develop a blood bank friendly assay. All the possible sources of variability and error were reduced, if not eliminated, by minimizing the number of steps involved in the assay. The cell culture parameters to be standardized were represented by: a) handling of the sample; b) culture media; c) growth stimulants; d) incubation conditions of the dish; e) scoring criteria and f) error-proof filing system as described below.

Handling of the samples

In preliminary experiments it was investigated whether the conditions used to store the sample would affect the results of the cultures. In particular, it was found that: a) the anticoagulant and the storage bag were biocompatible (i.e. they did not inhibit progenitor cell

growth); b) the results were not affected by the temperature of storage of the unit (4 °C, room temperature or 37 °C); c) the number of progenitor cells was stable for up to 48 h when the specimens were kept at room temperature before testing; d) the number of progenitor cells in a unit did not change if the assay was performed before or after freezing-thawing (variability within 10% of the mean in 100 indipendent determinations). Furthemore, once the unit was frozen, the number of progenitor cells did not decrease after long term storage (up to 1 year) (results not shown). Once all these parameters were established, the variability of the determination for a specific sample was investigated. Double blind replicate cultures of the same sample were performed for > 500 units. The variability between the two indipendent determinations of the same sample was found to be within 10% of the mean.

The distribution of the number of colonies per μl of blood observed in the first 100 samples ranged between 0 and 200. Colony determination is in the linear range of the assay (i.e. the number of colonies is directly proportional to the number of progenitor cells) between the value of 10 and 100 colony per dish. In the first 100 samples analyzed, > 98% of the units generate colony numbers within the linear range when either 2 or 4 μl of blood was cultured per assay. On the basis of all these results it was decided that the "standard" cord blood assay should include four replicate 1 μl culture dishes, two of which should be initiated with 2 μl and the other two with 4 μl of cord blood each. The blood should be taken directly from the collection bag and assayed without further manipulation.

Culture media

An in house culture kit was developed for cord blood banking. It was composed of batches of ≅150 tubes containing ready-to-use media (2.4 ml per tube) prepared with reverse osmosis grade pyrogen free water, pre-tested fetal bovine serum (30% v/v) and deionized albumin (1% v/v). The mixture is made semisolid with methylcellulose (0.8% wt/v) dissolved in Iscove's modified Dulbecco's medium. The batches are stable for more than 6 months if stored at -20 °C. At the time of use, the tubes are thawed (2 tubes per each cord blood sample to be tested) and the mixture of growth factors (in 0.1 ml) and the cord blood (5 or 10 μl per tube) added. After hand shaking, the culture mixture is plated with a syringe equipped with a blunt needle in replicate 35 mm petri dishes (1 ml per bacterial grade dish). The dishes are gently turned to distribute uniformly the media and incubated at 37 °C until scoring (see later). Recently, commercial kits for colony growth have been developed. We are currently comparing the efficiency and stability over time of the progenitor cell assay kit developed by us and of those recently developed from commercial sources.

Growth stimulants

Fetal and adult progenitor cells have different growth factor requirements [9]. Optimal growth of fetal progenitor cells is observed with only 1-2 growth factors while optimal growth of the corresponding adult cells requires the combination of at least 5 different growth factors [17, 18]. The switching between the two populations occurs at birth and perinatal cells have a growth factor requirement intermediate between those of the fetal and of the adult ones. Since the switching is asynchronous, there is the possibility that individual units have different growth factors requirements. For this reason, the cultures are stimulated with the mixture of growth factors necessary to grow adult cells. The growth factors used are: stem cell factor (SCF, 10 ng/ml), interleukin 3 (IL-3, 2×10^{-10} mol/l), granulocyte macrophage-colony stimulating factor (GM-CSF, 4.5×10^{-10} mol/l), granulocyte-colony stimulating factor (G-CSF, 2×10^{-10} mol/l) and erythropoietin (EPO, 1.5 U/ml). A fresh growth factor mixture is prepared every week from frozen pre-tested individual growth factor stocks and used within a week. Each growth factor is used at concentrations which are slightly higher than those which support optimal growth of adult progenitor cells [17, 18]. This fact increases the safety of the assay by buffering possible degradation that could occur during the storage.

Incubation conditions

All the four petri dishes (two replicate cultures with 2 μl and two with 4 μl of cord blood) prepared for a unit are stored in the same container (100 mm petri dish) clearly double marked with two bar code labels which identify the unit. The two labels, generated by computer, are located one within and one outside the container. The individual containers are incubated for 14 days at 37 °C in fully humidified CO_2 and O_2 monitored incubators. The storage space in the incubator is organized in such a way that the location of the containers is easily identified by day of plating. The air composition of the incubators is monitored weekly with the Fyrite apparatus by a technician and the values recorded.

Standardized scoring criteria

The number of colonies per dish must be enumerated by eye by dedicated personnel with the help of an inverted microscope. Although the criteria to enumerate the colonies are very well defined [17, 18], such a "human scoring" factor is thought to be the biggest source of variability and the scoring itself is the most difficult parameter to be standardized. However, in

contrast with the adult colonies, the colonies from cord blood are large colonies composed by so many cells that are visible by the naked eye (Fig. 1). Because of their size, they can be located without continuous change of focus. Therefore, as shown by Fig. 2, the experience of the investigator does not significantly affect the determination of the colony number. The number of colonies scored per dish decreases only slightly over a one week time when the dish is stored at 4 °C (results not shown).

Therefore, in the rare case in which the dishes cannot be scored at 14 days, they are removed from the incubator and stored in a cold room.

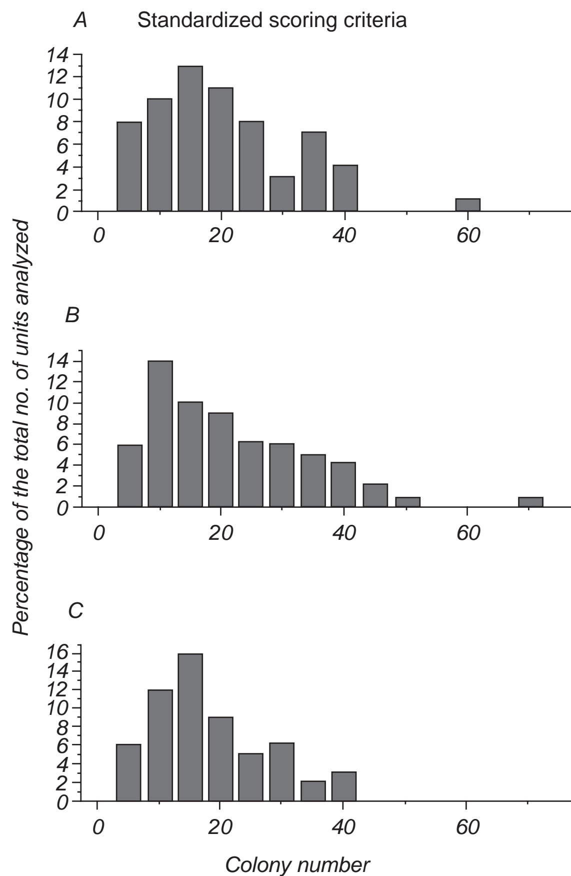

Fig. 2. - Variability of the progenitor cell content in cord blood units collected over one month according to the experience of the investigators scoring the cultures. The same dish has been scored by three separate investigators with either 4 years (A), 20 years (B) or 1 month (C) of scoring experience for a month. The X axe presents the number of colonies measured per ml of blood and the Y axe indicates the percentage of the units containing a certain number of colonies as scored by each investigator. There is no statistically significant difference in the average distributions of the number of progenitor cells scored in the cultures according to the experience of the investigator.

Error-proof filing system

A double (manual and computerized) filing of the results was adopted. The computerized filing was linked to an algorithm that corrects the values of the progenitor cells per dish, as scored by the technician, with the amount of anticoagulant present in the bag and the total volume of the unit. This algorithm ensures that the colony counts are automatically converted in total progenitor cell content. The data are stored in the computer linked to all the other information gathered for the same unit (summary from the clinical charts, virology data, HLA result, etc.).

Summary of the results of the standardization

Progenitor cell determination is not a novel technique and many laboratories are using progenitor cell determination in a transplantation setting as a “quality control” of the cells to be transplanted. However, researchers in the field are reluctant to use this assay for quantitative purposes because of problems they have experienced in standardizing the efficiency of the assay over time. The present manuscript is a “proof of principle” that, at least for a particular tissue, cord blood, conditions can be established to ensure that the performance of more than 2300 consecutive analysis remains stable over time. The report of this data should not be intended as an attempt to impose on any cord blood bank, either already existing or to be established in the future, any specific standardization method. Instead, it is intended to provide a backbone with which anybody wishing to do so may confront his/her own results. We would also be reluctant to extrapolate from these data any conclusion on the possibility to standardize adult progenitor cell determinations.

Using the criteria described in this paper, one technician has tested from February 1, 1993 to November 1997, 2386 out of 6352 cord blood samples stored at the New York Blood Center (≅ 40.7%). The average number of progenitor cells per ml of blood was found to be 40.88 ± 0.63.The progenitor cell determination was outside the linear range ($10 < CFC/ml < 100$) in only 2% of the samples. In 1.2% of the cases, the values were out of range because of colony counts below the minimal number required for reproducibility of scoring (< 0 colonies in the dish with 4 μl). We do not believe such a low progenitor cell content was due to technical failures. Instead, we think it is due to the fact that in ≅ 1% of the cases, cord blood does not contain significant number of progenitor cells.

The reproducibility of the assay over time remained very stable during all the course of the study. As shown in Fig. 3, the variability of the mean CFC content per ml of blood determined in 16 consecutive quarters (for a

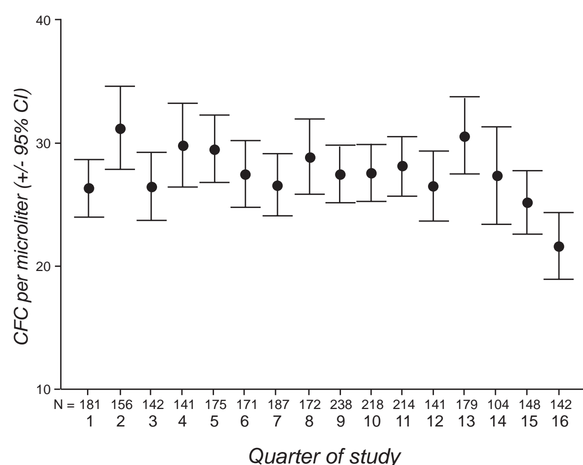

Fig. 3. - Average number of progenitor cells measured for ml of neonatal blood in consecutive quarters during the course of the study in the Cord Blood Program of the New York Blood Center (4 years). The N on the X axes specifies the number of samples tested in each quarter which was used to calculate the average number for that quarter.

total of 4 years) was within the range of variability of the quarterly mean. This fact ensured us not only that the culture conditions were stable over time, but also, more importantly, that the quality of the units stored at the Blood Center Bank in that particular period remained constant.

Over 2000 different units have been characterized for progenitor cell content during the course of the study. The high number of sample analyzed made possible to analyze the data for possible correlations between progenitor cell content and other parameters of the sample. The only significant ($p < 0.001$) correlation found was a positive correlation between number of progenitor cells and number of white blood cells per µl of blood ([5] and Fig. 4). Of all the other associations analyzed (length of the cord; weight of the baby; gestational age of the baby at the time of delivery; duration of the labor; race of the mother; type of delivery: cesarean section *vs* natural labor; etc.), the only one to reach significant levels was an inverse correlation between progenitor cell content and smoke of the mother during pregnancy (manuscript in preparation). Therefore none of these parameters can be used to predict the stem cell content of a unit and to exclude *a priori* a sample from storage.

It is more easy to evaluate white blood cell number than progenitor cell number. Therefore, on the basis of the close correlation between progenitor and white blood cell content of a cord blood units, the white blood cell dose was chosen as a guide in the first 562 transplants performed [2]. Among the very few cord blood units whose progenitor cells were below the limit of detection, there was a unit whose total volume (and therefore its white blood cell number) was among the highest collected (> 100 ml).This unit was used as a source of stem cells to transplant a patient who experienced the longest delay of engrafment for this type of transplants (white blood cell counts reached 500 in as long as 62 days). This result suggested us to compare by univariate and multivariate analysis which parameter (progenitor cell dose or nucleated cell dose) would correlate better with the post-transplant course. Indeed, the analysis of the 204 patients (out of a cohort of 562 transplanted patients) who received grafts which had been evaluated for CFC content, indicated that the CFC dose is a better predictor than nucleated cell dose of the speed of engraftment (both in terms of nucleated cells and platelets) [3]. Our experience indicates that, because of the tight correlation between the two parameters (Fig. 4), for most of the units the outcome of the transplant would be predicted equally well by total cell dose or progenitor cell dose. However, for the ≅ 1% of the units which do not contain progenitor cells, the parameter used to evaluate the dose could represent the difference between success and failure of the transplant. The safest solution would probably be not to include in the bank the low progenitor cord blood units.

A last important problem to be discussed in this setting is whether there is any correlation between progenitor cell content and $CD34^+$ cell content in cord blood units. This question arised from the report that in mouse the expression of the CD34 antigen is ontogenetically regulated and that most of the neonatal repopulating cells are $CD34^-$ [19]. Such an important and delicate question will be addressed in a manuscript in preparation on the characterization of more than 5000 cord blood units stored for transplantation. To make a long story short, the new manuscript will show that there is no correlation between progenitor cell content of a cord blood unit and

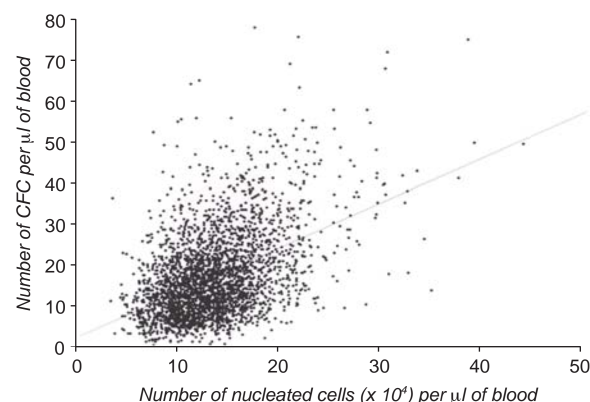

Fig. 4. - Correlation between the number of nucleated cells and of progenitor (CFC) cells contained in cord blood units. The correlation was evaluated in the first 500 units tested at the New York Blood Center.

its total number of CD34+ cells. However, there is a correlation, and it is very strong, between the number of a subset of the CD34+ cells, defined as the less bright, and progenitor cells. The cytofluorimeter determination of these cells may represent a surrogate progenitor cell assay.

In conclusion colony-forming assay provide an easily standardizable method to enumerate progenitor cell counts in placental/cord blood and the information obtained with this assay is important to predict the outcome of a cord blood transplant.

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REFERENCES

1. Rubinstein P, Rosenfield RE, Adamson JW, Stevens CE. Stored placental blood for unrelated bone marrow reconstitution. *Blood* 1993;81:1679-90.

2. Rubinstein P, Carrier C, Scaradavou A, Kurtzberge J, Adamson J, Migiaccio AR *et al.* Outcomes among 562 recipients of placental blood transplants from unrelated donors. *N Engl J Med* 1998; 339:1565-77.

3. Migliaccio AR, Adamson JW, Stevens CE, Dobrila NL, Carrier C, Rubinstein P. Cell dose and speed of engraftment in placental/ umbilical cord blood transplantation: graft progenitor cell content is a better predictor than nucleated cell quantity. *Blood* 2000;96:2717-22.

4. Gluckman E, Rocha V, Boyer-Chammard A, Locatelli F, Arcese W, Pasquini R *et al.* Outcome of cord blood transplantation from related and unrelated donors. *N. Engl J Med* 1997;337:373-481.

5. Rubinstein P, Taylor PE, Scaradavou A, Adamson JW, Migliaccio G, Emanuel D *et al.* Unrelated placental blood for bone marrow reconstitution: organization of the placental blood program. *Blood Cells* 1994;20:587-600.

6. Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* 2000;287:1442-6.

7. Zanjani ED, Pallavicini MG, Asceslao JL, Flake AW, Langlois RG, Reitsma M *et al.* Engraftment and long term expression of human fetal hemopoietic stem cells in sheep following transplantation *in utero. J Clin Inv* 1992;89:1178-88.

8. Dick JE. Human stem cell assays in immune-deficient mice. *Current Opinion Hematol* 1996 3:405-9.

9. Migliaccio AR, Papayannopoulou Th. In: Steinberg, MH, Forget BG, Higgs D, Nagel (Ed.). *Disorders of hemoglobin, genetics, pathophysiology, clinical management.* New York: Cambridge University Press; 2001. p. 72-94.

10. Krause DS, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility. *Blood* 1996;87:1-13.

11. Berenson RJ, Bensinger WI, Hill RS, Andrews RG, Garcia-Lopez J, Kalamasz DF *et al.* Engraftment after infusion of CD34+ marrow cells in patients with breast cancer or neuroblastoma. *Blood* 1991;77:1717-22.

12. Schiller G, Vescio R, Freytes C, Spitzer G, Sahebi F, Lee M, Wu CH, Cao J, Lee JC, Hong CH *et al.* Transplantation of CD34+ peripheral blood progenitor cells after high-dose chemotherapy for patients with advanced multiple myeloma. *Blood* 1995;86: 390-7.

13. Rubinstein P, Dobrila L, Rosenfield RE, Adamson JW, Migliaccio G, Migliaccio AR *et al.* Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. *Proc Natl Acad Sci USA* 1995;92:10119-22.

14. Phillips RL, Reinhart AJ, Van Zant G. Genetic control of murine hematopoietic stem cell pool sizes and cycling kinetics. *Proc Natl Acad Sci USA* 1992;89:11607-11.

15. Bol SJL. The recognition of early developmental stages in haematopoiesis. Ph. Thesis; University of Delft: W.D. Meinema B.V. The Netherlands; 1980.

16. Spitzer G, Verma DS, Fisher R, Zander A, Vellekoop L, Litam J *et al.* The myeloid progenitor cell - its value in predicting hematopoietic recovery after autologous bone marrow transplantation. *Blood* 1980;55:317-23.

17. Migliaccio G, Migliaccio AR, Adamson JW. *In vitro* differentiation of human granulocyte/macrophage and erythroid progenitors: comparative analysis of the influence of recombinant human erythropoietin, G-CSF, GM-CSF, and IL-3 in serum-supplemented and serum-deprived cultures. *Blood* 1988;72:248-56.

18. Migliaccio G, Migliaccio AR, Druzin ML, Giardina PJ, Zsebo KM, Adamson JW. Effects of recombinant human stem cell factor (SCF) on the growth of human progenitor cells *in vitro. J Cell Physiol* 1991;148:503-9.

19. Sato T, Laver JH, Ogawa M. Reversible expression of CD34 by murine hematopoietic stem cells. *Blood* 1999; 94:2548-54.