In vitro and *in vivo* megakaryocyte differentiation of fresh and *ex vivo* expanded cord blood cells: rapid and transient megakaryocyte reconstitution

STEFANIA BRUNO, MONICA GUNETTI, LORETTA GAMMAITONI, ALESSANDRA DANÈ, GIULIANA CAVALLONI, FIORELLA SANAVIO, FRANCA FAGIOLI, MASSIMO AGLIETTA, WANDA PIACIBELLO

Background and Objectives. Megakaryocyte (Mk) engraftment is often poor and delayed after cord blood (CB) transplantation. *Ex vivo* manipulations of the cells that will be infused may be a way to achieve better Mk engraftment. In this study we investigated the ability of different hematopoietic growth factor combinations to generate large numbers of Mk cells *ex vivo*.

Design and Methods. To find the best cytokine combination capable of generating large numbers of Mks, baseline CB CD34$^+$ (bCD34$^+$) cells and CD34$^+$ and CD34$^-$ cells, immunoselected after 4 weeks of expansion with thrombopoietin (TPO), stem cell factor (SCF) and Flt-3 ligand (FL) (eCD34$^+$, eCD34$^-$), were further cultured in the presence of different cytokine combinations (containing interleukin(IL)-3, SCF, TPO and IL-6). To evaluate Mk reconstitution *in vivo*, Mk-committed cells, generated during 10 days of *in vitro* culture, were injected into NOD/SCID mice and the kinetics of human platelet production was evaluated.

Results. TPO and SCF together were found to be sufficient to generate large numbers of Mk cells ($3\pm0.40 \times10^6/1\times10^5$ input bCD34$^+$ cells) from bCD34$^+$ cells; the addition of IL-3 and IL-6 did not further increase Mk production ($3.5\pm0.63\times10^6/1\times10^5$ input bCD34$^+$ cells). In contrast only one cytokine combination (IL-3+SCF+IL-6+TPO) induced a large Mk production from eCD34$^+$ and eCD34$^-$ cells ($0.16\pm0.04\times10^6/1\times10^5$ input eCD34$^+$ cells and $0.035\times10^6\pm0.012\times10^6/1\times10^5$ input eCD34$^-$ cells, respectively). In mice injected with Mk-committed cells derived from bCD34$^+$ or eCD34$^+$ cells, human platelets were first detected on day 3 and disappeared after 4 weeks; in mice injected with MK-committed cells derived from eCD34$^-$ cells, human platelets peaked at day 3, but disappeared quickly.

Interpretation and Conclusions. Fast Mk-engraftment can be obtained by *in vitro* selective lineage-commitment of baseline and *ex vivo* expanded CB cells.

Key words: cord blood, expansion, differentiation, megakaryocyte, NOD/SCID.

Haematologica 2003;88:379-387
http://www.haematologica.org/2003_04/88379.htm

From the Dept. of Oncological Sciences, University of Turin Medical School, Div. of Clinical Oncology, Institute for Cancer Research and Treatment, Candiolo, (SB, MG, LG, AD, GC, FS, MA, WP) and Pediatric Dept., University of Turin Medical School, Italy (FF).

Correspondence: Wanda Piacibello, M.D., University of Torino Medical School, Dept. of Oncological Sciences, Candiolo, Torino, Italy. E-mail: wanda.piacibello@ircc.it

High dose chemotherapy with autologous or allogeneic progenitor stem cell support is widely used in the treatment of hematologic and non-hematologic malignancies. The use of cord blood (CB) as a source of marrow repopulating cells in pediatric patients has been established.[1,2] However, two major limitations hamper the widespread use of CB as a source of hematopoietic stem cells for marrow replacement. First, although the number of hematopoietic progenitor/stem cells may be sufficient for full engraftment in children, *ex vivo* expansion of these cells might be necessary to engraft adults. Second, in comparison with bone marrow (BM) or mobilized peripheral blood (MPB), CB transplantation is characterized by a delayed engraftment and, in particular, a very slow platelet recovery.[2,3] Co-transfusion of large numbers of *ex vivo* generated human megakaryocyte (Mk)-committed cells, generated *ex vivo*, could be a new way to shorten the period of thrombocytopenia.

Several growth factor combinations have been tested to identify culture conditions that can support a large expansion of primitive repopulating stem cells. CB CD34$^+$ can be expanded in stroma-free cultures containing Flt-3 ligand (FL), thrombopoietin (TPO) and stem cell factor (SCF); this cytokine combination amplifies CB progenitors and precursors of all hematopoietic lineages,[4,5] without a concomitant loss, but rather an increase, of the *in vivo* repopulating ability of primitive stem cells. In fact it has been shown that NOD/SCID repopulating cells (SRC) are expanded more than 70-fold after 9 to 10 weeks of expansion.[6-9]

Nevertheless, it has not been established so far whether CB CD34$^+$ cells, after several weeks of expansion in this culture system, still retain not only the same *in vivo* repopulating ability, but also the same proliferation and differentiation potential towards the Mk lineage as the unmanipulated CD34$^+$ CB cells.

In this study the Mk differentiation potentials of baseline CB CD34$^+$ cells (bCD34$^+$) and those re-isolated after extensive expansion in liquid culture in the presence of FL, TPO and SCF (eCD34$^+$) were compared. Baseline and four-week expanded CD34$^+$ cells were further grown in the presence of various growth factor combinations containing megakaryocyte-active cytokines: interleukin (IL)-3,[10] SCF, IL-6[11,12] and TPO.[13-15] The Mk-differentiation capacity of the CD34$^-$ cell subpopulation, derived from bCD34$^+$ cells expanded for 4 weeks of culture in the presence of FL, SCF and TPO (eCD34$^-$), was also evalu-

ated. The non-obese diabetic severe combined immunodeficient (NOD/SCID) mouse model offers the possibility of analyzing both the short-term and the long-term repopulating ability and the differentiation and maturation potential of human hematopoietic tissues *in vivo*.[16-18] Mk-committed cells obtained from fresh and expanded cells were injected into NOD/SCID mice to evaluate the presence and persistence of human megakaryocytopoiesis in mouse BM and the speed of human platelet recovery (PLT) in mouse peripheral blood (PB).

Design and Methods

Recombinant human cytokines

The following recombinant purified human cytokines were used in these studies: recombinant human (rh) TPO and rhSCF were generous gifts from Kirin (Kirin Brewery, Tokyo, Japan); rh interleukin-6 (rhIL-6) was purchased from Peprothec Inc (NJ, USA), rh interleukin-3 (rhIL-3) was from Sandoz (Basel, Switzerland); rh FLT-3 ligand was kindly provided by SD Lyman (Immunex Corp, Seattle, WA, USA).

Human cells

CB samples were obtained, with written informed consent, at the end of full-term deliveries, after clamping and cutting of the cord, by draining the cord blood into sterile collection tubes containing the anticoagulant citrate-phosphate dextrose.

CD34+ cell purification

Mononuclear cells (MNC) were isolated from CB using Ficoll Hypaque (density 1077 g/cm^3; Nyegaard, Oslo, Norway) density centrifugation. The CD34$^+$ fraction was isolated with superparamagnetic microbead selection using a high-gradient magnetic field and a miniMACS column (Miltenyi Biotech, Gladbach, Germany). The efficiency of the purification was verified by flow cytometry counterstaining with a CD34-phycoerythrin (PE; HPCA-2, Becton Dickinson, San José, CA, USA) antibody. In the cell fraction containing purified cells, the percentage of CD34$^+$ cells ranged from 90 to 98%. The CD34$^+$ cells isolated at this step are named baseline CD34$^+$ cells (bCD34$^+$).

Ex vivo expansion cultures

CB CD34$^+$ cells (5×10^4/mL) in 10 mL of Iscove's modified Dulbecco's medium (IMDM, GIBCO Life Technologies, Milan) supplemented with 10% of fetal calf serum (FCS, Euroclone, Logan, UT, USA) were deposited on the bottom of tissue culture T_{75} flasks and maintained at 37°C in a fully humidified atmosphere with 5% CO_2. FL (50 ng/mL), TPO (10 ng/mL) and SCF (50 ng/mL) were added at the start of the culture and then twice a week. Every week the cells were resuspended and counted. Based upon cell number, the culture volume was doubled, by addition of new medium, serum and growth factors. At week 4, total cell volume was harvested after careful resuspension of cells, which were counted, washed and subjected to CD34 selection using the mini-MACS method to obtain two different sub-populations: eCD34$^+$ and eCD34$^-$ cells.

Liquid cultures for Mk

One hundred thousand bCD34$^+$, eCD34$^+$ and eCD34$^-$ cells were seeded in a total volume of 1 mL in IMDM plus 10% FCS with the following cytokine combinations: 1) TPO (20 ng/mL); 2) TPO+SCF (50 ng/mL); 3) IL-3 (5 ng/mL)+SCF+IL-6 (20 ng/mL); 4) IL-3+SCF+IL-6+TPO and cultured for 7, 14, 21 and 28 days. Each growth factor combination was added once a week. Cells were incubated at 37°C in a fully humidified atmosphere at 5% CO_2. The cells were counted and cell viability was evaluated by trypan blue dye exclusion; appearance of CD41$^+$ and CD34$^+$/41$^+$ cells, DNA content, and CFU-Mk output were evaluated at the indicated time points.

Megakaryocyte characterization

Flow cytometric analysis. Flow cytometric analysis was performed on a FACSVantage (Becton Dickinson) equipped with an argon ion laser, at an excitation wavelength of 488nm. At least 20,000 events were acquired for each analysis. Anti CD41 antibody (DAKO A/S, Denmark), directly conjugated with FITC, and anti CD34 antibody, directly conjugated with PE, were used to characterize the phenotype of cell samples. Cells were washed once in phosphate buffer saline (PBS) supplemented with 0.1% bovine serum albumin (BSA) (Sigma Chemical Co, Milan, Italy) and 0.01% sodium azide and then incubated for 30 minutes at 4°C with appropriate amounts of specific antibody. After washing with PBS/BSA, cells were resuspended in 0.2 mL PBS and analyzed by flow cytometry.

Mk ploidy. Cultured cells were washed in PBS before fixation in cold 80% ethanol. Cells were maintained for at least 24 hours at –20°C, washed in PBS/BSA. Thereafter, the cells were incubated for 2 hours at 4°C with propidium iodide (50 μg/mL) (Sigma Chemical Co, Milan, Italy) to stain the DNA in a solution containing RNase (200 μg/mL) (Sigma Chemical Co) and 0.1% Tween 20. Cell samples were analyzed on a FACSCVantage.

CFU-Mk assay. Two thousand CD34$^+$ CB cells of the initial cell suspension or suitable aliquots of the liquid cultures were seeded in plasma-clots as previously described.[19,20] Colony scoring was performed on day 12 by immunofluorescence microscopy after staining with a FITC-conjugated monoclonal antibody (MoAb) recognizing human CD41.

Injection of Mk-committed cells into NOD/SCID mice

NOD/LtSz scid/scid (NOD/SCID) mice were obtained from Jackson Laboratories (Bar Arbor, ME, USA), and maintained at the C.I.O.S animal facilities (Turin, Italy). All animals were handled under sterile conditions and maintained in cage microisolators. Sublethally irradiated (350 cGy of total body irradiation from a ^{137}Cs source) 6- to 8-week old mice were injected in the tail vein with the progeny of 5×10^5 bCD34$^+$, eCD34$^+$ and eCD34- cells induced to differentiate along the Mk lineage for 10 days of culture. Mice were sacrificed 1, 2, 3 and 4 weeks post-transplant and BM cells were flushed from femora and tibiae using a syringe and 26-gauge needle, for assessment of the number and types of human cells. No growth factors were administered to the animals.

Flow cytometric detection of human cells in murine BM. To analyze the levels of engraftment of human cells in the murine BM, the cells were resuspended at 1 to 2×10^6 cells/mL and incubated with mouse IgG (Fluka Chemika Biochemika, Buchs, Switzerland), to block non-specific binding to Fc receptors. Cells were then incubated with FITC- or PE-labeled MoAb specific for human CD for 30 minutes at 4°C to assess the population of human hematopoietic cells. Some cells from each suspension were similarly incubated with irrelevant (control) MoAbs labeled with FITC and PE. After staining, the cells were washed once in PBS/BSA. Contaminating red blood cells were eliminated with EDTA 10^{-4} mol/L, KHCO3 10^{-3} mol/L, NH_4CL 0.17 mol/L. The antibodies used were FITC-labeled antihuman CD41 (DAKO A/S Denmark), FITC CD71, PE CD34, PE CD19 (CALTAG Laboratory), PE CD33 (DAKO A/S Denmark) and CD45 TRI-COLOR (TC)-conjugated (CALTAG Laboratory).

Human platelet detection in NOD/SCID mouse peripheral blood (PB). Platelet appearance in murine PB 3, 7, 14, 21 and 28 days after transplantation was also assessed. Anticoagulated blood was incubated at room temperature with FITC-labeled anti mouse CD41 (CALTAG Laboratory) and PE-labeled anti human CD41 (CALTAG Laboratory) or isotype control for 5 minutes and analyzed immediately by flow cytometry. Data were acquired with a primary gate set on a dual parameter histogram of log forward light scatter and log side light scatter; 100,000 events were acquired. Background fluorescence was assessed with platelets labeled with the FITC- and PE-conjugated isotype control antibody. PB samples from untransplanted mice and from human donors were analyzed as additional controls.

Activation of human platelets by thrombin. Aliquots of mouse anti-coagulated PB (10 μL) were incubated with thrombin (ImmunoFrance S.A.) at a final concentration of 50 U/mL for 10 minutes. After this incubation the platelet CD62P (Caltag Laboratory) expression was assessed by flow cytometry. Live acquisition of 1,000 to 2,000 human platelet events was performed by gating human CD41$^+$ events in the platelet size range.

Statistical analysis

Basic statistical analysis such as mean values and standard deviations (SD) on fold-increase results, is shown. Differences were considered of statistical significance when the *p* value, calculated with Student's t-test, was <0.05. The correlation between the percentage of human CD41$^+$ cells in murine BM and the percentage of human circulating platelets in mice PB was made by a regression line, calculating the Pearson's coefficient (r).

Results

In vitro megakaryocyte differentiation from fresh and expanded cord blood CD34$^+$ cells

Baseline (b) and expanded (e) CD34$^+$ cells were cultured for 7, 14, 21 and 28 days in the presence of different cytokine combinations. To determine the number of Mk cells obtained, platelet surface marker CD41 (GPIIb) expression was measured by flow cytometry. CD41 is a platelet protein that is first expressed during megakaryocyte differentiation; furthermore, its expression increases along with megakaryocyte maturation, so the more mature megakaryocytes are CD41^{bright}.[21,22]

Figure 1A shows the absolute number of Mks (CD41$^+$ cells) obtained by seeding 1×10^5 bCD34$^+$ cells. At day 7 of culture, all cytokine combinations produced a significant (p<0.001) production of CD41$^+$ cells. At days 14 and 21, TPO+SCF and IL-3+SCF+IL-6+TPO induced a significant (p<0.001) and large production of CD41 cells (respectively 210 and 240-fold increase the initial number); there was not a significant difference between these two culture conditions (p>0.05). As depicted in Figure 1B, maximum expansion of Mk precursors (CD34$^+$CD41$^+$ cells) was obtained with SCF+TPO at day 7 with a 40-fold increase (p<0.001).

Figure 2A shows a representative cytofluorimetric analysis of CD41 expression on bCD34$^+$ cells cultured with TPO+SCF. This figure also shows the presence of dim and bright CD41$^+$ cells, indicating the presence of immature (CD41^{dim}) and more mature (CD41^{bright}) Mk cells.

Interestingly, different results were observed when eCD34$^+$ cells were grown with the same growth factor combinations. In all culture conditions Mk production was lower than that obtained from bCD34$^+$ cells. The growth factor combination containing IL-3+SCF+IL-6+TPO was necessary to produce a significant Mk-increase (40 fold the initial number; p<0.001) at 14 days of culture (the initial number was the number of CD41$^+$ cells measured after 4 weeks of expansion in the CD34$^+$ purified popula-

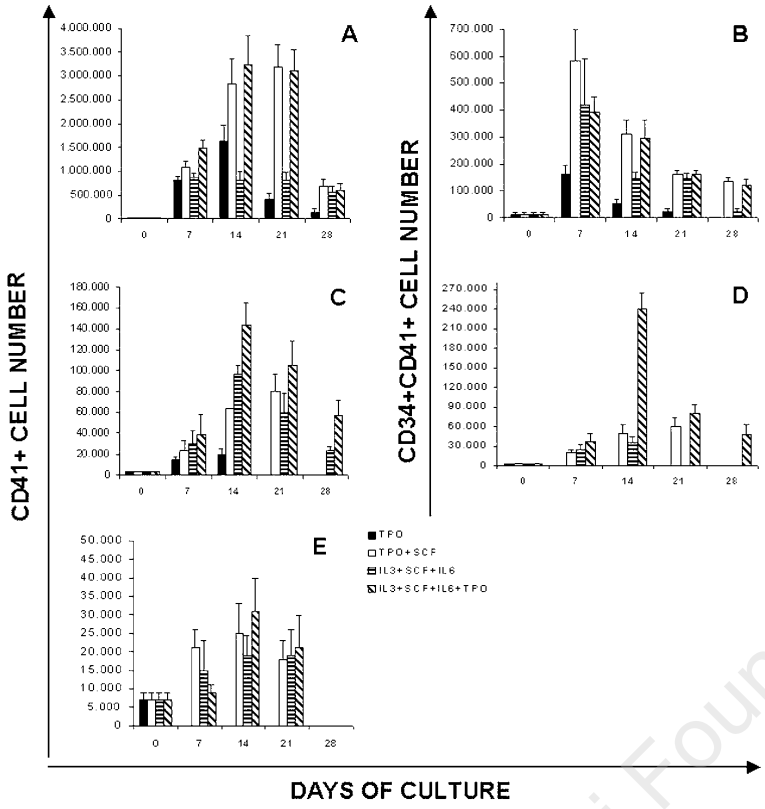

Figure 1. Kinetics of megakaryocyte differentiation of bCD34⁺, eCD34⁺ and eCD34⁻. cells. Absolute numbers of CD41⁺ cells (A) and CD34⁺/41⁺ cells (B) generated by 1×10⁵ initial bCD34⁺ cells; absolute number of CD41⁺ cells (C) and CD34⁺/41⁺ cells (D) generated by 1×10⁵ initial eCD34⁺ cells; absolute number of CD41⁺ cells (E) generated by 1×10⁵ initial eCD34⁻ cells. The different cell populations were cultured in quadruplicate wells for 7, 14, 21 and 28 days in the presence of the indicated cytokine combinations. Data are expressed as mean±SD from 4 wells per point; 10 separate experiments. CD34⁺ CD41⁺ cell number at T_0: A-B=13.5±7×10³/mL, C-D=3±0.77×10³/mL. CD41⁺ cell number at T_0: E=7±4.2×10³/mL.

tion) (Figure 1C). The kinetics of the appearance of more immature CD34⁺CD41⁺ cells (Figure 1D) demonstrated that IL-3+SCF+IL-6+TPO was, also in this case, significantly (p<0.001) better than the other growth factor combinations. Figure 2B shows a representative cytofluorimetric analysis of CD41 expression on eCD34⁺ cells cultured with IL-3+SCF+IL-6+TPO. In this case $CD41^{dim}$ cells and a few $CD41^{bright}$ cells can be observed, indicating the presence of a prevalently immature Mk population.

The ability of eCD34⁻ cells (isolated from expanded cells) to produce Mk cells was also evaluated. In the presence of TPO+SCF and IL-3+SCF+IL-6+TPO a large Mk-differentiated population (50% of the produced cells were CD41⁺ at 7 days of culture) was obtained; however, cell proliferation was scarce, as the total number of cells did not increase much (9-fold maximum) (Figure 1E). Figure 2C shows a representative cytofluorimetric analysis of CD41 expression on eCD34⁻ cells cultured with IL-3+SCF+IL-6+TPO. Also in this case only $CD41^{dim}$ cells can be seen.

The production of megakaryocytic colonies (CFU-Mk) by bCD34⁺ and eCD34⁺ cells was evaluated (Table 1). The maximum production of CFU-Mk was obtained with bCD34⁺ cells cultured with SCF+TPO for 7 days (41-fold increase of the initial number of CFU-Mk) and with eCD34⁺ cells cultured 14 days with IL-3+SCF+IL-6+TPO (28-fold increase), confirming the presence of Mk progenitors (CD34⁺ CD41⁺ cells) seen by flow-cytometry.

Analysis of ploidy of cultured megakaryocytes

To evaluate the degree of maturation of Mk cells we measured the ploidy of CD41⁺ cells. Cells were harvested at each time point and the DNA content was evaluated by flow cytometry. When bCD34⁺ cells were cultured with TPO+SCF or IL-3+SCF+IL-6+TPO 4N, 8N and some 16N and 32N CD41⁺ cells were obtained (Table 2 and Figure 3) during cell culture. By contrast, when eCD34⁺ and eCD34⁻ cells were cultured in the presence of all cytokine combinations, cells with only a 4N DNA content were obtained (Table 2 and Figure 3).

Short-term reconstitution of megakaryocytopoiesis in NOD/SCID mice by Mk-committed precursors

Based upon the kinetics of *in vitro* Mk production by the three CB populations, we chose a 10-day culture period to induce Mk commitment prior to *in vivo* transplantation.

Five groups of NOD/SCID mice were injected with all of the cells derived from:

- 5×10^5 bCD34⁺ cells that had been induced to differentiate for 10 days with TPO+SCF (total injected cells: $21\times10^6\pm3.5\times10^6$ of which the counts of

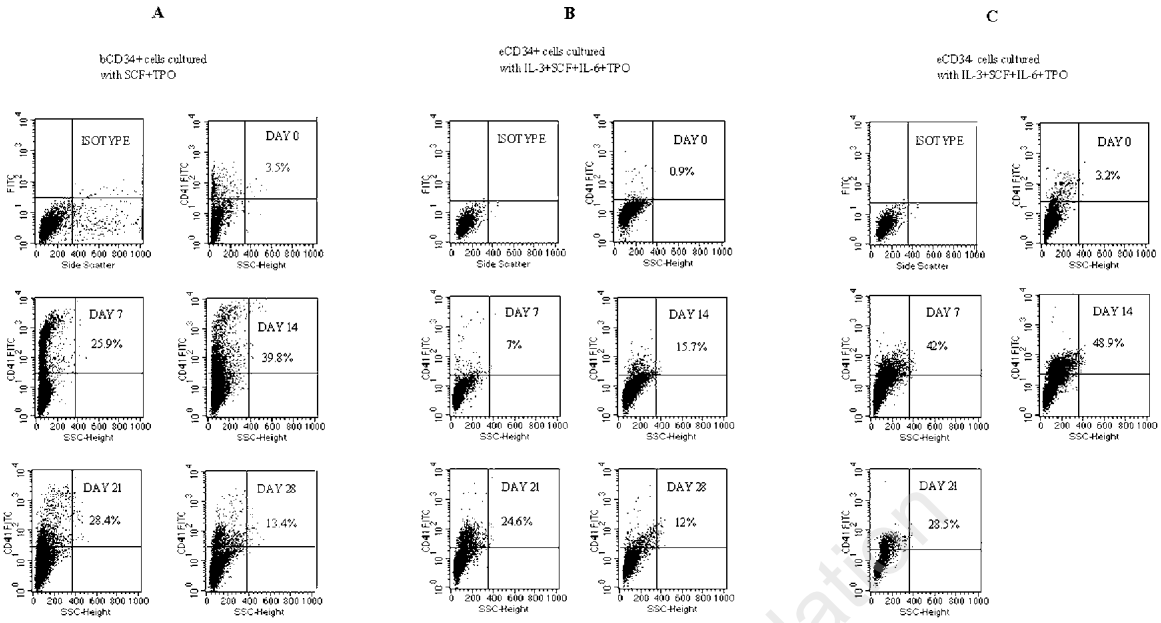

Figure 2. CD41 expression in megakaryocyte-induced differentiation cultures. Representative flow cytometric analysis of CD41 antigen expression on bCD34⁺ cells (A), eCD34⁺ cells (B), and eCD34⁻ cells (C) at start of culture (day 0) and after 7, 14, 21 and 28 days of culture in the presence of TPO+SCF (for baseline cells), and IL-3+SCF+IL-6+TPO (for expanded cells). The numbers in the top right quadrants show the percentages of CD41⁺ cells during the weeks of cultures.

CD41⁺ and CD34⁺CD41⁺ cells were, respectively, $9.78\times10^6\pm$ 1.7×10^6 and $2.19\times10^6\pm0.87\times10^6$): Group I;

- 5×10^5 bCD34⁺ cells that had been induced to differentiate for 10 days with IL-3+SCF+IL-6+TPO (total injected cells: $19\times10^6\pm2.7\times10^6$ of which the counts of CD41⁺ and CD34⁺CD41⁺ cells were, respectively, $11.86\times10^6\pm2.4\times10^6$ and $1.75\times10^6\pm0.63\times10^6$): Group II;
- 5×10^5 eCD34⁺ cells that had been induced to differentiate for 10 days with TPO+SCF (total injected cells: $3.4\times10^6\pm0.6\times10^6$ of which the counts of CD41⁺ and CD34⁺CD41⁺ cells were, respectively, $0.225\times10^6\pm0.1\times10^6$ and $0.33\times10^6\pm0.17\times10^6$): Group III;
- 5×10^5 eCD34⁺ cells that had been induced to differentiate for 10 days with IL-3+SCF+IL-6+TPO (total injected cells: $16.2\times10^5\pm2.9\times10^6$ of which the counts of CD41⁺ and CD34⁺CD41⁺ cells were, respectively, $0.52\times10^6\pm0.13\times10^6$ and $0.825\times10^6\pm0.22\times10^6$): Group IV;
- 5×10^5 eCD34⁻ cells that had been differentiated for 10 days with IL-3+SCF+IL-6+TPO (total injected cells: $0.7\times10^6\pm0.22\times10^6$ of which the count of CD41⁺ cells was $0.26\times10^6\pm0.1\times10^6$): Group V.

The capacity of Mk-committed cells to hasten Mk engraftment and platelet production was evaluated in these mice. The mice were sacrificed 1, 2, 3 and 4 weeks post-transplantation and the presence, in the mouse BM, of total human CD45⁺ cells as well as of human CD41⁺ cells was determined by

Table 1. CFU-Mk production during Mk-differentiation culture of bCD34⁺ and eCD34⁺ cells.

Starting population	*Growth factors*	*Days of culture*			
		7	*14*	*21*	*28*
bCD34⁺	TPO	4±2.3	2±0.9	0	0
	TPO+SCF	41±8.8°	23±5.9°	12.8±5.3*	0
	IL3+SCF+IL6	5±3.2	4.3±1.7	0	0
	IL3+SCF+IL6 +TPO	29±7.7°	19±7.1°	10.7±6.7*	0
eCD34⁺	TPO	0	0	0	0
	TPO+SCF	7.8±4.3	11±5.2*	9±5.4*	0
	IL3+SCF+IL6	11.2±6.5	11±6.1	0	0
	IL3+SCF+IL6 +TPO	19.8±8.9°	28±12.4°	13±6.5*	6±4.3

*The results show the mean±SD fold-increase in CFU-Mk number generated by culturing bCD34⁺ and eCD34⁺ cells. The mean of 10 separate experiments are shown. Time 0: CFU-Mk generated by 2×10^3 bCD34⁺: $0.27\pm0.13\times10^3$; by 2×10^3 eCD34⁺: $0.11\pm0.07\times10^3$; *p<0.05 as compared to the value at day 0; °p<0.001 as compared to the value at day 0.*

Table 2. DNA content in Mk cells obtained during cultures of baseline (bCD34⁺) and expanded (eCD34⁺ and eCD34) cells.

	Days of culture															
Growth factors	*7*				*14*				*21*				*28*			
	2N	*4N*	*8N*	*>8N*	*2N*	*4N*	*8N*	*>8N*	*2N*	*4N*	*8N*	*>8N*	*2N*	*4N*	*8N*	*>8N*
bCD34⁺ SCF+TPO	60.2±18	30±15	0	0	40±13.1	20±12.9	10±5	8±3	40.8±1	25.7±15	20±8	12.4±5	35±9.2	39±19	16±6	10±5
IL3+SCF+IL6+TPO	56.8±17	41±11	0	0	33±12	25.8±14	19±8	14±7.6	34±7.5	32.4±15	26±8	12±6.9	38.9±9	32±15.5	19±6	13±5.9
eCD34⁺ SCF+TPO	69±19.5	23±14	0	0	59±19.6	27±15.9	0	0	60±21.7	39.7±15	0	0	55±12.7	47±16.5	0	0
IL3+SCF+IL6+TPO	65±21.7	32±17.8	0	0	62±22.8	34±11.9	0	0	58±15.7	41.7±13.7	0	0	59±16.8	42.6±12.8	0	0
eCD34⁻ IL-3+SCF+IL-6+TPO					65±27.8	30.9±13.7	0	0	69±24.8	32±18.8	0	0				

The results show the mean ± SD of percentage of CD41⁺ cells with different DNA content (2N, 4N, 8N and >8N) during Mk-differentiating cultures of bCD34⁺, eCD34⁺ and eCD34⁻ cells. Data from 10 separate experiments are shown.

flow cytometry (Table 3). All groups of mice had engrafted by week 1. The levels of human cells were similar in mice of groups I, II and IV (CD45⁺%: 5.2±0.9, 8.9±2.3, 4.75±2.2, respectively) and higher than those of groups III and V (2.5±0.2, 1.6±0.2%, respectively). A high percentage of human CD41⁺ cells was found in the BM of mice belonging to groups I (2.3±0.7% in the total murine BM), II (1.9±0.5%) and IV (1.8±0.6%). The BM of group V contained human CD41⁺ cells, but the engraftment was transient, as at week 2 human Mk cells were no longer detectable. Conversely, in the BM of groups I, II and IV human Mk engraftment persisted for a longer period of time, in particular until week 4 for the mice belonging to groups I and IV. In no cases human engraftment was seen at 6 weeks post-transplantation (*data not shown*).

The presence of B lymphoid (CD19⁺), myeloid (CD33⁺) and erythroid (CD71⁺-glycophorin A⁺) human cells in the BM of mice injected with Mk-committed cells was also evaluated. In all groups of injected mice myeloid and erythroid human cells were found. Interestingly, cells belonging to the lymphoid lineage were found only in group II (*data not shown*).

Human platelet production in NOD/SCID mice injected with Mk-committed precursors

Kinetics of human platelet appearance in all groups of mice injected with Mk-committed cells is shown in Table 3. In groups I (mice injected with the progeny of 5×10^5 bCD34⁺ cells cultured with SCF+TPO) and II (bCD34⁺ cells cultured with IL-3+SCF+IL-6+TPO) a high percentage of platelets was found at days 3 and 5 post-transplantation (about 20%); the platelet production in group II decreased faster than that in group I, in which a good level of platelets persisted until day 21. In group III (eCD34⁺ cultured with SCF+TPO) human platelets peaked at day 5, although at lower levels (7%) and disappeared soon after. By contrast, in group IV (eCD34⁺ cells cultured with IL-3+SCF+IL-6+TPO) human platelets were found already on day 3 (6%) and peaked at day 14 (23%), when, in the other groups of mice, human platelets started to fall. Mk-committed cells derived from eCD34⁻ cells produced 8% platelets; this pro-

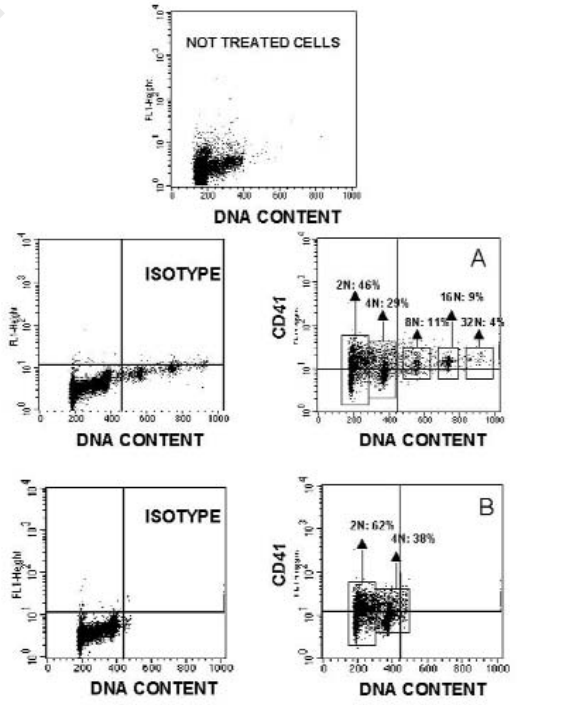

Figure 3. Analysis of DNA content in Mk-differentiation cultures. Representative ploidy analysis of cells from not-treated bCD34⁺, from bCD34⁺ cells cultured in the presence of TPO+SCF for 14 days (A), and from eCD34⁺cells cultured in the presence of IL-3+SCF+IL-6+TPO for 14 days (B). The isotype controls are shown.

Table 3. Megakaryocyte engraftment and platelet production in NOD/SCID mice transplanted with Mk-committed cells.

	Days after transplantation									
	3	5		7		14		21		28
	% Hu Plts PB	% Hu Plts PB	% BM	CD41⁺ % Hu Plts PB	% BM	CD41⁺ Hu Plts PB	% BM	CD41⁺ % Hu Plts PB	% BM	CD41⁺ % Hu Plts PB
Group I	15±5.7	22±5.7	2.3±0.7	27±6.9	1.5±0.6	19±5.3	1±0.3	11±4.8	0.5±0.25	3±2
Group II	12±4	23±7.6	1.9±0.5	17±4.6	1.3±0.4	12±6	0.5±0.3	6±3.9	0.3±0.1	1±0.5
Group III	*	7±2.3	*	1.5±0.7	*	*	*	*	*	*
Group IV	6±2.3	8±3.5	1.8 ±0.6	18±6.9	2.3±0.5	23±6.9	1.5±0.5	15±6.7	1±0.4	7±3.3
Group V	8±3.6	5±2.7	0.5±0.2	2±0.4	*	*	*	*	*	*

*Kinetics of in vivo Mk reconstitution and human platelet production in NOD/SCID mice transplanted with Mk-committed cells generated by 5×10⁵ bCD34⁺ cells cultured with SCF+TPO (group I) or IL-3+SCF+IL-6+TPO (group II), 5×10⁵ eCD34⁺ cells cultured with SCF+TPO (group III) or IL3⁺ SCF⁺ IL6⁺ TPO (group IV) and 5×10⁵ eCD34⁻ cells cultured with IL3+SCF+IL6+TPO (group V). Human Mk engraftment is expressed as the mean±SD of human CD41⁺ cells in total murine BM in animals that were sacrificed at the indicated time points. Human platelet value (Hu Plts) is expressed as mean±SD of human CD41⁺ platelets in murine PB at the indicated time points. Data obtained from 3 mice per group; 3 independent experiments. *:below detection limits.*

duction was transient and disappeared early.

The human platelets in mouse PB were functional as assessed by CD62P expression in response to thrombin stimulation *in vitro* (*data not shown*).

When statistical analysis was performed, a positive linear correlation was found between human Mk engraftment in murine BM and human platelet level in murine PB (group I r=0.99; group II r=0.98; group III r=0.99) (Figure 4).

Discussion

Chemotherapy-induced thrombocytopenia is a one of the major risk factors in cancer treatment. Transfusion of autologous *ex vivo* expanded Mk cells could be a new way to shorten the thrombocytopenic period, particularly after hematopoietic stem cell (HSC) transplantation.

In the first part of this work we studied the effect of various cytokine combinations on the differentiation of bCD34⁺ cells, in order to define the optimum cytokine combination for Mk expansion. Baseline (b) CB CD34⁺ cells produced the largest amount of CD41⁺ cells after 14 and 21 days of culture in the presence of TPO+SCF and IL-3+SCF+IL-6+TPO. Maximum production of less mature progenitors (CD34⁺CD41⁺ cells) was obtained at a much earlier time point (day 7) with TPO+SCF.

It has been demonstrated that *ex vivo* expansion of CB CD34⁺ cells with early-acting cytokines (FL, TPO and SCF) generates large quantities of primitive, *in vivo*-repopulating cells, and less primitive committed progenitors.[4-9] The use of already expanded cells could allow *in vitro* generation of even larger amounts of Mk-committed cells for clinical use. To assess whether expanded (e) CD34⁺ cells still retain differentiation potential towards the Mk lineage, the same cytokine combinations tested with bCD34⁺ were also tested with immunoselected eCD34⁺ and eCD34⁻ cells. Expanded CD34⁺ cells had a lower Mk differentiating ability than bCD34⁺. By contrast, eCD34⁻ cells produced a large proportion (>50%) of CD41⁺ cells. However, in this case the total cell count did not increase much, indicating that eCD34⁻ cells underwent differentiation without much further proliferation, as could be expected from culturing mature cells.

It is well known that CD41 is increasingly expressed during Mk differentiation; in particular a very high expression (CD41^{bright}) is present during the late stage of differentiation;[21-23] the absence of CD41^{bright} expressing cells during Mk-commitment cultures of expanded cells might indicate that expanded cells give rise only to a more immature Mk cell population, while baseline CD34⁺ cells can also produce more mature megakaryocytes.

Ploidy analysis, which evaluates the degree of maturation of CD41⁺ cells produced *in vitro*, shows that TPO+SCF and IL-3+SCF+IL-6+TPO induced formation of 2N, 4N, 8N and some 16N and 32N cells by bCD34⁺. The same cytokine combinations could not induce an increase of DNA content in cells derived from expanded cells; indeed in this case CD41⁺ cells with only a 4N DNA content appeared during the culture. The level of Mk polyploidy level has been shown to depend on the origin of the progenitors and presumably on their maturation profile.[24,25] In this work we show that expanded cells further induced to differentiate into the Mk lineage gave rise to megakaryocytes with a lower ploidy than did bCD34⁺ cells, indicating the immaturity of Mk obtained from previously expanded cells.

In the second part of our work we also examined the possibility of improving and accelerating

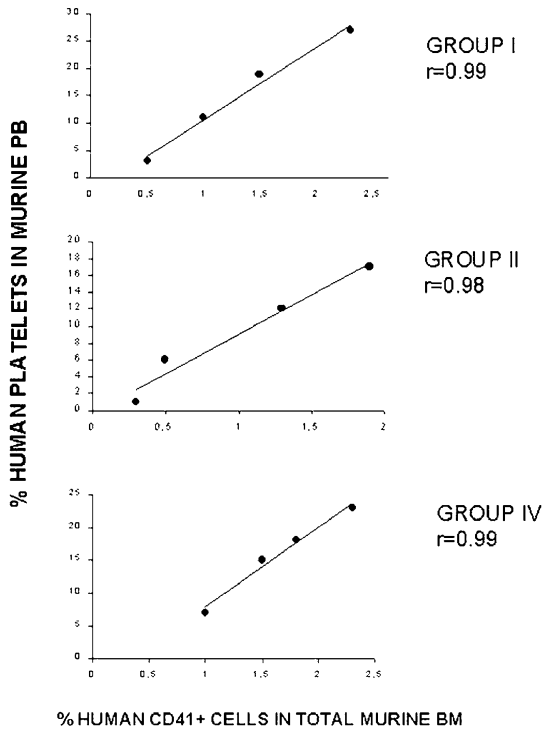

Figure 4. Correlation between human megakaryocyte engraftment in murine BM and the level of human platelets in murine PB. The regression line of a representative experiment (out of 4) is shown. The r coefficients in NOD/SCID groups I, II and IV are also shown.

human platelet recovery in NOD/SCID mice by increasing the number of Mk-committed cells generated by bCD34$^+$, eCD34$^+$ and eCD34$^-$ cells. As expected, we observed that Mk-committed cells derived from the three different CB populations only transiently engrafted the BM of NOD/SCID mice. In all cases human platelets were detectable already 3 days after transplantation and, injecting bCD34$^+$ and eCD34$^+$ cells further induced to Mk-commitment in the presence of IL-3+SCF+IL-6+TPO, human platelets persisted until week 4. Injection of bCD34$^+$ cells committed to differentiate along the Mk lineage with TPO+SCF supported long-lasting production of platelets, which disappeared only at week 4; by contrast, injection of eCD34$^+$ cells further cultured with TPO+SCF supported only a transient production of human platelets.

The *in vivo* Mk differentiation potential and the ability to differentiate completely into platelets in NOD/SCID mice, are retained by Mk-committed, baseline and expanded CD34$^+$ cord blood cells. Notably, human platelets retain normal *in vitro* thrombin activation.

The appearance of human platelets in the PB was not just a consequence of platelets infusion because:

a) platelets were not observed when Mk-committed cells were harvested from liquid cultures at the end of the 10-day period;

b) injection of human platelets into NOD/SCID mice led to the persistence of human platelets in the PB for no longer than 2 days (*data not shown*);

c) there was a strong linear correlation between levels of human platelets in the PB and levels of Mk engraftment in the BM.

So, we may hypothesize that the first wave of platelets in the PB is likely to be the consequence of *in vivo* final maturation of more mature (CD41^{bright}) cells formed *in vitro*. By that time, presumably, some Mks have been produced in the BM, where more immature CD34$^+$ and CD34$^+$CD41$^+$ progenitors start to proliferate and produce more mature CD41$^+$ cells that in turn produce platelets. This second wave of platelet production is long-lasting, as it persists for 4 weeks.

The *in vivo* results are in contrast with *in vitro* data, indicating that eCD34$^+$ cells seem to be incapable of producing large amounts of mature megakaryocytes. The environmental conditions in the NOD/SCID mouse model are capable of overcoming the Mk developmental block observed *in vitro* and allow functional platelet production. These data suggest that a different maturation pathway is triggered *in vitro* in basal and expanded cells. An alternative hypothesis is that there are some other factors *in vivo* that are important for Mk terminal maturation. The identification of these factors may be important for the implementation of clinical protocols.

References

1. To LB, Haylock DN, Simmons PJ, Juttner CA. The biology and clinical uses of blood stem cells. Blood 1997;89:2233-58.
2. Gluckman E, Locatelli F. Umbilical cord blood transplants. Curr Opin Hematol 2000;7:353-7.
3. Rubistein P, Carrier C, Scaradavou A. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. N Engl J Med 1998;339:1565-77.
4. Piacibello W, Sanavio F, Garetto L, Severino A, Bergandi D, Ferrario J, et al. Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. Blood 1997;89:2644-53.
5. Piacibello W, Sanavio F, Garetto L, Severino A, Danè A, Gammaitoni L, et al. Differential growth factor requirement of primitive cord blood hematopoietic stem cell for self-renewal and amplification vs proliferation and differentiation. Leukemia 1998;12:718-27.
6. Piacibello W, Sanavio F, Severino A, Danè A, Gammaitoni L, Fagioli F, et al. Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34$^+$ cord blood cells after ex vivo expansion: evidence for the amplification and self-renewal of repopulating stem cells. Blood 1999;93:3736-49.
7. Ueda T, Tsuji K, Yoshino H, Ebihara Y, Yagasaki H, Hisakawa H, et al. Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6 and soluble IL-6 receptor. J Clin Invest 2000;7:1013-21.
8. Piacibello W, Gammaitoni L, Bruno S, Gunetti M, Fagioli F, Cavalloni G, et al. Negative influence of IL3 on the expansion of human cord blood in vivo long-term repopulating stem cells. J Hematother Stem Cell Res 2000;9:945-56.

9. Bruno S, Gammaitoni L, Gunetti M, Sanavio F, Fagioli F, Aglietta M, et al. Different growth factor requirement for the ex-vivo amplification of transplantable human cord blood cells in a NOD/SCID mouse model. J Biol Reg Homeost Agent 2001;15:38-48.
10. Briddell RA, Brandt JE, Leemhuis TB, Hoffman R. Role of cytokines in sustaining long-term human megakaryocytopoiesis in vitro. Blood 1992;79:332-7.
11. Bruno E, Hoffman R. Effect of interleukin 6 on in vitro human megakaryocytopoiesis: its interaction with other cytokines. Exp Hematol 1989;17:1038-43.
12. Quesemberry PJ, McGrath HE, Williams ME, Robinson BE, Deacon DH, Clark S, et al. Multifactor stimulation of megakaryocytopoiesis: effects of interleukin 6. Exp Hematol 1991;19:35-41.
13. de Sauvage FJ, Carver-Moore K, Luoh SM, Ryan A, Dowd M, Eaton DL, et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. J Exp Med 1996;183:651-6.
14. Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Bailey MC, et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. Nature 1994;369:568-71.
15. Debili N, Wendling F, Katz A, Giuchard J, Breton-Gorius J, Hunt P, et al. The Mpl-ligand or thrombopoietin or megakaryocyte growth and differentiative factor has both direct proliferative and differentiative activities on human megakaryocyte progenitors. Blood 1995;86:2516-25.
16. Lowry PA, Shultz LD, Greiner DL, Hesselton RM, Kittler ELW, Tiarks CY, et al. Improved engraftment of human cord blood cells in NOD/LtSz-scid/scid mice after irradiation or multiple day injections into unirradiated recipients. Biol Blood Marrow Transplant 1996;2:15-23.
17. Güenechea G, Segovia JC, Albella B, Lamana M, Ramirez M, Regidar C, et al. Delayed engraftment of non-obese diabetic/severe combined immunodeficient mice transplanted with ex-vivo expanded human CD34+ cord blood cells. Blood 1999; 93:1097-105.
18. Perez L, Rinder HM, Wang C, Tracey JB, Maun N, Krause DS. Xenotransplantation of immunodeficient mice with mobilized human blood CD34+ cells provides an in vivo model for human megakaryocytopoiesis and platelet production Blood 2001;97:1635-43.
19. Aglietta M, Monzeglio C, Sanavio F, Apra F, Morelli S, Stacchini A, et al. In vivo effect of human granulocyte-macrophage colony stimulating factor on megakaryocytopoiesis. Blood 1991;77:1191-4.
20. Piacibello W, Garetto L, Sanavio F, Severino A, Fubini L, Stacchini A, et al. The effects of human FLT3 ligand on in vitro human megakaryocytopoiesis. Exp Hematol 1996;24:340-6.
21. Philips DR, Charo IF, Parise IV, Fitzgerald LA. The platelet membrane glycoprotein IIb-IIIa complex. Blood 1988;71:831-3.
22. Kanz L, Mielke R, Fauser A. Analysis of human hematopoietic progenitor cells for the expression of glycoprotein IIIa. Exp Hematol 1988;16:741-7.
23. Lepage A, Leboeuf M, Cazenave JP, La Salle C, Lanza F, Uzan G. The αIIβ β3 integrin and GPIb-V-IX complex identify distinct complex stages in the maturation of CD34+ cord blood cells to megakaryocyte. Blood 2000;96:4169-77.
24. Shipper LF, Brand A, Reniers NCM, Melief CJM, Willemze R, Fibbe W. Effect of thrombopoietin on the proliferation and differentiation of primitive and mature haematopoietic progenitors cells in cord blood. Br J Haematol 1998;101:425-35.
25. Mattia G, Vulcano F, Milazzo L, Barca A, Macioce G, Giampaolo A, et al. Different ploidy levels of megakaryocyte generated from peripheral or cord blood CD34+ cells are correlated with different levels of platelets release. Blood 2002; 99;888-97.

Pre-Publication Report & Outcomes of Peer Review

Contributions

WP was responsible for the study design, for the analysis of the data and for study co-ordination. SB, MG and LG worked extensively on the NOD /SCID mouse models, by injecting and sacrificing the animals, harvesting bone marrow and analyzing it by flow cytometry. FS and AD took care of the *in vitro* differentiation studies, selecting the optimum growth factor combinations and culture conditions to obtain Mk-oriented progenitors and cells. GC performed the ploidy analysis of differentiated cells. FS and SB also performed the platelet studies in the peripheral blood of NOD/SCID mice. MA and FF contributed to the critical reading of the manuscript.

Funding

Support for this work was provided by the Associazione Italiana per la Ricerca sul Cancro (AIRC; Milan, Italy) and from the Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR, Rome) to WP and to MA. MG is a recipient of a grant from the "G. Ghirotti Foundation" Piedmont section.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received September 26, 2002; accepted February 25, 2003.

In the following paragraphs, the Editor-in-Chief summarizes the peer-review process and its outcomes.

What is already known on this topic

Since megakaryocyte engraftment is often poor and delayed in cord blood transplantation, attempts have been made to use combinations of hematopoietic growth factors to improve it.

What this study adds

This study shows that human platelet recovery may be improved and accelerated in NOD/SCID mice by increasing the number of megakaryocyte-committed cells generated by cord-blood hematopoietic cells.

Caveats

There is no evidence that this approach may be useful in cord blood transplantation. By contrast, the available evidence indicates that standardized, unrelated donor cord blood transplantation is feasible not only in children but also in young adults (*N Engl J Med 2001;344:1815-22; Blood 2001;98:2332-8*).