

# Increase of CXCR4 Expression on Expanded Non-enriched Cord Blood CD34+ Cells Using MSCs

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## Abstract

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**Introduction:** A number of potential cell adhesion molecules, which mediate essential cell-to-cell or cell-to-matrix interactions, are expressed on the surface of CD34+ hematopoietic progenitor cells (HPCs), including integrins, CD44, and CXCR4. These molecules are essential for homing process. In this study, we compared the changes of expression of CD44 and CXCR4 on the CD34+ hematopoietic progenitor cells expanded on MSCs in the presence of cytokines.

**Material and Methods:** Cord blood CD34+ cells were expanded using human bone marrow mesenchymal stem cells and cytokines (TPO, SCF, FLT-3, IL-6, and IL-3), and then expression of CD44 and CXCR4 on CD34+ cells were evaluated by flow cytometric analysis.

**Results:** After 2 weeks of serum free culture of CD34+ cells in the presence of cytokines, the expression of CXCR4 on CD34+ cells was decreased 3.4 fold ( $p < 0.05$ ). In contrast, the expression of CXCR4 on CD34+ cells expanded on hMSCs was increased ( $p < 0.05$ ). The expression of CD44 on expanded CD34+ cells in both methods did not differ significantly.

**Conclusions:** Our results indicated that co-culture of cord blood stem cells on hMSCs significantly increased CXCR4 expression on cord blood CD34+ cells.

**Keywords:** cord blood stem cells, CXCR4, CD44, Expansion, hMSCs

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## Introduction

Hematopoietic reconstitution after stem cell transplantation depends on the successful homing and proliferation of sufficient primitive repopulating cells within the bone marrow. Homing and retention of hematopoietic stem cells within the bone marrow matrix depend on a variety of adhesion receptors (ARs), a process similar to transendothelial migration of leukocytes to inflammation sites (1). Initially, the cells are attracted to the marrow capillary endothelium by the chemokine stromal cell-

derived factor-1 (SDF-1), produced by bone marrow stromal cells (2). Interactions between stem cells and endothelial cell ligands then facilitate their transmigration from the circulation into the marrow. Migration experiments (3, 4, 5) and *in vivo* studies (4, 6, 7) have implicated very late antigen-4 (VLA-4), VLA-5, P-selectin, E-selectin, platelet endothelial cell adhesion molecule-1, CD44, and CXCR4 as crucial factors for homing and engraftment of stem cells.

*In vitro* expansion of CD34+ cells with cytokines is clinically important in cord blood (CB) transplantation and for retroviral gene therapy. However, cytokines not only induce cell division, but also drive stem cells to differentiate into more mature cells lacking the ability to repopulate (8, 9, 10). In phase I clinical studies, *in vitro*-expanded peripheral blood stem cells infused into myeloablated patients resulted in long-term engraftment failure (11). However, further research has indicated that the correct choice of cytokine combinations is crucial for the maintenance of cells repopulating the marrow. The use of stem cell factor (SCF), Flt-3-ligand (Flt-3), and thrombopoietin (TPO) has produced strong *in vitro* expansion of cytokine-cultured NOD/SCID mouse repopulating cells (12, 13). Even if repopulating cells are maintained in culture, engraftment defects will still occur if they cannot home correctly to the bone marrow or have reduced proliferative potential after homing. There is evidence of a dramatic reduction in the homing of colony-forming cells from cytokine-expanded cultures in animal models (14, 15, 16), together with altered adhesive characteristics (17, 18). These data point to an alteration in AR function in cultured stem cells. Joy Kahn et al reported that cells over-expressing CXCR4 exhibited significant increases in SDF-1-mediated chemotaxis and actin polymerization compared to control cells. A major advantage of CXCR4 over-expression was demonstrated by the ability of transduced CD34+ cells to respond to lower, physiologic levels of SDF-1 when compared to control cells, leading to improved SDF-1-induced migration and proliferation/survival, and finally resulting in significantly higher levels of *in vivo* repopulation of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice including primitive CD34<sup>+</sup>/CD38<sup>-low</sup> cells. These results suggest CXCR4 over-expression for improved definitive human stem cell motility, retention, and multilineage repopulation, which could be beneficial for *in vivo* navigation and expansion of hematopoietic progenitors (19).

This paper describes persistently increased expression of CXCR4 on CD34+ cells during co-culture of non-enriched cord blood progenitor/stem cells with MSCs and cytokines. These methods may be useful for hematopoietic reconstitution and successful

homing and proliferation of sufficient primitive repopulating cells after cord blood stem cell transplantation.

## Material and Methods

### *Umbilical cord blood cells (UCB)*

UCB was collected after obtaining consent from patients (n=13) scheduled to undergo Cesarean section. On average, 60 ml UCB was collected by gravity into a sterile container after cutting the distal end of the cord. Heparin (1000 U) was added to prevent coagulation. White blood cells (WBCs) were counted with hemocytometer using trypan blue. The sample was diluted with an equal volume of Hank's balanced salt solution (HBSS) (Sigma, St. Louis, MO), and layered onto Ficoll-Hypaque (Pharmacia-Amersham, Piscataway, NJ; d=1.077 g/ml) density gradients to deplete red blood cells (RBC) (20). The mononuclear cell interface was collected, diluted into three volumes of HBSS, and pelleted at 250 g for 10 minutes. The cell pellet was washed two more times and resuspended in either 5 ml of expansion media. When necessary, RBC-depleted UCB cells were stored frozen at -70 °C after suspending the cells in HBSS at a concentration of  $2 \times 10^8$  cells/ml, and adding an equal volume of pentastarch cryopreservative medium containing 10% dimethyl sulfoxide (DMSO)/8% HSA/12% pentastarch in normosol R, at a final cell concentration of  $10^8$  cells/ml. For further processing, frozen samples were thawed in a 37°C water bath.

### *Human mesenchymal stem cells culture*

Bone marrow aspirates were obtained from healthy donors after informed consent. The marrow was mixed with the same volume of PBS, and mononuclear cells were separated by density gradient centrifugation. Washed cells were resuspended in stroma medium (RPMI 1640, Dutch Modification, supplemented with 10% fetal calf serum [FCS], 2 mM L-glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 1.0 µmol/l hydrocortisone).

$5 \times 10^6$  cells were placed in 80-cm<sup>2</sup> flasks (Nunc; Wiesbaden, Germany) and maintained at 37°C and 5% CO<sub>2</sub>. Half the medium was exchanged twice a week. After two weeks, confluent adherent layers were passaged with trypsin-EDTA solution 1X (Sigma-Aldrich; Steinheim, Germany).

Trypsin was quickly inactivated with FCS-containing medium and centrifuged at 200 *g* for 10 min. The pellet was resuspended in stromal medium, and the cells of one 80-cm<sup>2</sup> culture vessel were transferred to six 25-cm<sup>2</sup> flasks or six-well plates for further expansion experiments. Second passage MSCs were seeded at 3 × 10<sup>3</sup> cells/cm<sup>2</sup>, grown to confluence in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) (GIBCO Life Technologies; Gaithersburg, MD), and irradiated (15 Gy, <sup>137</sup>Cs irradiation) prior to co-culture with UCB, to prevent MSC overgrowth.

#### Cytokines

Recombinant human flt-3 ligand (FL), recombinant human thrombopoietin (TPO), recombinant human stem cell factor (SCF, also known as mast cell growth factor [MGF] or c-kit ligand [KL]), recombinant human interleukin-6, and interleukin-3 were used in this study. All cytokines used were purchased from Stem cell Technology, Vancouver, BC, Canada.

#### Co-culture of cord blood hematopoietic cells and human mesenchymal stem cell (MSCs)

MSCs (6.0 × 10<sup>5</sup>) were plated in 75-cm<sup>2</sup> flasks in 20 ml of DMEM with 10% FBS supplemented antibiotics and incubated for one week at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. On the day of co-culture, the MSCs cells were washed with and re-cultured in the serum-free Stemspan medium (Stemcell Company, Canada) and then gamma-irradiated with a <sup>137</sup>Cs at a dosage of 1,500 cGy. Irradiated feeder cells were trypsinized and ten thousand cells transferred to 96-well microplate (nunc). 2 × 10<sup>5</sup> mononuclear cells were cultured on monolayers pre-established in 96-well microplate using 100 μL of Stemspan medium supplemented with combinations of human TPO (50 ng/ml, Stem Cell Company, Canada), human SCF (50 ng/ml), human FL (50 ng/ml), IL-6 (50 ng/ml), and IL-3 (50 ng/ml) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air for 2 weeks. Culture medium was replaced after 1 week of culture with a fresh medium containing cytokines.

#### Flow cytometry

Aliquots of cells were stained with fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal

antibodies in phosphate-buffered saline (PBS)/0.1% BSA at 4°C for 30 minutes. Analysis was performed using an EPICS XL flow cytometer (Coulter, Tokyo, Japan). Antibodies used were as follows: FITC-conjugated CD44 antibody (R&D system), FITC-conjugated CXCR4 (R&D system) and PE-conjugated CD34 (DAKO, Denmark). FITC- and PE-conjugated mouse IgG1 antibodies (DAKO, Denmark) were used as isotype-matched controls. Dead cells were gated out with a forward vs. side scatter window and propidium iodide staining.

## Results

#### Expansion culture of CB CD34<sup>+</sup> cells

We first examined the hematopoiesis-supporting effects of MSC cells. 2 × 10<sup>5</sup> MNC cells from cord blood were plated on an MSC cell layer under serum-free conditions with or without combinations of TPO, SCF, IL-3, IL-6 and FL.

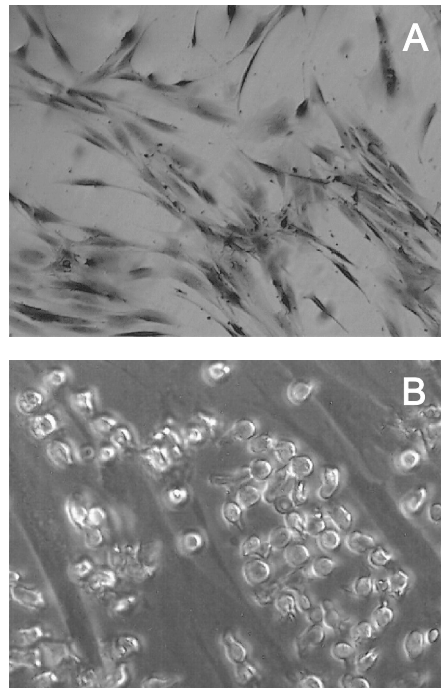
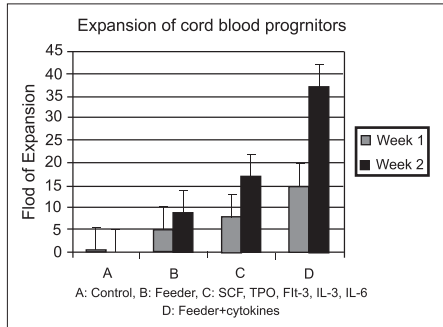


Figure 1. (A) Phase microscopy of human mesenchymal stem cells stained by haematoxylin (10x). (B) Appearance of proliferating cells in the co-culture of human cord blood CD34<sup>+</sup> cells on a monolayer of mesenchymal stem cells in the presence of TPO, SCF, IL-3, IL-6, and FL. (40x)

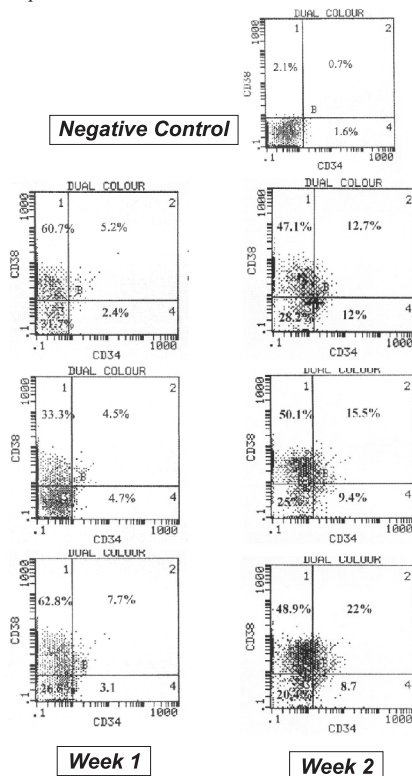
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Cells not adhering and adhering weakly to MSC cells were collected by gentle pipetting after 2 weeks of culture for analysis. Without cytokines, the mean number of total nucleated cells, CD34<sup>+</sup> cells, and CD34<sup>+</sup>CD38<sup>-</sup> cells after 2 weeks of culture was 2–

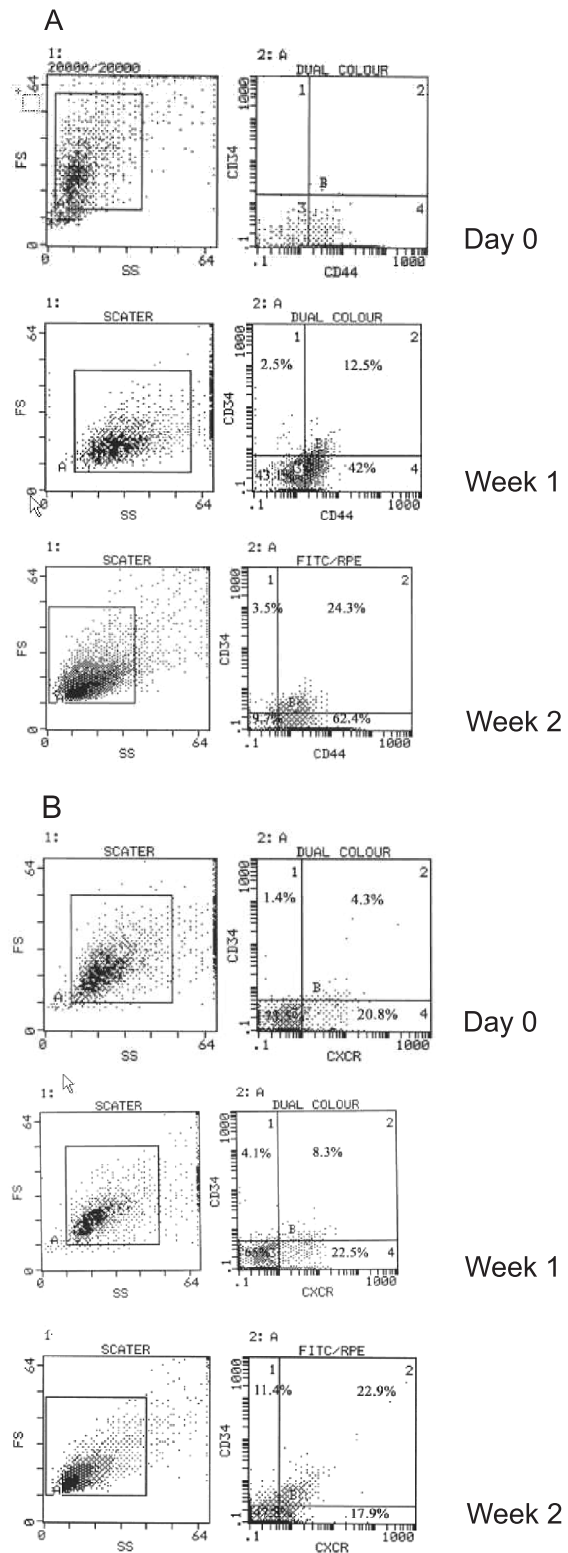
5 times the initial input number. In contrast, in the presence of TPO, not only total nucleated cells, CD34<sup>+</sup> cells, and CD34<sup>+</sup>CD38<sup>-</sup> cells, but also CFU-C, CFU-Mix, and HPP-CFC were significantly expanded.



**Figure 2A.** Expansion of human cord blood progenitors.  $2 \times 10^5$  human cord blood mononuclear cells were plated on a monolayer of human MSCs in the presence of combinations of cytokines (one culture for each group). After 2 weeks of culture, growing nonadherent hematopoietic cells and cells weakly attached to MSCs were collected by gentle pipetting and analyzed by flow cytometry and colony assay. Data represent mean  $\pm$  SD-fold increase compared to the initial value in four experiments performed on 13 separate cord blood donors.



**Figure 2B.** In vitro expansion of CD34<sup>+</sup> CB cells on MSCs.  $2 \times 10^5$  human cord blood mononuclear cells were plated on a monolayer of human MSCs in the presence of combinations of cytokines (one culture for each group). After 2 weeks of culture, growing nonadherent hematopoietic cells and cells weakly attached to MSCs were collected by gentle pipetting and analyzed by flow cytometry.



**Figure 3.** Flow cytometric analysis of CD44 (A) and CXCR4 (B) expression on fresh uncultured CB CD34<sup>+</sup> cells and after culture for 1 and 2 week with 50 ng/ml SCF, Flt3, TPO, IL-3, and IL-6 in serum-free media (Stem Span) co-cultured with MSCs.

The addition of SCF and/or FL to TPO further enhanced the expansion of nucleated cells and progenitors. Representative phase microscopy of MSC cells and growing hematopoietic cells in culture supplemented with TPO, SCF, IL-3, IL-6, and FL are shown in Figure 1. Although there were some differences in the degree of expansion among experiments, the maximum output of progenitors was consistently observed when stimulated with TPO, SCF, IL-3, IL-6, and FL. The results of four experiments are shown in Figure 2A and 2B.

	CD34+CD44+	CD34+CXCR4+
Control	92±6	72.6±5
Expansion in serum free	82±6.1	24.5±3.8*
Co-culture with MSCs	89.1±7	65.5±4.8

**Table 1.** The proportions of CD34<sup>+</sup> cells which were positive for CD44 and CXCR4 expression before and after 2 weeks of co-culture with MSCs and without co-culture in serum-free media. Control is day 1 of culture. Data represent mean ± SD of analysis of 13 separate cord blood donors.

## Discussion

The expression levels were within the ranges observed on other leukocytes: 80% or more CB CD34<sup>+</sup> cells were positive for CD44, SDF-1 receptor, and CXCR4, and nearly 50% were positive for VLA-5. Another study of AR expression on CD34<sup>+</sup> cells within whole blood showed similar proportions of positive cells, but lower levels of CD44 expression (median staining intensity <5) were reported, as the red cells were not removed before staining (21). There are several references to AR expression either on MNCs or purified CD34<sup>+</sup> cells (22, 23, 24, 25). However, these studies did not compare AR expression on fractionated cells with unprocessed cells. Our data showed that processing and purification of CB MNCs did not affect the expression of ARs on CD34<sup>+</sup>

cells.

There are two reports suggesting that AR expression on CD34<sup>+</sup> cells during cytokine culture is not altered (26, 27), while Chute *et al.*

reported that culture of bone marrow CD34<sup>+</sup> cells on porcine microvascular endothelial cells (PMVECs) with cytokines for 7 days produced a significant increase in the expression of VLA-4, CD58 (LFA-3), and L-selectin, but not of LFA-1 or CD44 (23). It is possible that different microenvironments and cytokine mixtures affect expression of individual ARs more or less. However, our experiments used culture conditions that are most likely to be used clinically with a growth factor combination that sustains repopulating cells. Recent studies have shown that engraftment of primitive human CD34<sup>+</sup> cells in NOD/SCID mice was dependent on the SDF-1 receptor and CXCR4, regardless of the initial level of surface expression (28, 29). Cells that are initially CXCR4 negative contain internal CXCR4 that can migrate to the surface under the right environment. Denning-Kendall *et al.* observed a much greater proportion of CXCR4 negative cells after cytokine culture (30). This may be due to a down-regulation of CXCR4 production, in which case there could be fewer internal CXCR4, or fluctuation of CXCR4 from the surface to the interior. We observed that co-culture of CB stem cells with MSCs after 2 weeks significantly increased CXCR4 cells.

## Conclusion

Given the reliance of engrafting cells upon migration toward SDF-1, an absolute increase in CXCR4 expression could have serious consequences on the engrafting capability of CD34<sup>+</sup> cells and increase of successful homing and stem cell transplantation.



## References

- Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994; 76: 301–314
- Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC: The chemokine SDF-1 is a chemoattractant for human CD34<sup>+</sup> hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34<sup>+</sup> progenitors to peripheral blood. *J Exp Med* 1997; 185:111–120
- Naiyer AJ, Jo DY, Ahn J, Mohle R, Peichev M, Lam G, Silverstein RL, Moore MA, Rafii S: Stromal derived factor-1-induced chemokinesis of cord blood CD34(+) cells (long-term culture-initiating cells) through endothelial cells is mediated by E-selectin. *Blood* 1999; 94: 4011–4019
- Peled A, Kollet O, Ponomaryov T, Petit I, Franitza S, Grabovsky V: The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature

- human CD34 (+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood* 2000; 95: 3289–3296
5. Yong KL, Watts M, Shaun Thomas N, Sullivan A, Ings S, Linch DC: Transmigration of CD34<sup>+</sup> cells across specialized and nonspecialized endothelium requires prior activation by growth factors and is mediated by PECAM-1 (CD31). *Blood* 1998; 91:1196–1205.
6. Van der Loo JC, Xiao X, McMillin D, Hashino K, Kato I, Williams DA: VLA-5 is expressed by mouse and human long-term repopulating hematopoietic cells and mediates adhesion to extracellular matrix protein fibronectin. *J Clin Invest* 1998; 102: 1051–1061
7. Frenette PS, Subbarao S, Mazo IB, von Andrian UH, Wagner DD: Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. *Proc Natl Acad Sci USA* 1998; 95: 14423–14428
8. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE: Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci USA* 1997; 94: 5320–5325
9. Bryder D, Jacobsen SE: Interleukin-3 supports expansion of long-term multilineage repopulating activity after multiple stem cell divisions in vitro. *Blood* 2000; 96: 1748–1755
10. Piacibello W, Gammaitoni L, Bruno S: 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: 945–956
11. Holyoake TL, Alcorn MJ, Richmond L, Farrell E, Pearson C, Green R: CD34 positive PBPC expanded ex vivo may not provide durable engraftment following myeloablative chemoradiotherapy regimens. *Bone Marrow Transplant* 1997; 19: 1095–1101
12. Piacibello W, Sanavio F, Severino A: 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: 3736–3749
13. Ueda T, Tsuji K, Yoshino H, Ebihara Y, Yagasaki H, Hisakawa H, Mitsui T: 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; 105:1013–1021.
14. Szilvassy SJ, Bass MJ, Van Zant G, Grimes B: Organ-selective homing defines engraftment kinetics of murine hematopoietic stem cells and is compromised by ex vivo expansion. *Blood* 1999; 93: 1557–1566
15. Spiegel A, Kollet O, Peled A: Both human leukemic pre-B ALL and normal cord blood CD34<sup>+</sup> cells home into NOD/SCID mice in a SDF-1/CXCR4-dependent manner but differ in their signaling and activation. *Blood* 2001; 98: 313
16. Kerre TC, De Smet G, De Smedt M, Offner F, De Bosscher J, Plum J, Vandekerckhove B: The behavior of long-term cultured human hematopoietic stem cells early after transplantation into NOD/SCID mice provides explanations for their impaired long-term engraftment in this model. *Blood* 2001; 98: 645a
17. Giet O, Huygen S, Beguin Y, Gothot A: Cell cycle activation of hematopoietic progenitor cells increases very late antigen-5-mediated adhesion to fibronectin. *Exp Hematol* 2001; 29: 515–524
18. Yamaguchi M, Ikebuchi K, Hirayama F, Sato N, Mogi Y, Ohkawara J: Different adhesive characteristics and VLA-4 expression of CD34(+) progenitors in G<sub>0</sub>/G<sub>1</sub> versus S+G<sub>2</sub>/M phases of the cell cycle. *Blood* 1998; 92: 842–848
19. Kahn J, Byk T, Jansson-Sjostrand L, Petit I, Shivtiel S: Overexpression of CXCR4 on human CD34<sup>+</sup> progenitors increases their proliferation, migration, and NOD/SCID repopulation. *Blood* 2004; 103: 2942-2949
20. Denning-Kendall PA, Horsley H, Donaldson C, Bradley B, Hows JM: Different behaviour of fresh and cultured CD34<sup>+</sup> cells during immunomagnetic separation. *Br J Haematol* 1999; 105: 780–785
21. Timeus F, Crescenzo N, Basso G, Ramenghi U, Saracco P, Gabutti V: Cell adhesion molecule expression in cord blood CD34<sup>+</sup> cells. *STEM CELLS* 1998; 16: 120–126
22. Asosingh K, Renmans W, Van der Gucht K, Foulon W, Schots R, Van Riet I, De Waele M. Circulating CD34<sup>+</sup> cells in cord blood and mobilized blood have a different profile of adhesion molecules than bone marrow CD34<sup>+</sup> cells. *Eur J Haematol* 1998; 60: 153–160
23. Chute JP, Saini AA, Kampen RL, Wells MR, Davis TA: A comparative study of the cell cycle status and primitive cell adhesion molecule profile of human CD34<sup>+</sup> cells cultured in stroma-free versus porcine microvascular endothelial cell cultures. *Exp Hematol* 1999; 27: 370–379
24. Hattori Y, Kato H, Nitta M, Takamoto S: Decrease of L-selectin expression on human CD34<sup>+</sup> cells on freeze-thawing and rapid recovery with short-term incubation. *Exp Hematol* 2001; 29:114–122
25. Roy V, Verfaillie CM: Expression and function of cell adhesion molecules on fetal liver, cord blood and bone marrow hematopoietic progenitors: implications for anatomical localization and developmental stage specific regulation of hematopoiesis. *Exp Hematol* 1999; 27: 302–312
26. Dravid G, Rao SG: Ex vivo expansion of stem cells from umbilical cord blood: expression of cell adhesion molecules. *STEM CELLS* 2002; 20: 183–189
27. Reems JA, Mielcarek M, Torok-Storb B: Differential modulation of adhesion markers with ex vivo expansion of human umbilical CD34<sup>+</sup> progenitor cells. *Biol Blood Marrow Transplant* 1997; 3: 133–141
28. Rosu-Myles M, Gallacher L, Murdoch B, Hess DA, Keeney M, Kelvin D: The human hematopoietic stem cell compartment is heterogeneous for CXCR4 expression. *Proc Natl Acad Sci USA* 2000; 97: 14626–14631
29. Kollet O, Spiegel A, Peled A, Petit I, Byk T, Hershkovitz R, Guetta E: Sorted human cord blood CD34<sup>+</sup>CXCR4<sup>+</sup> cells contain internal CXCR4 which can oscillate in vivo and rescue low SDF-1 dependent repopulation in NOD/SCID mice. *Blood* 2001; 97: 3283-3291
30. Denning-Kendall P, Singha S, Bradley B, Hows J Cytokine Expansion Culture of Cord Blood CD34<sup>+</sup> Cells Induces Marked and Sustained Changes in Adhesion Receptor and CXCR4 Expressions. *Stem Cells* 2003; 21: 61-70

