



## Mobilized peripheral blood CD34<sup>+</sup> cells express more amphotropic retrovirus receptor than bone marrow CD34<sup>+</sup> cells

MARCO BREGNI, MASSIMO DI NICOLA, SALVATORE SIENA, NADIA BELLI, MARCO MILANESI, SUZY SHAMMAH, FERNANDO RAVAGNANI, ALESSANDRO M. GIANNI

The Cristina Gandini Transplantation Unit, Division of Medical Oncology, and the Division of Immunohematology, Istituto Nazionale Tumori, Milan, Italy

### Abstract

**Background and Objective.** The increased susceptibility to gene transfer by amphotropic retroviral vectors of mobilized peripheral blood (PB) CD34<sup>+</sup> cells compared to their bone marrow (BM) counterparts may depend, among other factors, on the level of expression of the amphotropic receptor on the progenitor cell. Using a previously described flow cytometry strategy, we have studied retrovirus binding to mobilized CD34<sup>+</sup> cells, derived from cancer patients treated with high-dose chemotherapy and growth factor(s), that are efficiently transduced by N2 retrovirus vector.

**Design and Methods.** We measured the binding of the retrovirus to the cells using a rat monoclonal antibody reactive with the gp70 envelope glycoprotein, common to all replication-defective amphotropic retroviruses. Antibody-virus-cell complexes were indirectly labeled and analyzed by flow cytometry. We compared the binding of PA317-N2 vector to CD34<sup>+</sup> cells derived from steady-state BM, steady-state PB and mobilized PB from cancer patients treated with high-dose chemotherapy and cytokine.

**Results.** The fluorescence intensity of mobilized CD34<sup>+</sup> cells was approximately one log higher than that of steady-state BM or PB CD34<sup>+</sup> cells, indicating that the expression of the amphotropic receptor was increased. Moreover, the virus binding was proportional to the gene transfer rate, as assessed by G418 resistance into mobilized PB-derived CFU-GM. The increase in fluorescence intensity appeared to be restricted to CD34<sup>+</sup> cell subset, neither CD2<sup>+</sup> nor CD14<sup>+</sup> cells bound the virus in an appreciable amount.

**Interpretation and Conclusions.** Virus binding, as assessed by indirect immunofluorescence assay, is increased in mobilized CD34<sup>+</sup> cells. The increased binding may contribute to their high susceptibility to retrovirus vector infection.

©1998, Ferrata Storti Foundation

Key words: stem cells, CD34, gene transfer, retroviruses, amphotropic receptor

Correspondence: Marco Bregni, M.D., Istituto Nazionale Tumori, via Venezian 1, 20133 Milan, Italy.  
Phone: international +39-2-2390717 • Fax: international +39-2-2390678.

In preclinical and clinical studies, replication-defective amphotropic retroviruses have been most commonly used as vehicles for transducing genes into hematopoietic progenitors.<sup>1</sup> Mobilized peripheral blood progenitors are better targets of retroviral-mediated gene transfer than bone marrow steady-state progenitors, as demonstrated by PCR-assessed transfer rate and G418 resistance into single CFU-GM colonies.<sup>2,3</sup> The increased susceptibility to virus infection of mobilized progenitors may depend, among other factors, on higher expression of the amphotropic receptor used for virus entry. Recently, the human retrovirus receptor has been cloned, and its function was demonstrated by its susceptibility to virus infection of receptor-negative cell lines upon ectopic expression of the receptor cDNA.<sup>4</sup> To our knowledge, direct measurement of the receptor expression levels on target cells is not available as specific monoclonal antibodies for the receptor have yet to be developed. However, Crooks and Kohn recently reported a flow cytometry assay for measuring the binding of retrovirus to the cell using the rat monoclonal antibody 83A25, reactive with the viral envelope gp70 glycoprotein, common to all murine leukemia viruses.<sup>5</sup> By employing this approach, they were able to demonstrate that the culture of CD34<sup>+</sup> bone marrow cells with IL-3, IL-6 and stem cell factor increases the virus binding by two to fourfold, and concluded that increased receptor expression may be one mechanism by which cytokines enhance gene transfer into bone marrow CD34<sup>+</sup> cells.<sup>6</sup>

Using this flow cytometry strategy, we have studied retrovirus binding to mobilized CD34<sup>+</sup> cells derived from cancer patients treated with high-dose chemotherapy and growth factor(s), efficiently transduced by the N2 retrovirus vector.<sup>2</sup> We have found that their fluorescence intensity is increased in comparison to that of steady-state BM and PB CD34<sup>+</sup> cells. In addition, we found a significant correlation between the virus binding and the rate of gene transfer, indicating that this assay can predict the transduction efficiency into target cells.

## Materials and Methods

### Target cells

Hematopoietic cells utilized in these experiments were taken from: 1) leukapheresis cell suspensions collected for autografting from non-Hodgkin's lymphoma and breast cancer patients in the recovery-phase after high-dose cyclophosphamide therapy (HD-CTX) and cytokine treatment;<sup>7</sup> 2) bone marrow aspirates from cancer patients with non-hematologic malignancies undergoing staging procedures with biopsy-proven normal bone marrow; 3) leukapheresis cell suspensions from normal volunteers undergoing apheresis for platelet donation. All patients and donors were informed and gave oral consent to the procedure. Hematopoietic cells from leukapheresis and from bone marrow suspensions were utilized without further processing. In some experiments, CD34<sup>+</sup> cells were selected by a high gradient magnetic cell separation system (MACS; Miltenyi Biotech, Bergish Gladbach, Germany), according to the instructions of the manufacturer. Prior to exposure to the viral supernatant, either unmanipulated or CD34<sup>+</sup> selected cells were resuspended in Iscove's modified Dulbecco medium supplemented with 10% heat-inactivated fetal bovine serum (IMDM/10%) plus Steel factor 50 ng/mL and IL-3 75 ng/mL. They were cultured at 37°C in 5% CO<sub>2</sub> for 48 hours. Jurkat cell line, which expresses high amounts of the amphotropic receptor, was utilized as a positive control for retroviral binding.

### Monoclonal antibodies

83A25 is a IgG<sub>2</sub> rat monoclonal antibody reactive with gp70 envelope protein, common to all replication-defective amphotropic viruses.<sup>5</sup> An isotype-matched rat anti-mouse SCF antibody (Genzyme, Cambridge, MA, USA) was utilized as an irrelevant control. Fluorescein-conjugated CD34 HPCA-2, CD2 Leu-5b, CD14 Leu-M3 and CD19 Leu-12 antibodies (Becton Dickinson, Mountain View, CA, USA) were utilized according to the manufacturer's instructions.

### Retroviral vectors

The N2 retroviral vector, which carries the neo gene that confers resistance to G418, was produced by the PA317 packaging cell line. Producer cells were grown in IMDM/10%. Viral titer was approximately 10<sup>7</sup> cfu/mL on the NIH<sub>3</sub>T<sub>3</sub> cell line. Fresh viral supernatant was 0.45 μm filtered from confluent cultures and immediately utilized. An ecotropic replication-defective retrovirus (ψ2 ALDO A1),<sup>8</sup> that does not bind to amphotropic receptor, was utilized as negative control.

### Flow cytometry analysis of retrovirus binding

The virus binding assay has already been described.<sup>6</sup> Briefly, target cells were incubated with fresh viral supernatant at a multiplicity of infection of 10:1 in the

presence of protamine sulphate 5 μg/mL (Sigma, St. Louis, MO, USA) for 2 hours at 37°C. Thereafter, all procedures were carried out on ice. After washings in PBS 1% FBS 0.01% sodium azide, the cells were incubated for 20 minutes with 83A25 or with an irrelevant antibody, washed, and incubated with biotinylated goat anti rat antiserum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After further washings, the cells were incubated for 20 minutes with R-phycoerythrin-conjugated streptavidin (PE) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), washed and incubated for 20 minutes with FITC-conjugated CD34 antibody. In some experiments, FITC-conjugated CD2, CD14 or CD19 antibodies were employed in place of CD34. After final washings, samples were analyzed on a FACScan (Becton Dickinson, Mountain View, CA, USA) equipped with Lysis II version 1.0 software. All cells were acquired, with the exclusion of erythrocytes when present in the sample. At least 10,000 events were analyzed. PE fluorescence was evaluated on a FITC-positive CD34<sup>+</sup> cell population after appropriate gating. PE mean fluorescence intensity was calculated by histogram statistics function (Lysis II Software, Becton Dickinson, Mountain View, CA, USA). The increase in mean fluorescence intensity over irrelevant controls was utilized as a measure of retrovirus binding to target cells.

### Correlation between virus binding and gene transfer into mobilized progenitors

Mobilized PB cells from a cancer patient (UPN 95/809) undergoing chemotherapy with HD-CTX and filgrastim were collected by leukapheresis at day +11 after chemotherapy administration. A sample of leukapheresis cell suspension was cultured for 48 hours in IMDM/10% with SCF and IL-3. Thereafter, aliquots of cells were infected with serial dilutions of N2 supernatant (undiluted; 1:5; 1:10; 1:100 dilutions) for 3 hours at 37°C with protamine 5 μg/mL. Half of each cell aliquot was utilized for virus binding assay (see above). The other half was plated in methylcellulose for G418 resistance, as already described.<sup>2</sup> Briefly, 5,000 cells from each sample were plated with or without G418 (1.0 mg/mL active dose), in Methocult H4230 medium (Stem Cell Technologies, Vancouver, CA, USA), with 10% 5637 conditioned medium. After 14 days in the culture, the percentage of G418-resistant colonies was evaluated by comparing the numbers of colonies grown in the presence of G418 to that of untreated controls. The relation between the mean fluorescence intensity of samples stained with 83A25 and the percentage of G418-resistant colonies was evaluated by linear regression and correlation analysis using a commercially available computer program (Cricket Graph III, Cricket Software, Philadelphia, PA, USA).

## Results

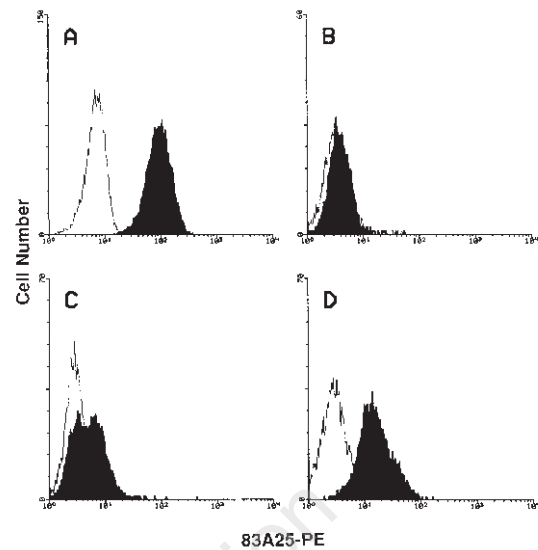
CD34<sup>+</sup> selected cells from steady-state peripheral blood, steady-state bone marrow and mobilized

peripheral blood were cultured for 48 hours in medium plus cytokines, infected with N2 retrovirus vector, and analyzed for virus binding by 83A25 staining. As shown in Figure 1, the PE fluorescence intensity of the mobilized CD34<sup>+</sup> cells was one log higher than that of the steady-state PB or BM CD34<sup>+</sup> cells, indicating that the virus binding to the amphotropic receptor is increased in the former cells (Panel D versus Panel B and C, respectively). These data were confirmed in 17 patients who underwent HD-CTX chemotherapy and cytokine(s) as primary treatment for their disease (high-risk breast cancer or diffuse large cell lymphoma). Taken together, the PE mean fluorescence intensity of the CD34<sup>+</sup> cells from mobilized PB after culture was increased 6.5 fold over negative controls, versus 2.4 fold in the steady-state BM34<sup>+</sup> cells. Virus binding was not influenced by different diseases (non-Hodgkin's lymphoma vs breast cancer) or by different cytokine treatment (G-CSF vs GM-CSF) (data not shown). Specificity was assessed by substituting 83A25 antibody with an irrelevant antibody against rat stem cell factor and by infecting CD34<sup>+</sup> cells with an ecotropic supernatant (not shown). Positive control consisted of Jurkat cells, which express high amounts of the amphotropic receptor, and stained brightly with 83A25, as already reported<sup>6</sup> (Figure 1, Panel A).

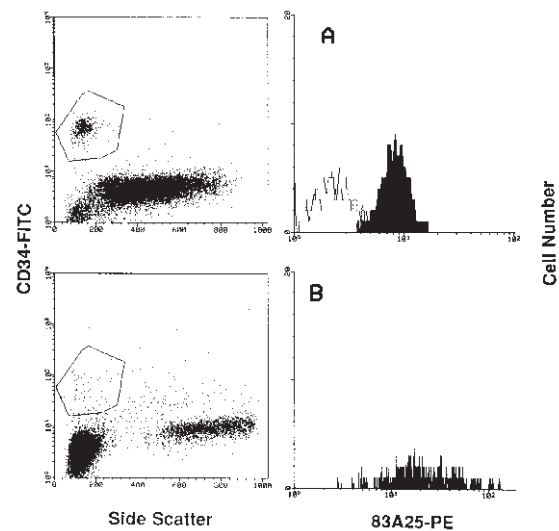
Virus binding was also studied on fresh, uncultured cells from leukapheresis cell suspension and from bone marrow buffy coat. As shown in Figure 2, mobilized CD34<sup>+</sup> cells remarkably bound the retrovirus. No binding was observed on uncultured bone marrow cells.

In order to understand whether a correlation exists between the amount of virus binding and the gene transfer rate into target cells, a viral supernatant was serially diluted and then utilized to infect mobilized PB cells after a 48-hour incubation with cytokines. A cell aliquot was stained with 83A25 antibody, and another aliquot was plated in methylcellulose to assay for G418-resistant colonies (see *Materials and Methods*). As illustrated in Figure 3 and Table 1, the mean fluorescence intensity of the samples decreased with supernatant dilutions, and the percentage of G418-resistant colonies decreased accordingly. The correlation coefficient between the two variables ( $r=0.869$ ) confirmed that the virus binding is an important factor determining the efficiency of the infection.

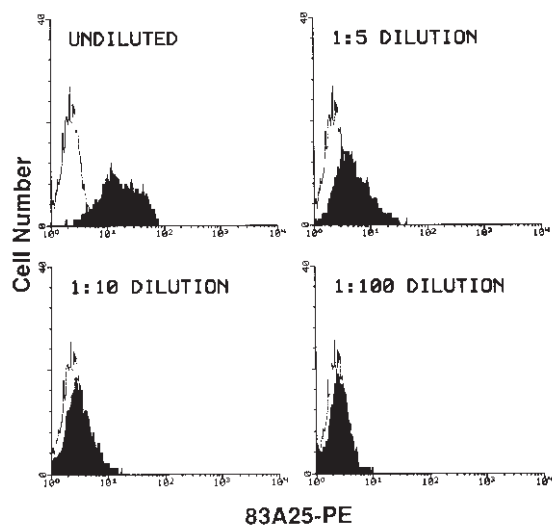
Finally, we studied the virus binding to non-CD34<sup>+</sup> cell subsets present in mobilized peripheral blood suspensions. As reported in Figure 4, the increase in fluorescence intensity appeared to be restricted to CD34<sup>+</sup> cells. Neither CD2<sup>+</sup> (T lymphocytes) nor CD14<sup>+</sup> (monocyte-macrophage) cells bound the virus in an appreciable amount, while a minimal increase of virus binding was observed on CD19<sup>+</sup> (B lymphocytes) cells.



**Figure 1.** Binding of PA317-N2 retrovirus vector to Jurkat and CD34<sup>+</sup> cells from different sources. Cells were infected with N2 vector for 2 hrs, labeled with 83A25 antibody, and stained with streptavidin-phycoerythrin (PE). The retrovirus binding is detected by PE fluorescence intensity (see *Materials and Methods* for details). White area: negative control (irrelevant antibody); black area: staining with 83A25 antibody. A: Jurkat cells (positive control) B: steady-state PB CD34<sup>+</sup> cells. C: steady-state BM CD34<sup>+</sup> cells. D: mobilized PB CD34<sup>+</sup> cells.



**Figure 2.** Binding of PA317-N2 retrovirus vector to CD34<sup>+</sup> cells from fresh, unseparated steady-state BM and from mobilized PB (see legend to Figure 1 for details). Left panels: CD34<sup>+</sup> cells from mobilized PB (up) and steady-state BM (down) are identified by green fluorescence (gate). Right panels: 83A25-PE fluorescence intensity of gated CD34<sup>+</sup> cells. In Panel B (steady-state BM CD34<sup>+</sup> cells) the negative control is indistinguishable from the positive sample.



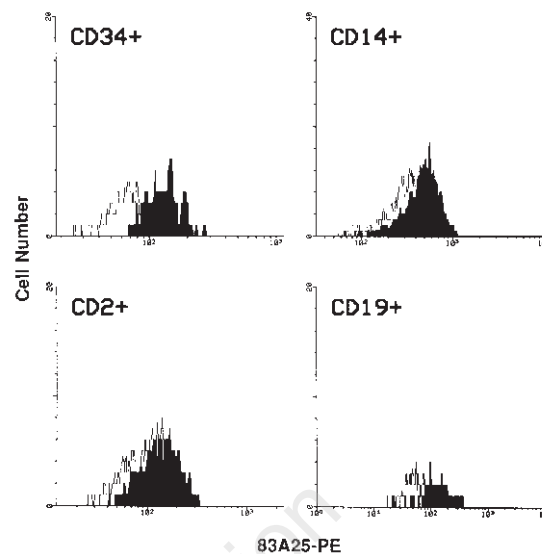
**Figure 3.** Binding of PA317-N2 retrovirus vector to mobilized CD34<sup>+</sup> cells after infection with serial dilutions of N2 supernatant. The retrovirus binding is detected by phycoerythrin (PE) fluorescence intensity (see legend to Figure 1 for details).

**Table 1.** 83A25-PE mean fluorescence intensity of CD34<sup>+</sup> cells and percentage of G418-resistant CFU-GM from mobilized PB cells infected with serial dilutions of N2 supernatant.

Dilution of N2 supernatant	83A25-PE mean fluorescence intensity	G418-resistant CFU-GM (%)
No dilution	20.86	20.0
1:5	5.96	8.2
1:10	3.50	11.2
1:100	2.73	1.1
Mock	3.39	0

## Discussion

Using a flow cytometry approach, we have shown that virus binding is increased in mobilized PB CD34<sup>+</sup> cells compared with steady-state BM CD34<sup>+</sup> cells. Our results confirm and expand earlier observations indicating that growth factors incubation increases the binding of retrovirus vector to BM CD34<sup>+</sup> cells,<sup>6</sup> and the expression of amphotropic receptor mRNA in PB CD34<sup>+</sup> cells.<sup>9</sup> An analogous observation has been reported in mouse hematopoietic stem cells, where low efficiency of transduction by amphotropic retroviral vectors correlated with low levels of the amphotropic receptor expression.<sup>9</sup> We have also



**Figure 4.** Binding of PA317-N2 retrovirus vector to CD34<sup>+</sup>, CD14<sup>+</sup>, CD2<sup>+</sup> and CD19<sup>+</sup> cell subsets from mobilized PB suspensions (see legend to Figure 1 for details).

shown that virus binding to mobilized CD34<sup>+</sup> cells occurs immediately after harvesting for clinical cell autografting, while bone marrow CD34<sup>+</sup> cells require growth factors incubation as a pre-requisite for virus binding. This finding suggests that an additional advantage of mobilized PB progenitors in a gene transfer clinical setting is the possibility of avoiding laborious *ex vivo* incubations prior to virus infection. Furthermore, we show that retrovirus binding in mobilized PB CD34<sup>+</sup> cells is significantly correlated with their transduction efficiency. This finding is in agreement with previous reports<sup>2,3</sup> showing that mobilized progenitors are more efficiently infected than bone marrow counterparts, and helps explain their high susceptibility to retrovirus infection. It is also interesting to notice that the virus binding is detectable only on CD34<sup>+</sup> cells from mobilized PB, and not on either monocyte-macrophage nor on T cell subsets. These data are in keeping with other investigators' observations that T lymphocytes are relatively refractory to retrovirus infection. These analyses also suggest a mechanism by which they can escape retroviral-mediated gene transfer.

Another attempt to explain the different susceptibility to retrovirus infection among progenitors of different sources (BM, steady-state PB, mobilized PB) is based on their cell cycle kinetics. For example, mobilized PB progenitors are mitotically more active than BM cells and thus more efficiently transduced.<sup>10</sup> However, other studies addressing the issue of the proliferative status of mobilized PB CD34<sup>+</sup> cells gave the following controversial results: 96% of mobilized

PB CD34<sup>+</sup> cells were found in G<sub>0</sub>/G<sub>1</sub> phases of the cell cycle or, conversely, only 4-7% of PB progenitors were in S phase.<sup>11,12</sup> These data, apparently contradicting the knowledge that retroviral infection is dependent on active replication of the target cells,<sup>13</sup> may be explained by considering virus binding and proviral integration as two distinct events. Retrovirus binding is dependent on the expression of the amphotropic receptor on the cell surface of the target cell; after translocation of the viral genome into the cell, integration of the provirus will take place only if the cell undergoes S-phase. PB CD34<sup>+</sup> cells are ready to progress into S-phase under cytokine stimulation.<sup>12</sup> Thus, the presence of cytokines in the culture medium (e.g., IL-3, IL-6, SCF) inducing CD34<sup>+</sup> cell cycling<sup>14</sup> is presumably the factor that promotes virus integration.

In conclusion, our data indirectly indicate that the expression of the amphotropic receptor is an important factor in the series of events that lead to retrovirus-mediated gene transfer into hematopoietic progenitors. Further knowledge of the biology of the amphotropic receptor may allow genetic manipulation aimed at optimizing gene transfer into CD34<sup>+</sup> cells.

#### Contributions and Acknowledgements

MB designed the study, contributed to the flow cytometry data and to PCR analysis and wrote the paper. MDN, SS, NB, and SSh carried out flow cytometry and PCR analysis. SS also collaborated in the study design and in the writing of the paper. FR coordinated CD34<sup>+</sup> cell collection by leukapheresis. AMG gave intellectual and financial support. All the Authors gave their critical contribution to the manuscript and approved its final version.

#### Funding

Supported in part by grant No. 94.01273.PF39 from Consiglio Nazionale delle Ricerche, and by Associazione Italiana per la Ricerca sul Cancro to AMG.

#### Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

#### Manuscript processing

Received on October 3, 1997; accepted on November 13, 1997.

#### References

- Mulligan RC. The basic science of gene therapy. *Science* 1993; 260:926.
- Bregni M, Magni M, Siena S, et al. Human peripheral blood hematopoietic progenitors are optimal targets of retroviral-mediated gene transfer. *Blood* 1992; 80:1418-22.
- Nolta JA, Smogorzewska EM, Kohn DB. Analysis of optimal conditions for retroviral-mediated transduction of primitive human hematopoietic cells. *Blood* 1995; 86:101-10.
- van Zeijl M, Johann SV, Closs E, et al. A human amphotropic retrovirus receptor is a second member of the gibbon ape leukemia virus receptor family. *Proc Natl Acad Sci USA* 1994; 91:1168-72.
- Evans LH, Morrison RP, Malik FG, et al. A neutralizable epitope common to the envelope glycoprotein of ecotropic, polytropic, xenotropic, and amphotropic murine leukemia viruses. *J Virol* 1990; 64:6176-83.
- Crooks GM, Kohn DB. Growth factors increase amphotropic retrovirus binding to human CD34<sup>+</sup> bone marrow progenitor cells. *Blood* 1994; 82:3290-7.
- Gianni AM, Bregni M, Siena S, et al. Recombinant human granulocyte-macrophage colony stimulating factor reduces hematologic toxicity and widens the clinical applicability of high-dose cyclophosphamide treatment in breast cancer and non-Hodgkin's lymphoma. *J Clin Oncol* 1990; 8:768-78.
- Shammah S, Magni M, Schirò R, et al. Induction of cyclophosphamide resistance by aldehyde dehydrogenase gene transfer into hematopoietic cells. *Gene Ther* 1995; 2:(Suppl. 1):S12.
- Orlic D, Girard LJ, Jordan CT, et al. The relative level of expression of retrovirus receptor mRNA in mouse and human primitive hematopoietic stem cells predicts the efficiency of gene transfer. *Blood* 1995; 86:628a.
- Nolta JA, Kohn DB. Comparison of the effects of growth factors on retroviral vector-mediated gene transfer and the proliferative status of human hematopoietic progenitor cells. *Hum Gene Ther* 1990; 1:257-68.
- Roberts AW, Metcalf D. Noncycling state of peripheral blood progenitor cells mobilized by granulocyte colony-stimulating factor and other cytokines. *Blood* 1995; 86:1600-5.
- Lemoli RM, Tafuri A, Fortuna A, et al. Cycling status of CD34<sup>+</sup> cells mobilized into peripheral blood of healthy donors by recombinant human granulocyte colony-stimulating factor. *Blood* 1997; 89:1189-96.
- Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol Cell Biol* 1990; 10:4329-34.
- Carlo Stella C, Tabilio A. Stem cells and stem cell transplantation. *Haematologica* 1996; 81:573-87.