[haematologica]
2004;89:462-470

Efficient gene transfer into primitive hematopoietic progenitors using a bone marrow microenvironment cell line engineered to produce retroviral vectors

JONATHAN S. DANDO
FRANCESCA FICARA
SARA DEOLA
MARIA GRAZIA RONCAROLO
CLAUDIO BORDIGNON
ALESSANDRO AIUTI

A B S T R A C T

Background and Objectives. Effective gene transfer into human hematopoietic stem/progenitor cells is a compromise between achieving high transduction efficiency and maintaining the desired biological characteristics of the target cell. The aim of our work was to exploit the stromal microenvironment to increase gene transfer and maintenance of hematopoietic progenitors.

Design and Methods. The murine bone marrow stromal cell line MS-5, known to support primitive human progenitors, was modified into an amphotropic packaging cell, by the stable introduction of DNA coding for retroviral structural proteins, and a viral vector encoding a marker gene. The gene transfer efficiency of the recombinant virus was evaluated by flow cytometry, *in vitro* assays for committed (CFC) and primitive (LTC-CFC) progenitors, as well as a clonal assay for B and NK lymphoid progenitors.

Results. The new packaging cell line (NEXUS) produced equivalent levels of virus as did the established GP+Am12 system, also under serum-free conditions. On average 30% of human mobilized peripheral blood $CD34^+$ cells were transduced by a single exposure to NEXUS supernatant, representing a three-fold increase over GP+Am12-based technology. Gene transfer into both committed and primitive progenitors increased on average two-fold using NEXUS retroviral supernatant. Furthermore, $CD34^+ CD38^{low}$ early progenitor cells purified from umbilical cord blood were efficiently transduced with NEXUS retroviral vector and gave rise to a high frequency of marked B and NK lymphocytes.

Interpretation and Conclusions. Our data show that that an established bone marrow stromal cell can be engineered to enhance the genetic modification of primitive hematopoietic and lymphoid progenitors using a clinically relevant method.

Key words: stromal cell, $CD34^+$ cells, gene therapy, packaging cell.

From the San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Via Olgettina 58, Milan 20132, Italy.

JSD and FF contributed equally to the manuscript.

Correspondence:Alessandro Aiuti M.D., PhD, HSR-TIGET, Via Olgettina 58, Milan 20132, Italy E -mail: a.aiuti@hsr.it

Hematopoietic stem/progenitor cells (HSPC) are responsible for the lifelong maintenance and generation of the hematopoietic system, and therefore represent an ideal target for gene therapy approaches for blood borne disorders.[1-3] Genetic modification of HSPC is dependent on the physical removal of the cells from the native microenvironment,[4] and exposure to virus under defined and most favorable *in vitro* culture conditions.[5-7] However, even optimized cytokine-mediated *in vitro* culture techniques cannot substitute for the fine control provided by the hematopoietic microenvironment. Stromal cells represent the ideal environment for all the requirements of the HSPC, providing both soluble[8,9] and membrane[10] or extra-cellular matrix associated molecules,[11] which play essential roles in programming and maintaining the hematopoietic cell. The microenvironment has also been demonstrated to be involved in genetic modification by mediating increased gene transfer into human HSPC. The inclusion of a stromal cell layer during transduction of bone marrow (BM) or mobilized peripheral blood (MPB)-derived $CD34^+$ cells, with or without exogenous cytokines, resulted in higher gene transfer into long-term culture colony forming cells (LTC-CFC).[12,13] Long-term engraftment of *bnx* mice by transduced BM or MPB progenitors could only be achieved if the gene transfer protocol was performed in the presence of stroma and exogenous cytokines.[14] More recently, the addition of soluble factors secreted by murine stromal cell lines was shown to increase gene transfer into myeloid progenitor cells from MPB by 50%.[15] Moreover, the use of extra-cellular matrix molecules also produced by the hematopoietic microenvironment facilitated

enhanced gene transfer into HSPC,[16] a result which appears to be mediated by increased interaction between the target cell and the virus.[17] MS-5 is a bone marrow stromal cell line established from an irradiated murine Dexter culture.[18] It is phenotypically undifferentiated[19] and has been documented to support and expand early human hematopoietic progenitors, as measured by the LTC-IC assay.[20,21] It was recently demonstrated that MS-5 supports other early human hematopoietic cells, such as lymphohematopoietic progenitor cells.[22] MS-5 has also been used in gene transfer experiments, increasing the transduction efficiency of murine colony forming units- spleen (CFU-S).[23] MS-5 had negligible effects on human HSPC gene transfer when used in direct contact co-culture, but did increase the number of LTC-CFC-derived colonies.[24]

In an attempt to optimize gene transfer into the HSPC, we wanted to combine the gene transfer vehicle and the favorable effect of the hematopoietic microenvironment into one system, providing the HSPC with the most advantageous *in vitro* environment for gene transfer. To this aim, the MS-5 cell line was selected for development into a type C retroviral packaging cell line, and tested for its ability to modify human HSPC.

Design and Methods

DNA constructs, plasmids, and transfection of cell lines

All DNA for stable transfection was prepared using the cesium chloride density gradient based method. Transfection of the MS-5 cell line was performed using the synthetic polymer, polyethylenimine 25K (Sigma Chemicals, St. Louis, USA), and the stable generation of the packaging cell is described in the text. Individual clones were generated using sterile cloning cylinders (Sigma Chemicals, St. Louis, USA).

Culture of adherent cells

All standard cell lines described and the developed packaging cells were cultured using IMDM media (Biowhittaker, Verviers, Belgium) containing 10% v/v FCS (Mascia Brunelli, Milan, Italy), 2 mM L-glutamine (Gibco Brl, Paisley, UK), and penicillin/streptomycin (100 units/mL), named IMDM complete media.

Retroviral supernatant production, and quality controls

All producer cell lines were assayed over a 12-week period to test for constant production of viral supernatants, as measured by bulk transduction and end point titration. Cells were seeded at 5×10^4 cells/cm^2, and received specific and timed media exchanges over two days, prior to the retroviral production phase. Supernatants were produced in IMDM complete media or serum free X-VIVO 10 (Biowhittaker, Verviers, Belgium) media for 6-10 hours, filtered and stored at -80°C until further use. End point titer and bulk gene transfer was performed using NIH 3T3 as the target cell and calculated by both ΔNGFr expression and neomycin resistance, as previously described.[25,26] At the point at which stable production was observed, selective pressure with G418 was removed. Producer cells were frozen in aliquots which were thawed at 3-month intervals to ensure that all experiments were performed with similar producer lines. The producer cell lines did not show any variability in virus production at time points after this. Supernatants were routinely tested using both the marker rescue and S+L- methods for replication competent retrovirus, using published methods.[27]

Source and purification of human mobilized peripheral blood CD34$^+$ cells

Hematopoietic targets were CD34$^+$ cells obtained from granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood (MPB) of healthy donors after informed consent, or CD34$^+$CD38^{low} cells obtained from umbilical cord blood (UCB) collected from discarded placentas at HS Raffaele, Milan, Italy. Human CD34$^+$ cells were purified using the MiniMACS MULTISORT (Miltenyi Biotec, Bologna, Italy) purification device, with cell purity typically being between 85 and 95%, while CD34$^+$CD38^{low} cells were purified using a FACStar cell sorting device (Becton Dickinson-BD, San Josè, USA, USA), with a purity >99.8%.

Gene transfer into CD34$^+$ cells

Twenty five thousand human CD34$^+$ cells were prestimulated for 24 hours in 24-well plates, in X-VIVO 10 media containing the cytokines stem cell factor, (SCF) (R&D Systems, Minneapolis, USA), Thrombopoietin (TPO), and Flt-3 Ligand (both from PeproTech, London, UK) all at 50 ng/mL, named X-VIVO 10$^+$ media. The cells were exposed once to retroviral supernatant supplemented with cytokines, and placed on Retronectin (Takara Biomedicals, Shiga, Japan) coated plates, in a final volume of 1 mL. The multiplicity of infection (MOI) was on average 2 and equivalent MOI were used for different packaging cells. Eighteen hours later the cells received an exchange of media being returned to X-VIVO 10$^+$ medium. CD34$^+$CD38^{low} cells were transduced in an identical way except that a 48-hour pre-stimulation period was allowed, and only 5000 cells were used per condition, seeded in 96-well plates. Gene transfer by direct contact co-culture or in transwell plates was performed by seeding and growing the producer cells as described for supernatant production. Mixing experiments with conditioned media were performed by re-suspending CD34$^+$ cells in a five-fold concentration of

X-VIVO 10+ media at a concentration of 125,000 cells/mL. The cell suspension (200 μL) was added to 400 μL of conditioned media or serum-free media, and 400 μL of viral supernatant. All gene transfer was analyzed either by FACS 48 hours later[28] or by functional assays.

Phenotypic analysis of human hematopoietic cells

Forty-eight hours after viral exposure cells were collected, counted, and were washed once in PBS containing 0.5% w/v sodium azide, 1.5% w/v BSA (HC FACS), and labeled with a PE-conjugated anti-CD34 antibody (BD) and a tricolor-conjugated anti-CD45 antibody (Caltag, Burlingame, USA). Analysis of transduced CD34+ cells was performed using a biotinylated anti-NGFr specific antibody revealed by FITC-conjugated streptavidin (Vector, Burlingame, CA, USA). The cells were washed once after 30 min, and analyzed on a BD FACScan.

In vitro hematopoietic assays (CFU-C and LTC-CFC)

To test for gene transfer into colony-forming unit-cells (CFU-C) 3000 cultured CD34+ cells were plated in the absence or presence of G418 (1 mg/mL final active concentration) in 2.1% methylcellulose medium.[29] Colonies were counted using standard microscopic procedures and gene transfer efficiency calculated as the proportion of colonies growing in the presence of G418. Gene transfer into long term culture-colony forming cells (LTC-CFC)[29] was assayed by plating 20,000 MPB-transduced CD34+ cells on a preformed monolayer of MS-5[18] stromal cells, in Myelocult medium (Stem Cell Technologies Inc. Vancouver, Canada). At week 5, the cells were plated as for the CFU-C assay, in the absence or presence of G418 (1.2 mg/mL final active concentration), and colonies were counted two weeks later. The number of CFC generated gives an indirect, but consistent, measurement of the content of LTC-IC, because the average number of colonies generated by each LTC-IC under identical culture conditions is reproducibly the same. For all colony-forming assays no colonies were observed in the untransduced samples in the presence of G418.

In vitro lymphohematopoietic progenitor assays

Following a modification of a previously published protocol,[22] transduced CD34+ cells were plated at 3 cells per well in a 96-well plate containing MS-5 cells at 5×10^3 cells per well. In preliminary experiments, it was determined that this cell concentration allowed measurement of clonal frequency of progenitors. Cells were grown in lymphoid differentiation medium which was composed of RPMI 1640 (Biowhittaker, Belgium) medium containing 5% v/v FCS, 10% v/v human serum, IL-2 (Roche, Milan, Italy) at 250 units/mL, TPO, Flt-3 Ligabnd, SCF (R&D), IL-7 (Peprotech), and IL-15 (R&D), at 20 ng/mL, 20mM HEPES (Gibco Brl, UK), penicillin/streptomycin, and L-glutamine. For 5 weeks the medium was demi-depleted weekly and refreshed with an equal volume of lymphoid medium. The cells were stained with either anti-human CD56-PE (BD) or anti-human CD19-PE (Immunosource, Los Altos, USA), and anti-human NGFr, as described for CD34+ cell transduction, after staining and gating with an FITC-conjugated anti-human CD45 (BD) antibody.

Statistical analysis

Statistical analysis was performed using the two-tailed student's t test. Differences were considered statistically significant if $p<0.05$.

Results

Generation and characterization of packaging cells developed from the MS-5 stromal cell line

The murine stromal cell line MS-5 was modified into a split-function amphotropic retroviral packaging cell line using two separate expression cassettes coding for the amphotropic envelope, under Moloney-Leukemia Virus (MLV) LTR promoter, and the MLV Gag-pol proteins, respectively.[30] These retroviral structural coding sequences represent the absolute minimal sequence requirement for correct protein expression and assembly.[30] The amphotropic envelope was co-transfected with a plasmid conferring hygromycin resistance (64 μg/mL toxicity), and selected for a clone expressing high envelope by amphotropic interference. The selected clone was co-transfected with the MLV Gag-pol construct, and a plasmid conferring puromycin resistance (15 μg/mL toxicity). Clones were isolated and screened for the simultaneous secretion of retroviral proteins from both the envelope and Gag-pol genes, as measured by an ELISA-based method.[30] Alternatively, in a first step the MLV Gag-pol expression cassette was introduced first by co-transfection with puromycin, and then the envelope construct by co-transfection with a plasmid conferring zeocin resistance (20 μg/mL toxicity). In total, 9 out of 87 clones demonstrated high levels of viral protein secretion, which were then converted into stable producer lines by super-infection with an ecotropic supernatant containing the SFCMM-2 vector, and G418 selection (0.8 mg/mL). The SFCMM-2 vector enables measurement of gene transfer by both surface expression of the truncated nerve growth factor receptor (NGFr) and by neomycin resistance.[31] In parallel, GP+Am12 packaging cells were superinfected with the same ecotropic supernatant, followed by G418 selection to ensure the creation of a stable pool, prior

Table 1. Testing of viral levels by bulk gene transfer (NGFr expression) on ten independent lots.

Lot#	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Mean*	*SD*
GP+Am12	81.8	60.7	71.8	74.1	84.6	90.9	85.4	67.0	93.8	95.6	80.6	11.7
NEXUS 17/9	69.2	84.9	86.4	56.6	65.4	81.9	83.1	98.7	85.6	90.1	80.2	12.6
NEXUS 22-10	75.9	95.2	89.1	92.7	68.9	68.5	63.9	83.4	87.1	87.6	81.2	11.1

Bulk gene transfer efficiencies into NIH 3T3 by the NEXUS producers. Supernatant was inoculated onto NIH 3T3 at a 1/2 dilution and measured by anti NGFr FACS analysis to quantify bulk gene transfer efficiency. Results from ten independent supernatant lots are shown, represented as % of gene transfer. Limiting dilution experiments confirmed that there were no significant differences in titer between the producers tested.

to further cloning. Preliminary screening revealed two clones which transduced NIH 3T3 to levels equivalent to those of GP+Am12 (one clone derived by the introduction of envelope first, and one clone which had the Gag-pol introduced first), which were then further analyzed for replication competent retrovirus (RCR) using both the Marker Rescue and S+L- assays. Setting the sensitivity at one colony or syncytium, the two producers, 17/9 and 22-10, were tested over a 14-week period, for the presence of RCR. Neither producers generated RCR of any tropism during the test period (*data not shown*). The cell lines had a doubling time of approximately 24 hours in subconfluent conditions and could be easily expanded to amounts required for large scale production. The new packaging cell lines were named NEXUS (from the Latin, *Nexus Logica*).

Testing of viral levels by bulk gene transfer (NGFr expression) on ten independent lots (Table 1) showed that the two clones produced levels of vector equivalent to those of GP+Am12. Measurement of end-point titer into NIH 3T3 resulted in comparable levels of infectious particles for NEXUS 17/9 ($17\pm6\times10^5$ CFU/mL) and NEXUS 22-10 ($10\pm1\times10^5$ CFU/mL) with respect to Gp+Am12 ($10\pm4\times10^5$ CFU/mL) (n=10).

NEXUS-based producer supernatants increase gene transfer into human mobilized peripheral blood CD34⁺ cells

Vector preparations produced from the NEXUS cells were used to transduce mobilized peripheral blood (MPB) derived CD34⁺ hematopoietic target cells a single time in the presence of the cytokines SCF, TPO, and Flt3-Ligand. As shown in Figure 1A, NEXUS 22-10 transduced almost 30%, NEXUS 17/9 nearly 20%, while GP+Am12 transduced only 6% percent of the CD34⁺ cell population. Analysis of gene transfer efficiencies from eight different experiments showed three- and two-fold increases in gene transfer for the NEXUS 22-10 ($p \leq 0.0001$) and 17/9 respectively ($p \leq 0.0001$) in comparison to GP+Am12 (Figure 1B). The rate of CD34⁺ cell differentiation was faster with all retroviral supernatants than with untransduced cells. There was a tendency towards a faster loss of CD34 expression in the presence of NEXUS than in the presence of GP+Am12, but the overall proportion of differentiated CD34⁻ cells was not significantly different (Figure1A and *data not shown*).

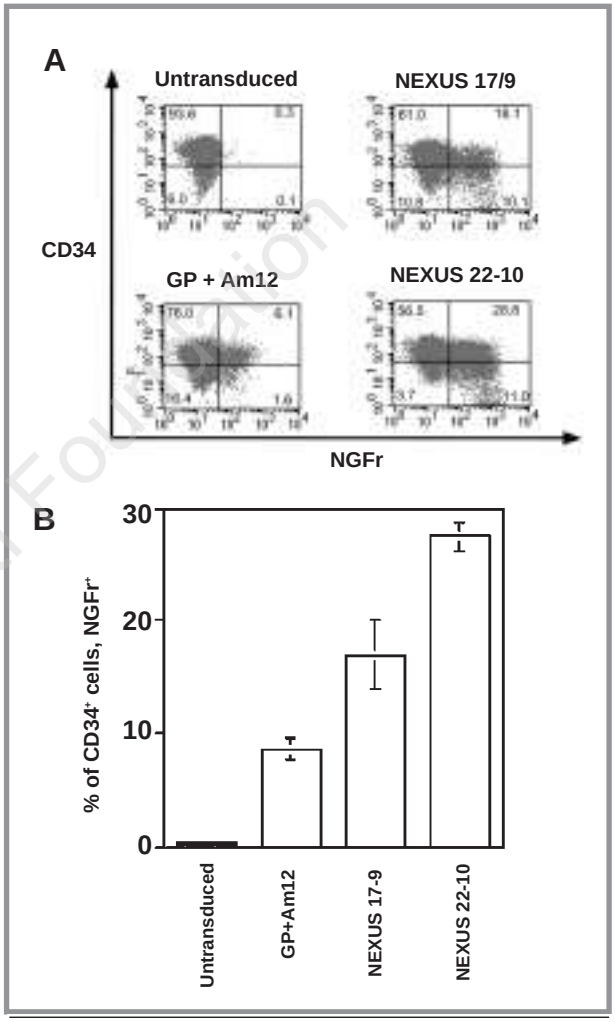

Figure 1. Gene transfer into MPB CD34⁺ cells using NEXUS supernatant. A: Flow cytometry analysis of a representative gene transfer experiment into MPB-derived CD34⁺ cells. Target cells were transduced once as described in the Methods and assayed for NGFr expression at day 4. Both of the NEXUS vector preparations gave rise to significant increases in gene transfer into CD34⁺ cells when compared to GP+Am12 vector preparations. B: Comparison of transduction efficiency of MPB CD34⁺ cells using NEXUS and GP+ Am12 vectors. Cells were transduced and assayed as described. Data are mean±SD from eight different experiments.

Even after sub-cloning of the NEXUS 22-10 producer pool, attempting to further increase HSPC gene

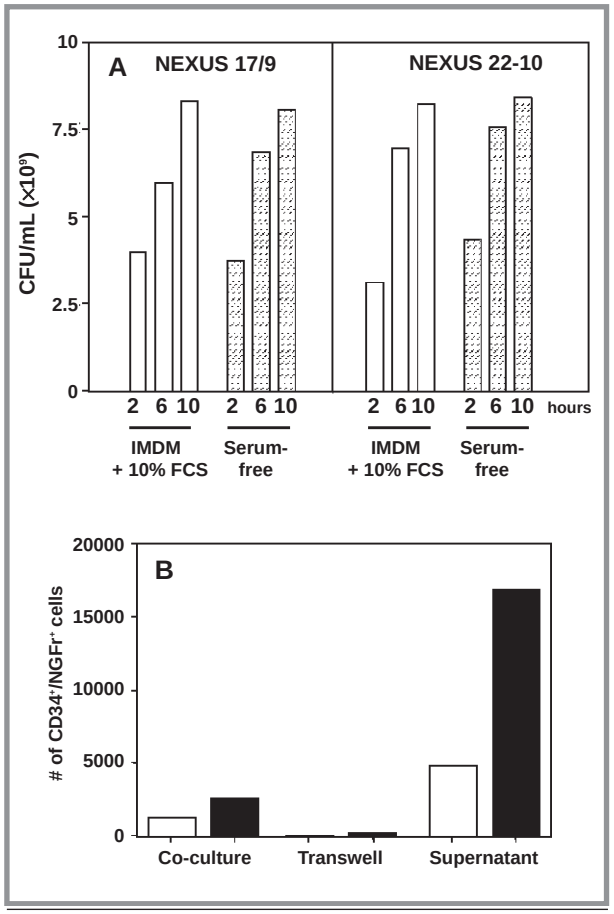

Figure 2. Influence of different supernatant production and transduction conditions. A: Titer on NIH 3T3 of supernantant produced with standard serum-containing medium (IMDM + 10%FCS) or X-VIVO 10 medium (serum-free). Supernatant was collected from the two NEXUS packaging cell lines after 2, 6 or 10 hours. B: Comparison of human MPB CD34⁺ cell gene transfer under different transduction conditions, using either Gp+Am12 (empty bars) or NEXUS 22-10 (filled bars) producer cell lines. One representative experiment out of three is shown.

transfer by selecting high-titer clones, no increases in CD34⁺ gene transfer were observed, and therefore all further experiments were performed using the original pool. Similar results were also obtained after subcloning the GP+Am12 pool (*data not shown*).

Remarkably, NEXUS supernatant produced under serum-free conditions retained equivalent titer and gene transfer efficiency as compared to FCS-containing medium (Figure 2A and *data not shown*). To compare different methods of vector presentation, MPB-derived CD34⁺ cells were exposed to vector preparations by either direct contact with the producer cell pools, by separation from the producer cell pools using a microporous filter (transwell), or by cell-free supernatant. In all experiments, cell-free supernatants gave rise to the highest level of gene transfer for both GP+Am12 and NEXUS, the latter being superior (Figure 2B).

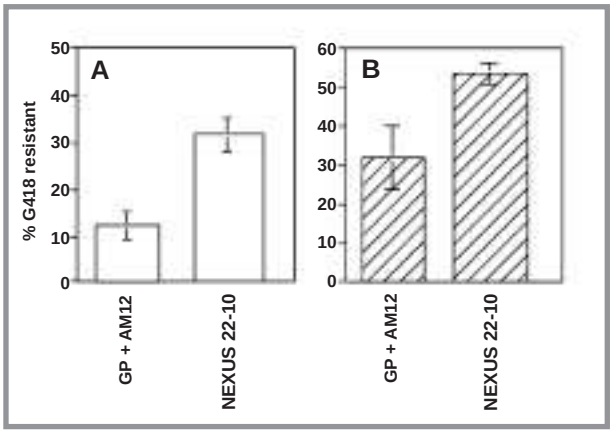

Figure 3. Increased gene transfer into committed and early *in vitro* hematopoietic progenitors. Percent transduction of (A) CFU-C, and (B) LTC-CFC was measured by neomycin resistance, comparing NEXUS 22-10 to GP+Am12. Data are mean ± SD from eight different experiments.

NEXUS increases gene transfer into early hematopoietic progenitors

We next wanted to test whether NEXUS supernatant could also give rise to increased gene transfer into committed hematopoietic progenitor cells as measured using the *in vitro* CFU-C. In addition, gene transfer into early *in vitro* progenitors was studied by analysis of CFU-C derived from long-term culture of transduced CD34⁺ cells, on a stromal cell layer (LTC-CFC assay). Using equivalent titer vector preparations, NEXUS gave a three-fold increase into CFU-C ($p = 0.002$, Figure 3A), and a 55% increase in gene transfer efficiency into LTC-CFC ($p = 0.02$, Figure 3B) as compared to GP+Am12.

To address whether soluble factors present in the NEXUS supernatant were influencing the growth and gene transfer of early and committed hematopoietic progenitors *in vitro*, conditioned media were prepared from both MS-5 and NIH 3T3 cells, and mixed with GP+Am12 or NEXUS vector preparations, prior to CD34⁺ cell transduction. Consistent with the above findings, NEXUS supernatant gave rise to a three- and two-fold increase in the number of transduced CFU-C and LTC-CFC, respectively, compared to GP+Am12 supernatant ($p = 0.002$, Figures 4A and 4B). The inclusion of MS-5 conditioned medium prior to transduction with NEXUS further increased the number of transduced committed and early progenitors, resulting in a doubling of the number of G418-resistant LTC-CFC, compared to no conditioned medium or NIH 3T3 supernatant ($p = 0.02$). In addition, the number of transduced early progenitors was three-fold higher in the presence of NEXUS plus MS-5 conditioned media than in the presence of GP+Am12 plus MS-5 conditioned media ($p = 0.01$) (Figure 4B).

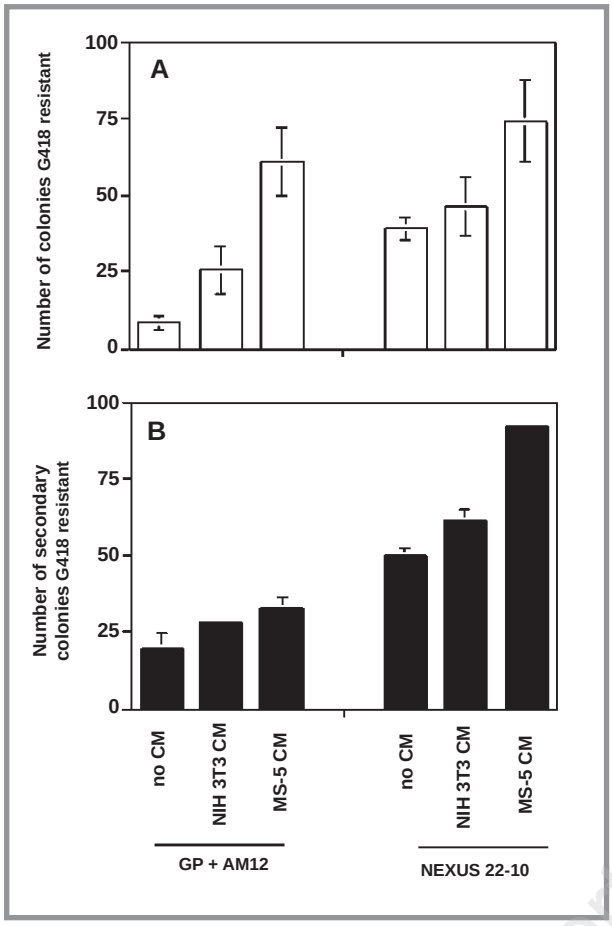

Figure 4. Effect of conditioned media on gene transfer into committed and early hematopoietic progenitors. Following exposure of CD34⁺ cells to vector preparations mixed with serum-free or conditioned media, the cells were plated in standard CFU-C assays (panel A) or 5 weeks on a stromal layer followed by CFU-C assays (panel B). Data represent the mean number of (A) G418-resistant colonies (±SD) or (B) G418 resistant LTC-CFC derived colonies (from at least three different experiments).

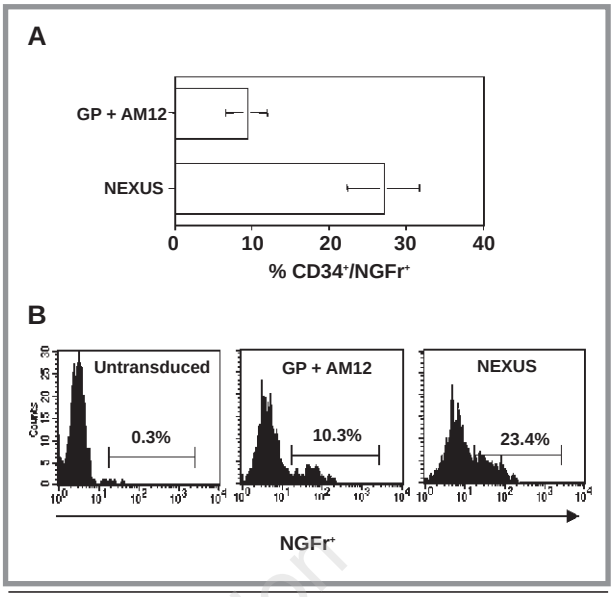

Figure 5. Effective gene transfer into CD34⁺ CD38^{low} cells derived from umbilical cord blood. Following cell sorting purification, CD34⁺ CD38^{low} cells were pre-stimulated for 48 hours and transduced as described in *Design and Methods*. A: mean gene transfer efficiency into CD34⁺/CD38^{low} cells measured by NGFr expression (n=5). B: representative FACS analyses of NGFr expression in transduced cells gated for the CD34⁺ CD38^{low} phenotype.

NEXUS increases gene transfer into primitive CD34⁺/CD38^{low} hematopoietic progenitors, which can give rise to marked NK and B cells

To confirm that the NEXUS-based preparations were transducing primitive hematopoietic cells *in vitro*, CD34⁺ CD38^{low} cells were purified from umbilical cord blood by cell sorting, and exposed once to either GP+Am12 or NEXUS vector. Overall, NEXUS supernatants resulted in significantly higher gene transfer into CD34⁺ CD38^{low} cells (almost three-fold increase), than did GP+Am12 (Figure 5A), including cells which maintained the CD34⁺ CD38^{low} primitive phenotype after culture (Figure 5B).

The ability of NEXUS supernatants to transduce early *in vitro* hematopoietic progenitor cells of different lineages was tested by plating transduced CD34⁺CD38^{low} cells in a lymphoid differentiation assay on preformed stromal monolayers in the presence of cytokines. This assay allows the differentiation of lymphohematopoietic progenitors into myeloid, B (CD19⁺), and natural killer (NK, CD56⁺) cells. As shown in Table 2, NEXUS transduction resulted in a higher frequency of wells containing CD56⁺/NGFr⁺ and CD19⁺/NGFr⁺ transduced cells than did GP+Am12. Taken together, these data demonstrate that a higher proportion of primitive *in vitro* progenitors is transduced by using NEXUS-derived supernatant.

Discussion

The generation of MLV-based retroviral packaging cells has historically been performed using murine cells, and specifically NIH 3T3 as the standard cell,[32, 33] engineered to secrete amphotropic viral particles. These have been utilized in successful clinical trials,[3, 7, 31,34,35] although other viral tropisms may offer a greater clinical efficacy.[36,37] Importantly, the two NEXUS producers that transduced MPB CD34⁺ cells at high frequency proved not to generate transmissible replication competent retrovirus of any known type-C tropism, even after 14 weeks of continuous culture.

Gene transfer efficiency into MPB primitive HSPC cells, as measured by FACS 48 hours after transduction,

Table 2. Frequency of gene transfer into lymphohematopoietic progenitors.

	Frequency of CD19⁺ cells	*Frequency of NGFr⁺/CD19⁺cells*	*Frequency of CD56⁺ cells*	*Frequency of NGFr⁺/CD56⁺ cells cells*
GP+Am12	36.6% (26/71)	3.8% (1/26)	80.3% (57/71)	26.3% (15/57)
NEXUS	20.7% (12/58)	41.7% (5/12)	65.5% (38/58)	76.3% (29/38)

$CD34^+CD38^{low}$ exposed to viral supernatant were seeded at 3 cells per well in a standard in vitro lymphohematopoietic assay. After 5 weeks the wells containing hematopoietic cells were measured for expression of the NK marker (CD56) and the B-cell marker (CD19), in conjunction with NGFr expression, to determine whether lymphohematopoietic progenitor cells had been transduced. Numbers in brackets indicate the number of wells positive for the indicated markers and the total wells grown from which the frequency was determined. One representative experiment from three is shown.

represented an improvement in gene transfer efficacy relative to GP+Am12, confirming previous observations that the hematopoietic microenvironment can be used to increase gene transfer into hematopoietic cells.[12-15] Furthermore our results with GP+Am12 are similar to those of other reports,[3,7,19] although differences observed may be due to the source of $CD34^+$ cells, the length of pre-stimulation and of viral exposure, the culture medium and cytokine conditions, and the assays used for analysis of gene transfer. In accordance with the observation that co-culture with MS-5 during gene transfer does not significantly increase gene transfer efficacy,[24] we found that cell-free vector preparations represented the optimal mode of vector presentation for both NEXUS and GP+Am12 during the gene transfer protocol. The hematopoietic supportive capacity and the beneficial effect on gene transfer of NEXUS may be attributed to its documented secretion of early-acting known and unknown hematopoietic growth factors.[38-41] In addition, extra-cellular matrix component, such proteoglycans secreted by this cell line[42] might act as cytokine-presenting molecules or sites of co-localization of progenitor cells and retroviral particles. Further studies will be required to assess the contribution of these and other molecules on the NEXUS improved gene transfer into HSPC.

Our data suggest that: a) soluble factors from the NEXUS packaging cell (and its parental cell line MS-5) further improve gene transfer into clonogenic progenitors; b) retroviral vector particles derived from the stromal cell line transduce with higher efficiency early *in vitro* hematopoietic progenitors. The effect mediated by NEXUS on committed progenitors seemed to be mediated mainly by soluble factors, concurring with the reports on this characteristic of this cell line.[38,43] On the other hand, the enhancing effect on transduction of early progenitors was not reproduced by the simple addition of MS-5 conditioned medium to GP+Am12. Thus, it is intriguing to speculate that the inclusion of membrane associated molecules of the stromal cell line in the budding retrovirus may increase the interaction between the vector particle and the target cell, as recently demonstrated for other cell types and viral systems.[44,45]

This new system might be easily developed into a clinical producer, since the gene transfer experiments were performed in serum-free media, and NEXUS supernatant produced with serum-free media gave similar titer and gene transfer efficiency as compared to FCS-containing media.

The application of NEXUS could be particularly pertinent for immunodeficiencies[34,46] considering that the NEXUS viral exposure resulted in a higher yield of genetically marked NK and B cells than does existing gene transfer technology. We have recently demonstrated that a human endothelial-like cell line, redefined as a human bladder cancer derived epithelial cell line,[47] could be engineered into a stable packaging cell with increased gene transfer into hematopoietic progenitors.[48]

The present study represents the formal proof of the principle that an established bone marrow stromal cell, with well characterized hematopoietic supportive function[20,21,22] can be engineered to enhance the genetic modification of primitive hematopoietic and lymphohematopoietic cells in a clinically relevant method. Further studies are required to evaluate the ability of NEXUS to transduce *in vivo* reconstitutive hematopoietic stem cells, using clinically relevant animal models. Additionally, our approach might be applied to human stromal cell lines, although these are more difficult to generate.[48] Our preliminary data indicate that immortalized human bone marrow stromal cell clones can be stably grown in culture and converted into retroviral packaging cell lines (*Dando, unpublished*).

JD, FF and AA: conception of the study, and design of experiments. JD, conduction of experiments and responsibility for Figures 1, 3, 4, and Table 1; FF conduction of experiments and responsibility for Figures 2 and Figure 5, purification of umbilical cord blood, studies on lymphoid progenitors (Table 2); SD purification of mobilized peripheral blood and studies on myeloid progenitors. FF, SD, CB and MGR interpreted the data and revised the article. All authors approved the final version of the manuscript. JD and AA drafted the paper, FF par-

ticipated substantially in revising the manuscript. AA is the author primarily responsible for the publication.

We are very grateful to Dr. A. Lever for his constant input of helpful comments and suggestions during the course of this study. We would also like to thank Dr R. Rigg and E. Bohnlein for providing us with reagents necessary for completing this study, and critique of the manuscript. Dr. K. Itoh is thanked for the MS-5 cell line. Dr. C. Traversari is thanked for providing the retroviral vectors, Dr. R. Furlan for the antibiotic resistant plasmids, and Dr. M. Bregni for the blood samples. The Authors reported no potential conflicts of interest.

This research was supported by grants from the Fondazione Telethon, Italy, EEC grants BIO4 CT97 5074 and QLK3-CT-1999-00859, and Ricerca Finalizzata 2001 (Ministero della Salute).

Manuscript received July 7, 2003. Accepted January 26, 2004.

References

1. Bregni M, Magni M, Siena S, Di Nicola M, Bonadonna G, Gianni AM. Human peripheral blood hematopoietic progenitors are optimal targets of retroviral-mediated gene transfer. Blood 1992;80:1418-22.
2. Dunbar CE, Cottler-Fox M, O'Shaughnessy JA, Doren S, Carter C, Berenson R, et al. Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. Blood 1995; 85:3048-57.
3. Abonour R, Williams DA, Einhorn L, Hall KM, Chen J, Coffman J, et al. Efficient retrovirus-mediated transfer of the multidrug resistance 1 gene into autologous human long-term repopulating hematopoietic stem cells. Nat Med 2000; 6: 652-8.
4. Funk PE, Kincade PW, Witte PL. Native associations of early hematopoietic stem cells and stromal cells isolated in bone marrow cell aggregates. Blood 1994;83: 361-9.
5. Knaan-Shanzer S, Valerio D, van Beusechem VW. Cell cycle state, response to hemopoietic growth factors and retroviral vector-mediated transduction of human hemopoietic stem cells. Gene Ther 1996; 3:323-33.
6. Hennemann B, Conneally E, Pawliuk R, Leboulch P, Rose-John S, Reid D, et al. Optimization of retroviral-mediated gene transfer to human NOD/SCID mouse repopulating cord blood cells through a systematic analysis of protocol variables. Exp Hematol 1999;27:817-25.
7. Hesdorffer C, Ayello J, Ward M, Kaubisch A, Vahdat L, Balmaceda C, et al. Phase I trial of retroviral-mediated transfer of the human MDR1 gene as marrow chemoprotection in patients undergoing high-dose chemotherapy and autologous stem-cell transplantation. J Clin Oncol 1998;16:165-72.
8. Kittler EL, McGrath H, Temeles D, Crittenden RB, Kister VK, Quesenberry PJ. Biologic significance of constitutive and subliminal growth factor production by bone marrow stroma. Blood 1992;79:3168-78.
9. Verfaillie CM. Soluble factor(s) produced by human bone marrow stroma increase cytokine-induced proliferation and maturation of primitive hematopoietic progenitors while preventing their terminal differentiation. Blood 1993;82:2045-53.
10. Slanicka Krieger M, Nissen C, Manz CY, Toksoz D, Lyman SD, Wodnar-Filipowicz A. The membrane-bound isoform of stem cell factor synergizes with soluble flt3 ligand in supporting early hematopoietic cells in long-term cultures of normal and aplastic anemia bone marrow. Exp Hematol 1998;26:365-73.
11. Hurley RW, McCarthy JB, Verfaillie CM. Direct adhesion to bone marrow stroma via fibronectin receptors inhibits hematopoietic progenitor proliferation. J Clin Invest 1995;96:511-9.
12. Wells S, Malik P, Pensiero M, Kohn DB, Nolta JA. The presence of an autologous marrow stromal cell layer increases glucocerebrosidase gene transduction of long-term culture initiating cells (LTCICs) from the bone marrow of a patient with Gaucher disease. Gene Ther 1995;2:512-20.
13. Xu LC, Kluepfel-Stahl S, Blanco M, Schiffmann R, Dunbar C, Karlsson S. Growth factors and stromal support generate very efficient retroviral transduction of peripheral blood CD34$^+$ cells from Gaucher patients. Blood 1995;86:141-6.
14. Nolta JA, Smogorzewska EM, Kohn DB. Analysis of optimal conditions for retroviral-mediated transduction of primitive human hematopoietic cells. Blood 1995; 86:101-10.
15. Schiedlmeier B, Buss EC, Veldwijk MR, Zeller WJ, Fruehauf S. Soluble bone marrow stroma factors improve the efficiency of retroviral transfer of the human multidrug resistance 1 gene to human mobilized peripheral blood progenitor cells. Hum Gene Ther 1999;10:1443-52.
16. Hanenberg H, Xiao XL, Dilloo D, Hashino K, Kato I, Williams DA. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nat Med 1996;2:876-82.
17. Hanenberg H, Hashino K, Konishi H, Hock RA, Kato I, Williams DA. Optimization of fibronectin-assisted retroviral gene transfer into human CD34+ hematopoietic cells. Hum Gene Ther 1997;8:2193-206.
18. Itoh K, Tezuka H, Sakoda H, Konno M, Nagata K, Uchiyama T, et al. Reproducible establishment of hemopoietic supportive stromal cell lines from murine bone marrow. Exp Hematol 1989;17:145-53.
19. Remy-Martin JP, Marandin A, Challier B, Bernard G, Deschaseaux M, Herve P, et al. Vascular smooth muscle differentiation of murine stroma: a sequential model. Exp Hematol 1999;27:1782-95.
20. Issaad C, Croisille L, Katz A, Vainchenker W, Coulombel L. A murine stromal cell line allows the proliferation of very primitive human CD34^{++}/CD38$^-$ progenitor cells in long-term cultures and semisolid assays. Blood 1993;81:2916-24.
21. Bennaceur-Griscelli A, Tourino C, Izac B, Vainchenker W, Coulombel L. Murine stromal cells counteract the loss of long-term culture-initiating cell potential induced by cytokines in CD34($^+$)CD38($^{low/neg}$) human bone marrow cells. Blood 1999;94:529-38.
22. Robin C, Bennaceur-Griscelli A, Louache F, Vainchenker W, Coulombel L. Identification of human T-lymphoid progenitor cells in CD34$^+$ CD38^{low} and CD34$^+$ CD38$^+$ subsets of human cord blood and bone marrow cells using NOD-SCID fetal thymus organ cultures. Br J Haematol 1999; 104:809-19.
23. Goncalves F, Dubart A, Lacout C, Vainchenker W, Dumenil D. Stromal cells maintain the radioprotective capacity of CFU-S during retroviral infection. Gene Ther 1996;3:761-8.
24. Marandin A, Dubart A, Pflumio F, Cosset FL, Cordette V, Chapel-Fernandes S, et al. Retrovirus-mediated gene transfer into human CD34$^+$38^{low} primitive cells capable of reconstituting long-term cultures in vitro and nonobese diabetic-severe combined immunodeficiency mice in vivo. Hum Gene Ther 1998;9:1497-511.
25. Forestell SP, Bohnlein E, Rigg RJ. Retroviral end-point titer is not predictive of gene transfer efficiency: implications for vector production. Gene Ther 1995; 2: 723-30.
26. Rudoll T, Phillips K, Lee SW, Hull S, Gaspar O, Sucgang N, et al. High-efficiency retroviral vector mediated gene transfer into human peripheral blood CD4$^+$ T lymphocytes. Gene Ther 1996;3:695-705.
27. Forestell SP, Dando JS, Bohnlein E, Rigg RJ. Improved detection of replication-competent retrovirus. J Virol Methods 1996;60:171-8.
28. Ruggieri L, Aiuti A, Salomoni M, Zappone E, Ferrari G, Bordignon C. Cell-surface marking of CD(34$^+$)-restricted phenotypes of human hematopoietic progenitor cells by retrovirus-mediated gene transfer. Hum Gene Ther 1997;8:1611-23.
29. Aiuti A, Friedrich C, Sieff CA, Gutierrez-Ramos JC. Identification of distinct elements of the stromal microenvironment that control human hematopoietic stem/progenitor cell growth and differentiation. Exp Hematol 1998;26:143-57.
30. Rigg RJ, Chen J, Dando JS, Forestell SP, Plavec I, Bohnlein E. A novel human amphotropic packaging cell line: high titer, complement resistance, and improved safety. Virology 1996;218:290-5.
31. Bonini C, Ferrari G, Verzeletti S, Servida P, Zappone E, Ruggieri L, et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. Science 1997;276:1719-24.
32. Miller AD, Buttimore C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol Cell Biol 1986;6:2895-902.
33. Markowitz D, Goff S, Bank A. Construction and use of a safe and efficient amphotropic packaging cell line. Virology 1988;167:400-6.
34. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 2000;288:669-72.
35. Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A, et al. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Sci-

ence 2002;296:2410-3.
36. Glimm H, Kiem HP, Darovsky B, Storb R, Wolf J, Diehl V, et al. Efficient gene transfer in primitive CD34+/CD38lo human bone marrow cells reselected after long-term exposure to GALV-pseudotyped retroviral vector. Hum Gene Ther 1997; 8:2079-86.
37. Kelly PF, Vandergriff J, Nathwani A, Nienhuis AW, Vanin EF. Highly efficient gene transfer into cord blood nonobese diabetic/severe combined immunodeficiency repopulating cells by oncoretroviral vector particles pseudotyped with the feline endogenous retrovirus (RD114) envelope protein. Blood 2000;96:1206-14.
38. Kobari L, Dubart A, Le Pesteur F, Vainchenker W, Sainteny F. Hematopoietic-promoting activity of the murine stromal cell line MS-5 is not related to the expression of the major hematopoietic cytokines. J Cell Physiol 1995;163:295-304.
39. Itoh K, Friel J, Laker C, Zeller W, Just U, Bittner S, et al. The role of soluble growth factors in inducing transient growth and clonal extinction of stroma cell dependent erythroblastic leukemia cells. Leukemia 1997;11:1753-61.
40. Mitjavila MT, Filippi MD, Cohen-Solal K, Le Pesteur F, Vainchenker W, Sainteny F. The Mpl-ligand is involved in the growth-promoting activity of the murine stromal cell line MS-5 on ES cell-derived hematopoiesis. Exp Hematol 1998; 26:124-34.
41. Han W, Ye Q, Moore MA. A soluble form of human Delta-like-1 inhibits differentiation of hematopoietic progenitor cells. Blood 2000;95:1616-25.
42. Drzeniek Z, Siebertz B, Stocker G, Just U, Ostertag W, Greiling H, et al. Proteoglycan synthesis in haematopoietic cells: isolation and characterization of heparan sulphate proteoglycans expressed by the bone-marrow stromal cell line MS-5. Biochem J 1997;327:473-80.
43. Nishi N, Ishikawa R, Inoue H, Nishikawa M, Yoneya T, Kakeda M, et al. In vitro long-term culture of human primitive hematopoietic cells supported by murine stromal cell line MS-5. Leukemia 1997;11 Suppl 3:468-73.
44. Ott DE, Coren LV, Copeland TD, Kane BP, Johnson DG, Sowder RC 2nd, et al. Ubiquitin is covalently attached to the p6Gag proteins of human immunodeficiency virus type 1 and simian immunodeficiency virus and to the p12Gag protein of Moloney murine leukemia virus. J Virol 1998;72:2962-8.
45. Hammarstedt M, Wallengren K, Pedersen KW, Roos N, Garoff H. Minimal exclusion of plasma membrane proteins during retroviral envelope formation. Proc Natl Acad Sci U S A 2000;97:7527-32.
46. Aiuti A, Vai S, Mortellaro A, Casorati G, Ficara F, Andolfi G, et al. Immune reconstitution in ADA-SCID after PBL gene therapy and discontinuation of enzyme replacement. Nat Med 2002;8:423-5.
47. Brown J, Reading SJ, Jones S, Fitchett CJ, Howl J, Martin A, et al. Critical evaluation of ECV304 as a human endothelial cell model defined by genetic analysis and functional responses: a comparison with the human bladder cancer derived epithelial cell line T24/83. Lab Invest 2000; 80:37-45.
48. Dando JS, Roncarolo MG, Bordignon C, Aiuti A. A novel human packaging cell line with hematopoietic supportive capacity increases gene transfer into early hematopoietic progenitors. Hum Gene Ther 2001; 12:1979-88.
49. Deryugina EI, Muller-Sieburg CE. Stromal cells in long-term cultures: keys to the elucidation of hematopoietic development? Crit Rev Immunol 1993;13:115-50.