Gene Transfer Into Human Bone Marrow Hematopoietic Cells Mediated by Adenovirus Vectors

By Tsutomu Watanabe, Charles Kuszynski, Kazuhiko Ino, Dean G. Heimann, H. Michael Shephard, Yoshinori Yasui, Daniel C. Maneval, and James E. Talmadge

Human bone marrow mononuclear cells (BMMNCs) and enriched CD34-positive (CD34+) cells were transduced with adenovirus vectors encoding Escherichia coli β-galactosidase gene. Transductions were carried out by 24-hour coinoculation with adenovirus vectors at different multiplicities of infections (moi). Efficacy of gene transfer into BM cells and expression of the gene product (ie, β-galactosidase) were studied using X-Gal histochemical staining and flow cytometric analysis. X-Gal staining demonstrated that the percentage of positive cells at moi of 5 to 500 was 3.4% to 34.5% for BMMNCs and 6.0% to 20.0% for enriched CD34+ cells. Similar results (1.5% to 35.7% for BMMNCs and 5.4% to 24.2% for enriched CD34+ cells) were obtained with flow cytometric analysis using fluorescein di-β-D-galactopyranoside (FDG). Multicolor flow cytometry analysis, which included FDG, demonstrated that BM progenitors (CD34+ or CD34+CD38-) and natural killer cells (CD56-), granulocytes, and monocytes all expressed the adenovirus transgene. To ascertain the effects of adenovirus vectors on normal BM progenitors, the numbers of colony-forming unit-granulocyte/macrophage (CFU-GM), burst-forming unit-erythocyte (BFU-E), and high-proliferative potential-colony-forming cells (HPP-CFC) after 24-hour coinoculation with adenovirus vectors were determined. When BMMNCs or enriched CD34+ cells were incubated with adenovirus vectors at moi of 5 and 50, no significant differences in the numbers of CFU-GM, BFU-E, and HPP-CFC were observed compared with the uninfected control cells. However, the numbers of CFU-GM were significantly (P < .01) decreased when BMMNCs or enriched CD34+ cells were incubated with adenovirus vectors at a moi of 500, compared with the uninfected control cells. The adenovirus infected cells, purified by cell sorting for FDG expression, were capable of growing in culture and gave rise to various colonies (ie, CFU-GM, BFU-E, and HPP-CFC). These data indicate that recombinant adenovirus vectors can be used to transfer genes to human BM hematopoietic cells with expression of the exogenous gene at a high transduction efficiency.

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Materials and Methods

Recombinant Adenovirus Vectors

The recombinant, replication-deficient adenovirus vectors encoding E.coli β-galactosidase gene (Ad/β-gal) were generously provided by Canji, Inc (San Diego, CA). The viruses were prepared, purified, and titered as previously described.13 Briefly, in Ad/β-gal a portion of the E1a and E1b region of human adenovirus type 5 was replaced with the β-galactosidase gene under the transcriptional control of human cytomegalovirus (CMV) promoter using standard cloning procedures. Ad/β-gal was produced in the 293 human embryonic kidney cell line and purified by ultracentrifugation through two cesium chloride gradients. The titers of viral stocks were determined by a limiting dilution assay on 293 cells. Titters of the viral stocks used were 2.9 to 5.8 × 10^10 infectious units/mL as measured by titration assay on 293 cells. Titters of the viral stocks used were 2.9 to 5.8 × 10^10 infectious units/mL as measured by titration assay on 293 cells.
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Fig 1. Demonstration of β-galactosidase expression in human bone marrow cells infected with Ad/β-gal by X-Gal histochemical staining. Diffuse stained blue cells (indicated by arrows) were counted as positive. 34.5% of cells expressed β-galactosidase on this slide.

Human Bone marrow Cells and Enrichment of CD34+ Cells

Human BM cells were obtained from normal donors or patients who were undergoing autologous BM harvesting without histologic evidence of tumor cell infiltration in the BM after informed consent was obtained. The BM mononuclear cells (BMMNCs) were isolated on Ficoll-Hypaque gradients, and CD34+ cells were separated on a Ceprate (Cell-Pro Inc, Bothell, WA) immunoaffinity column according to the manufacturer’s protocol.

Transduction of Human Bone Marrow Cells

BMMNCs or enriched CD34+ cells were plated in 2 mL of Iscove’s Modified Dulbecco’s Medium (IMDM) containing 10% fetal bovine serum (FBS) in 24-well flat bottom plates at 2 x 10^6 cells per well. The cells were incubated in triplicate with Ad/β-gal at different MOI (500, 50, 5, and 0). Viral stock was diluted with Ca2+-Mg2+-free phosphate-buffered saline (PBS). Twenty-four-hour coincubation was utilized because prior results revealed improved rates of infection with this protocol (data not shown). After 24-hour incubation, the cells were harvested and assessed for transduction efficiency and colony-forming cell (CFC) assays.

Determination of Transduction Efficiency by β-Galactosidase Gene Expression

X-Gal histochemical staining. After 24-hour incubation with Ad/β-gal, cells (1 x 10^6) were washed once with PBS to remove free viral particles and fixed with 1:10 (3.7%) formaldehyde (Sigma Chemical Co, St Louis, MO) solution in PBS for 6 to 8 minutes. The cells were washed twice with PBS after fixation and then were stained using a staining solution containing 1.2 mmol/L MgCl2 (Research Organic Inc, Cleveland, OH), 3 mmol/L K,Fe(CN)6 (Sigma Chemical Co), and 1 mmol/L of the X-Gal stain (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; US Biochemical, Cleveland, OH) at room temperature overnight. Cytospin preparations were prepared using standard techniques (Cytospin 3; Shandon Inc, Pittsburgh, PA). Diffusely stained blue cells were counted as positive for β-galactosidase gene expression.

Table 1. Frequency of Cells Expressing β-Galactosidase by FACS Analysis and X-Gal Histochemical Staining

<table>
<thead>
<tr>
<th></th>
<th>MOI: 500</th>
<th>MOI: 50</th>
<th>MOI: 5</th>
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<tr>
<td>FDG (+) Cells by FACS Analysis (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMMNC#1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BMMNC#2</td>
<td>6.7 ± 4.6</td>
<td>11.7 ± 6.5</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>BMMNC#4</td>
<td>30.0 ± 19.5</td>
<td>35.7 ± 20.0</td>
<td>9.5 ± 1.3</td>
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<tr>
<td>BMMNC#5</td>
<td>7.7 ± 0.5</td>
<td>14.9 ± 2.8</td>
<td>7.8 ± 3.4</td>
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<tr>
<td>Enriched CD34+ #2</td>
<td>24.2 ± 1.5</td>
<td>13.1 ± 1.3</td>
<td>9.2 ± 1.0</td>
</tr>
<tr>
<td>Enriched CD34+ #3</td>
<td>18.1 ± 1.4</td>
<td>10.8 ± 1.2</td>
<td>5.4 ± 0.4</td>
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<tr>
<td>Positive Cells by X-Gal Stain (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMMNC#1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BMMNC#2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BMMNC#4</td>
<td>19.7 ± 1.6</td>
<td>12.4 ± 1.8</td>
<td>3.8 ± 1.2</td>
</tr>
<tr>
<td>BMMNC#5</td>
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<td>15.0 ± 2.3</td>
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<td>13.3 ± 2.4</td>
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</tr>
<tr>
<td>Enriched CD34+ #3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviations: FDG, fluorescein di-β-galactopyranoside; MOI, multiplicity of infection; BMMNC, bone marrow mononuclear cell; ND, not determined.

* Data are % positively stained cells and represent the mean of independent experiments ± SD.
† The negative control cells after 24-hour incubation in media had <0.5% positive cells by X-Gal staining.
Flow cytometry analysis. After 24-hour incubation with Ad/β-gal, cells were washed once with PBS to remove free viral particles and were prepared for flow cytometry. Cells were suspended with phycoerythrin (PE) and biotin-allophycocyanin (APC)- (or cytochrome) conjugated specific monoclonal antibodies or PE/biotin-APC-conjugated irrelevant antibody of the same isotype in PBS. The antibodies used were as follows: T11-PE (CD2), My4-biotin (CD14), B4-biotin (CD19) (all from Coulter Immunology, Hialeah, FL); LeuM7-PE (CD13), HPCA2-PE (CD34), Leu19-PE (CD56) (all from Becton Dickinson, San Jose, CA); and cytochrome anti-human CD38 (Pharmingen, San Diego, CA). After 30 minutes of incubation in the dark at 4°C and then being washed with PBS containing 2% bovine serum albumin, the cells (1 X 10^6 in 50 µL of PBS) were stained with fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes, Inc, Eugene, OR) as described elsewhere4,15 and as per the manufacturer’s recommendations. Briefly, a 2 mmol/L solution of FDG as a substrate in 98:1:1 H2O/DMSO/ethanol was mixed with an equal volume (50 µL) of cell suspension, which was prewarmed at 37°C. After a 1-minute incubation at 37°C, an equal volume (50 µL) of ice-cold two times-strength PBS was added. The samples were maintained at 4°C for 3 to 4 hours before analysis by flow cytometry. Flow cytometry was performed with a FACStar flow cytometer (Becton Dickinson). Data analysis was performed with the Lysys II analysis software (Becton Dickinson). Uninfected cells not incubated with FDG served as the negative control for FDG fluorescence and were used to set the upper boundary of the negative cell autofluorescence range. Cells with FDG fluorescence above this boundary were considered to be FDG positive (+).

For BMMNCs or enriched CD34' cells, the frequency of FDG positive cells was calculated by subtracting the frequency of FDG (+) cells in the uninfected cells with respect to each antibody, from that observed in the infected cells. Averages of a series of multicolor flow cytometry studies (ie, FDG v CD2, CD13, CD14, CD19, or CD56 for BMMNCs [n = 5], and FDG v CD34 or CD38 for enriched CD34' cells [n = 2]) were obtained. Side-scatter characteristics and CD14 fluorescence were used to discriminate between granulocyte and monocyte populations. For the analysis of CD34'CD38' cell populations, appropriate numbers (>400) of cells were acquired in a window gate based on CD34 and CD38 expression. The frequency of FDG (+) cell for each particular phenotype population was calculated as follows:

For example (for CD2' phenotype population),

\[
\text{Frequency} \% = \frac{\% \text{CD2'} \text{FDG}^+ \text{of Infected Cells}}{\% \text{CD2'} \text{FDG}^+ \text{of Uninfected Cells} - \% \text{CD2'} \text{FDG}^+ \text{of Uninfected Cells}}
\]

The frequencies of FDG (+) cell for phenotype fractions were examined in three separate BMMNC samples and two enriched CD34' cell samples.

FDG positive cell sorting by flow cytometry. Enriched CD34' cells (6 to 8 X 10^6 cells) after incubation with Ad/β-gal (at a moi of 50) were labeled with FDG as described above with modification (In these experiments, 800 to 1,200 µL of cell suspension and 800 to 1,200 µL of 2 mmol/L FDG solution were used). Using a dual-laser FACStar plus instrument and an automated deposition unit, cells expressing a high level of fluorescence intensity were isolated in a single step. A FDG (-) cell fraction was also obtained. After being washed twice with Hank’s Balanced Salt Solution (HBSS) containing 25 µg/mL of ammonium B (Life Technologies Inc, Grand Island, NY) and 40 µg/mL of gentamicin (Life Technologies Inc), cells were plated for CFC assays.
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RESULTS

The Frequency of BMMNC Infected by Adenovirus Vectors

Exposure of BMMNCs to Ad/β-gal for 24 hours at moi of 5 to 500 resulted in expression of the β-gal gene (Fig 1) in 3.4% to 34.5% of the cells by X-Gal histochemical staining, and 1.5% to 35.7% by fluorescence-activated cell sorter (FACS) analysis using FDG (Table 1). A dose-response relationship between viral dose (ie, MOI) and the frequency of β-gal-positive cells was observed at a MOI of 50 in three of three experiments. Double- or triple-color staining of the BM cells using lineage-specific monoclonal antibodies after 24-hour incubation with Ad/β-gal allowed the analysis of the phenotypic frequencies of FDG (+) cells (Fig 2). All cell lineages appeared to be transduced with Ad/β-gal (Fig 3). The data in Fig 3 are a composite of all the studies and incorporate the results that are shown from a single study in Fig 2.

The Frequency of Enriched CD34⁺ Cells Infected by Adenovirus Vectors

Flow cytometry analysis using the anti-CD34 antibody HPCA-2-PE showed that 61.7% to 81.7% of the cells purified were positive for CD34 expression. Two-color analysis of enriched CD34⁺ cells is shown in part in the bottom four panels of Fig 2. Exposure of enriched CD34⁺ cells to Ad/β-gal for 24 hours at MOIs of 5 to 500 resulted in expression of the β-gal gene in 6.0% to 20.0% of the cells by X-Gal histochemical staining, and 5.4% to 24.2% by FACS analysis using FDG (Table 1). A dose-response relationship between viral dose and the frequency of β-gal-positive cells was seen for X-Gal staining and FACS analysis. Double- or triple-color staining of enriched CD34⁺ cells after 24-hour incubation with Ad/β-gal showed that the transduction efficiency of CD34⁺ cells and primitive progenitor cells (ie, CD34⁺CD38⁻ fraction) was 10.6% to 25.2% and 7.9% to 22.9%, respectively, at MOIs of 5 to 500 (Fig 3).

The Cytotoxicity of Ad/β-gal to Bone Marrow Progenitors

After 24-hour incubation with Ad/β-gal, nonadherent cells in the culture were harvested. There was no difference in the numbers of nonadherent cells between the infected cells containing 25% FBS, 10% AB human plasma, 100 units/mL penicillin, 100 μg/mL streptomycin, 5 × 10⁻⁵ mol 2-ME, 200 units/mL rHu IL-3, 200 units/mL rHu GM-CSF, and 200 units/mL rHu G-CSF. After a 21-day incubation, cell aggregates having a diameter of greater than 1 mm were counted as HPP-CFC. The number of progenitor cells per well was calculated as follows:

**Progenitor Cells/Well**

\[
\text{No. of Progenitors/Plate} \times \frac{\text{No. of Cells/Well}}{\text{No. of Cells/Plate}}
\]

Statistics

Gene transfer experiments and CFC assays were performed in triplicate except for multicolor flow cytometry analysis. Statistical analysis were performed by two-tailed Student’s t-test. A P value <.05 was considered to indicate statistical significance.

CFC Assays

For colony-forming unit-granulocyte/macrophage (CFU-GM), 1 × 10⁵ BMMNCs or 1 to 2 × 10⁶ enriched CD34⁺ cells were cultured in 1 mL of 0.3% agar-IMDM containing 25% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 5 × 10⁻⁵ mol 2-mercaptoethanol (2-ME), 200 units/mL recombinant human interleukin-3 (rHu IL-3; Biosource International, Camarillo, CA), 200 units/mL recombinant granulocyte colony-stimulating factor (rHu GM-CSF; Immunex Corp., Seattle, WA), and 200 units/mL recombinant granulocyte/macrophage colony-stimulating factor (rHu G-CSF; Amgen Inc., Thousand Oaks, CA). For burst-forming units-erythroid (BFU-E), 1 × 10⁵ BMNCs or 1 to 2 × 10⁶ enriched CD34⁺ cells were cultured in 1 mL of 1.2% methylcellulose-IMDM containing 25% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 5 × 10⁻⁵ mol 2-ME, 200 units/mL rHu IL-3, and 2 units/mL recombinant erythropoietin (rHu EPO; Amgen Inc.). Cells were cultured in 35 mm plates and incubated for 14 days at 37°C in a humidified atmosphere containing 5% CO₂ in air. After a 14-day incubation, colonies consisting of 50 cells or more were counted as CFU-GM, and more than three cluster of hemoglobinized cells were counted as BFU-E under an inverted microscope. For high proliferative potential—colony-forming cell (HPP-CFC), 5 × 10⁵ BMNCs or 500 to 1,000 enriched CD34⁺ cells were cultured in 1 mL of 0.3% agar-IMDM containing 25% FBS, 10% AB human plasma, 100 units/mL penicillin, 100 μg/mL streptomycin, 5 × 10⁻⁵ mol 2-ME, 200 units/mL rHu IL-3, 200 units/mL rHu GM-CSF, and 200 units/mL rHu G-CSF. After a 21-day incubation, cell aggregates having a diameter of greater than 1 mm were counted as HPP-CFC. The number of progenitor cells per well was calculated as follows:
Fig 4. The effects of 24-hour incubation with Ad/β-gal on cell numbers per well and progenitor cells per well in experiments using BMMNCs.

*P < .05; **P < .01.

Clonogenic Potential of FDG-Positive Cells Sorted by Flow Cytometry

After cell sorting of enriched CD34+ cells (purity 70.2% to 81.7%), populations of 88% to 96% pure FDG (+) cells that were 87% to 95% viable (trypan blue exclusion) were obtained (Fig 6). FDG (-) cells that were 93% to 100% viable and 95% to 98% pure were also obtained. The colony-forming properties of the sorted FDG (+) and FDG (-) CD34+ cells are shown in Table 2. A decreased number of CFU-GM was observed in the FDG (+) fraction compared with the uninfected control cells (P < .01). However, the inhibition of other colony assays was not observed in the same experiment (BFU-E and HPP-CFC assays). In other experiments there were no significant differences in the colony numbers obtained from FDG (+) and FDG (-) fractions.

DISCUSSION

Gene transfer to BM hematopoietic cells is an attractive theoretical option for the treatment of hematologic and congenital disorders. Potential gene therapy approaches include induced expression of multiple drug resistance gene, MDR1 and the p53 tumor suppressor gene. Another approach is to enhance expression of proteins such as growth factors and interleukins. Retroviruses as a vector to deliver genes into the hematopoietic system have been extensively studied, but their efficacy is limited to actively replicating target cells. The quiescent nature of human BM cells
limits the utility of retroviruses for efficient gene transfer in this system. In addition, the stem cell transduction efficiencies of retroviruses for human BM cells are generally <10%. However, recent studies using both growth factors and stromal cells have reported higher transduction efficiencies with retroviruses. Thus, ex vivo expansion of retrovirus-infected cells might be necessary to ensure sufficient expression of gene. Furthermore, pretreatment of target cells with hematopoietic growth factors such as GM-CSF and IL-3 or the addition of detergents such as protamine and polybrene during co-incubation of target cells with retrovirus-producing cells are needed to improve transduction efficiency. In contrast, adenovirus vectors can infect both quiescent and dividing cells without manipulation, thereby, facilitating gene transduction ex vivo. This property might be an advantage for the use of adenovirus vectors in the clinical application of gene transfer, although adenovirus-mediated gene transfer is transient and might not be suitable for the treatment of congenital gene defects.

Human BM consists of heterogeneous cells expressing a variety of cell maturation and lineage markers in different stages of the cell cycle. Retrovirus entry into cells is dependent on the expression of the appropriate viral receptors on the target cells. Because receptors are expressed at varying levels on different cell types, this results in a variable infection efficiency by adenovirus vectors. However, receptors can be induced by pretreatment of hematopoietic growth factors. Our data demonstrate that adenovirus vectors infect all hematopoietic cellular subpopulations, although to a variable frequency ranging from 10% to 40%. These data are consistent with other reports demonstrating adenovirus-mediated transduction of quiescent cells as well as activated cells, including fully differentiated hematopoietic cells. It might be possible to select the target cell population and to transfer the specific gene of choice using adenovirus vectors. Alternatively, the use of tailored promoters may enhance the specificity of gene expression. This could broaden the utility of adenovirus vectors for clinical application.

The potential toxicity of adenovirus vectors to the target cells still remains a concern. In the present study, the growth of progenitor colonies was significantly inhibited after coinoculation with adenovirus vectors at a high MOI (500). The apparent differential toxicity between CFU-GM and HPP-CFC may be associated with the difference in commitment between these two populations and the limited number of cell division that a CFU-GM is capable of. Because the infection by an adenovirus may slow or limit division and is not found in daughter cells, it would reduce the number of colonies especially in a cell that is only capable of two to three additional cell divisions. Whereas, in a cell capable of a greater number of cell divisions the effect would be as apparent as appears to be the case between CFU-GM and HPP-CFC. We have not determined the causes of this inhibi-
The reaction is induced with the production of cytokines such as interferons and viability. This observation suggests that the function of an immune response directed against the cells. Further, these viral proteins may lead to the induction of an immune response directed against the cells. Also, at the initial phase of adenovirus infection, an inflammatory reaction is induced with the production of cytokines such as IL-6 and IL-8, which are implicated in this reaction.

In the present study, interestingly, we observed the highest transduction at a MOI of 50 by FACS analysis using FDG in three of five experiments, which is inconsistent with the results of X-Gal staining. However, the fluorescence assay using FDG is believed to be more sensitive than the histochemical assays. Nonetheless in the FDG assays, the enzymatically liberated fluorochrome must be retained within the cells, and the staining procedure must maintain cell function and viability. This observation suggests that the function of cells infected at a high MOI (500) might be impaired, consistent with results from CFC assays. However, we demonstrated that the population of cells (transduced at a MOI of 50) obtained after cell sorting for FDG expression were capable of growing in culture and giving rise to various colonies (ie, CFU-GM, BFU-E, and HPP-CFC), suggesting that the function of transduced cells (at a MOI of 50) is not impaired. Significant gene vector infection and gene expression is seen at a MOI of 50. However, the transduction efficiency is likely to be better at a higher MOI, and future studies examining MOIs between 50 and 500 are ongoing. Future studies might also focus on the use of different promoters that might improve the expression of genes.

Thus, the present study showed that recombinant adenovirus vectors can be used for gene transfer to the hematopoietic system ex vivo with expression of the exogenous gene. Further, the virus titers needed to attain the desired biologic effects have limited effects on normal hematopoietic function. The short-term infection and expression in hematopoietic cells by adenovirus vectors of suppressor proteins such as p53 or the retinoblastoma gene product have significant potential for use as a purging agent. In addition, this is an ideal way to target the delivery of hematopoietic cytokines, particularly hematopoietic cytokines with a systemic toxicity, for site-specific secretion in the marrow. This approach could increase hematopoietic recovery after myeloablative and myelosuppressive chemotherapeutic protocols. Lastly, transduction by adenovirus vectors may provide a way to deliver antigens for specific stimulation of the immune system at sites of lymphoid and hematopoietic function.

ACKNOWLEDGMENT

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REFERENCES


Table 2. Clonogenic Potential of FDG-Positive Cells Sorted by Flow Cytometry

<table>
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<tr>
<th>Cell Fraction</th>
<th>CFU-GM (&gt;50 cells at day 14)</th>
<th>BFU-E (&gt;3 clusters at day 14)</th>
<th>HPP-CFC (&gt;1 mm at day 21)</th>
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<tbody>
<tr>
<td>Exp 1</td>
<td>40.7 ± 8.2</td>
<td>ND</td>
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</tr>
<tr>
<td></td>
<td>42.0 ± 15.6 (P = .902)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FDG (+)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FDG (-)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Exp 2</td>
<td>20.0 ± 6.0</td>
<td>26.0 ± 8.5 (P = .579)</td>
<td>8.7 ± 2.3 (P = .422)</td>
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<tr>
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<td>44.4 ± 5.7 (P = .005)</td>
<td>30.8 ± 3.4 (P = .422)</td>
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<td>Exp 3</td>
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</tr>
<tr>
<td>FDG (-)</td>
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</tbody>
</table>

Enriched CD34+ cells were used in all experiments. BM cells were infected with Ad/β-gal at a MOI of 50. All samples were plated in triplicate. Results represent the mean number of colonies per 105 cells ± SD. P values vs FDG (-) fraction.

Abbreviations: FDG, fluorescein di-β-galactopyranoside; ND, not determined.
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Gene transfer into human bone marrow hematopoietic cells mediated by adenovirus vectors [see comments]

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