In vivo selection of MGMT(P140K) lentivirus–transduced human NOD/SCID repopulating cells without pretransplant irradiation conditioning

See the related Commentary beginning on page 1478.

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Infusion of transduced hematopoietic stem cells into nonmyeloablated hosts results in ineffective in vivo levels of transduced cells. To increase the proportion of transduced cells in vivo, selection based on P140K O^6-methylguanine-DNA-methyltransferase (MGMT[P140K]) gene transduction and O^6-benzylguanine/1,3-bis(2-chloroethyl)-1-nitrosourea (BG/BCNU) treatment has been devised. In this study, we transduced human NOD/SCID repopulating cells (SRCs) with MGMT(P140K) using a lentiviral vector and infused them into BG/BCNU–conditioned NOD/SCID mice before rounds of BG/BCNU treatment as a model for in vivo selection. Engraftment was not observed until the second round of BG/BCNU treatment, at which time human cells emerged to compose up to 20% of the bone marrow. Furthermore, 99% of human CFCs derived from NOD/SCID mice were positive for provirus as measured by PCR, compared with 35% before transplant and 11% in untreated irradiation-preconditioned mice, demonstrating selection. Bone marrow showed BG-resistant O^6-alkylguanine-DNA-alkyltransferase (AGT) activity, and CFUs were stained intensely for AGT protein, indicating high transgene expression. Real-time PCR estimates of the number of proviral insertions in individual CFUs ranged from 3 to 22. Selection resulted in expansion of one or more SRC clones containing similar numbers of proviral copies per mouse. To our knowledge, these results provide the first evidence of potent in vivo selection of MGMT(P140K) lentivirus–transduced human SRCs following BG/BCNU treatment.

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Introduction

Correction of hematologic disorders by gene therapy has been hampered by the inability to transduce sufficient numbers of hematopoietic stem cells (HSCs) to exert a phenotypic change. Gene transfer using oncoretroviral vectors in human clinical trials has frequently resulted in less than 1% of peripheral blood or bone marrow cells containing the transgene (1, 2), although gene marking in the 10% range has been reported (3). Development of lentiviral vectors, which for various reasons have a greater propensity to transduce nondividing cells than do oncoretroviral vectors (4–6), has generated excitement due to the relative ease at which gene transfer to 80% or more NOD/SCID repopulating cells (SRCs) can be achieved using short-term culturing conditions considered to be superior to those used for oncoretroviral transduction (7–9). However, in the clinical setting of autologous transplantation without myeloablative conditioning, it is likely that in vivo gene marking of peripheral blood and bone marrow cells will be substantially less, and therefore correction of some hematologic disorders, such as the hemoglobinopathies, may still be out of reach.

Methods to increase the proportion of transduced HSCs in the bone marrow have therefore been under investigation and usually rely on transduction of a drug-resistance gene followed by chemotherapeutic treatment to mediate selection. O^6-methylguanine-DNA-methyltransferase (MGMT) has been extensively studied, because its gene product, O^6-alkylguanine-DNA-alkyltransferase (AGT), functions to repair alkylated DNA. The chemotherapeutic chloroethylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) is a potent stem cell toxin, and myelosuppression is the clinically observed dose limiting toxicity associated with treatment. O^6-benzylguanine (BG) inactivates endogenous AGT, increasing

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Nonstandard abbreviations used: hematopoietic stem cell (HSC); NOD/SCID repopulating cell (SRC); O^6-methylguanine-DNA-methyltransferase (MGMT); O^6-alkylguanine-DNA-alkyltransferase (AGT); 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU); O^6-benzylguanine (BG); 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloro)-3-nitrosourea (ACNU); self-inactivating (SIN); lentiviral vector stem cell factor (SCF); thrombopoietin (TPO); irradiation (IR); linear amplification–mediated PCR (LAM-PCR).

sensitivity to alkylating agents; however, as we have previously shown, transduction of BG-resistant MGMT mutants (G156A or P140K) to human hematopoietic progenitors using oncoretroviral or lentiviral vectors increases their resistance to combination BG/BCNU treatment in vitro (10, 11). In the murine model, we and others have shown that bone marrow transduced with MGMT(P140K) or MGMTx(G156A) can be enriched in vivo to 100% after rounds of BG/BCNU, BG/temozolomide, or BG/1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloro)-3-nitrosourea (BG/ACNU) treatment (12–16). Notably, Davis et al. (13) have shown that in vivo enrichment of retrovirus–MGMT(G156A)–transduced HSCs can occur in a nonmyeloablative setting from undetectable levels to 100% in bone marrow–derived CFUs, representing up to 940-fold enrichment.

These results have been encouraging, but gene transfer studies using murine HSC models have not been predictive of the human system, as shown by clinical trials. Murine HSCs are more easily transduced by oncoretroviral vectors, and study of hematopoietic reconstitution by highly transduced HSCs in fully myeloablated animals does not closely mimic the clinical setting. Development of the NOD/SCID mouse model for human HSCs has allowed assessment of gene transfer to primitive human hematopoietic progenitors in a more relevant system of presumably greater predictive value.

Lentiviral vectors are rapidly becoming the vectors of choice for HSC gene therapy, but their in vivo use for drug-resistance gene therapy has not been evaluated. Neither has there been a direct evaluation of the frequency of lentiviral integration in primitive human hematopoietic progenitors undergoing selection. In this study, we demonstrate in vivo selection of human SRCs transduced by a lentiviral vector with MGMT(P140K). Importantly, NOD/SCID mice were conditioned with BG/BCNU before transplantation with human $CD34^+$ cells, mimicking a potential clinical application. Rounds of BG/BCNU treatment selected for human MGMT(P140K)–transduced SRCs over both human and endogenous murine hematopoietic progenitors, resulting in increased human-cell engraftment, bone marrow AGT activity, and expression of AGT in human CFUs. Using real-time PCR, we determined that repopulation was by SRCs containing 3–22 proviral copies. Multiple vector insertions occurred despite low-MOI transduction conditions.

Methods

Vector. The vector used was a VSV-G–pseudotyped, second-generation HIV-based lentivirus with self-inactivating (SIN) long terminal repeats (17), containing a MGMT(P140K) transgene run from an internal CMV promoter. The MGMT(P140K) transgene and central polypurine tract/central termination sequence (cPPT/CTS) sequences (DNA flap) were inserted into the transfer plasmid pHR′CMV.SIN as previously described (11, 18). The woodchuck hepatitis virus post-transcriptional regulatory element was incorporated 3′ of the transgene by blunt-end ligation into a unique SmaI restriction site.

Vector (lentiviral vector MGMT(P140K), LV–MGMT(P140K)) was produced by transient transfection of 293T cells with component plasmids. Briefly, 293T cells were seeded into 15-cm plates 1 day before cotransfection with plasmids pCMVΔR8.91 (packaging), pMD.G (envelope), and pHR′f.C.P140K.W.SIN (transfer) as previously described (11). The titer of collected supernatant was determined by dilution on 293T cells, and transduction was analyzed by flow cytometric determination of AGT expression. The same unconcentrated virus lot was used for all transductions and had an expression titer of 2×10^7 per milliliter.

$CD34^+$ cell isolation and transduction. Human umbilical cord blood was obtained through the Comprehensive Cancer Center Stem Cell Facility, Case Western Reserve University, from normal deliveries of mothers who gave informed consent at Rainbow Babies and Children's Hospital (Cleveland, Ohio, USA). Ficoll-Paque density centrifugation (Pharmacia Biotech AB, Uppsala, Sweden) was used to obtain mononuclear cells. A Miltenyi MiniMACS $CD34^+$ cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used to enrich for $CD34^+$ cells.

$CD34^+$ cells were prestimulated overnight in Iscove's medium containing 10% FBS (Invitrogen Corp., Carlsbad, California, USA), 2 mM GlutaMAX (Invitrogen Corp.), 100 ng/ml stem cell factor (SCF; a gift of Amgen Inc., Thousand Oaks, California, USA), 100 ng/ml Flt-3L (Immunex, Seattle, Washington, USA), and 50 ng/ml thrombopoietin (TPO; R&D Systems Inc., Minneapolis, Minnesota, USA). At 1 day after isolation, cells were transduced with LV–MGMT(P140K) in DMEM containing 10% heat-inactivated FBS, 2 mM GlutaMAX, 8 μg/ml Polybrene (Sigma-Aldrich), and SCF, Flt-3L, and TPO as above. Cell density was 4×10^5 per milliliter, and MOI was 2. Transduction cultures were centrifuged (spinoculated) at 600 *g* for 45 minutes at room temperature before incubation at 37°C in a 5% CO_2 humidified environment.

Following a 1-day transduction, cells were washed in serum-free Iscove's medium; then they were transplanted into NOD/SCID mice, or the in vitro transduction level was analyzed by flow cytometry of mass culture or PCR analysis of CFUs.

Transplants and treatments in NOD/SCID mice. Six- to eight-week old NOD.CB17-$Prkdc^{SCID}$/J (NOD/SCID) mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were housed under specific pathogen–free conditions. Beginning at transplant, water was supplemented with Sulfatrim (Alpharma Inc., Baltimore, Maryland, USA). One day before transplant, mice were injected intraperitoneally with 30 mg/kg BG dissolved in 40% polyethylene glycol (Union Carbide Corp., Danbury, Connecticut, USA), 60% PBS (pH 8.0), followed at 1 hour by 10 mg/kg BCNU dissolved in ethanol and diluted in PBS. Irradiated control mice were given 250 cGy radiation from a Cs^{137} source and transplanted the same day. All mice were transplanted via the tail vein. Subsequent treatments

were with 30 mg/kg BG and 7.5 mg/kg BCNU, prepared and administered as described above. Mice were sacrificed 8–10 weeks after transplant, and bone marrow was flushed from clipped femurs for analysis.

Flow cytometry. AGT staining using mT3.1 antibody (Kamiya Biomedical Co., Seattle, Washington, USA) was done as previously described (11).

$CD34^+$ staining of human cells was done with phycoerythrin-conjugated anti-CD34 antibody (BD Biosciences Pharmingen, San Diego, California, USA). For identification of human cells in NOD/SCID bone marrow, cells were washed, blocked with normal mouse IgG (Caltag Laboratories Inc., Burlingame, California, USA), and stained with CyChrome-conjugated anti–human CD45 (BD Biosciences Pharmingen).

CFC assay. Following transduction, 1,200 cultured cells were added to 4 ml MethoCult (StemCell Technologies, Vancouver, British Columbia, Canada) containing hemin, SCF, IL-3, GM-CSF, and erythropoietin as previously described (11), to generate CFUs for analysis of the in vitro transduction level by PCR. These conditions stimulate human CFU formation only (our unpublished observation). CFUs were plucked 13 days after plating and processed for PCR analysis by digestion with proteinase K. Human CFUs from NOD/SCID mouse bone marrow were generated in a similar manner, except that 10^4–10^6 nucleated bone marrow cells were plated.

For determination of recoverable murine CFCs in bone marrow of treated mice, 3×10^5 cells were plated in 4 ml MethoCult containing hemin, rat SCF, murine IL-3, 1.2% spleen cell–conditioned medium, and erythropoietin, as previously described (13).

AGT activity assay. DNA was quantified in bone marrow extracts by staining with Hoechst dye and comparison of fluorescence measured on a fluorometer with a standard curve of calf-thymus DNA of known concentration. AGT activity was determined in bone marrow extracts, as previously described, by measurement of release of tritiated methyl groups from [^{3}H]methylnitrosourea-treated calf-thymus DNA substrate (19). Alkylated [^{3}H-methyl]O^6-methylguanine and N^7-methylguanine were separated by HPLC and quantified by liquid scintillation. AGT activity was expressed as fmol O^6-methylguanine removed per microgram DNA. BG-resistant AGT activity was determined by incubation of cell extracts with 50 μM BG for 30 minutes, before addition of DNA substrate.

Immunocytochemistry. CFUs were washed and cells dispersed in PBS. One drop of cell suspension was dried on a glass slide and fixed in 4% paraformaldehyde. Slides were incubated with 0.3% H_2O_2 in methanol, washed, and incubated in 10% normal goat serum. Slides were then incubated with anti-AGT antibody mT3.1 overnight followed by HRP-conjugated goat anti-mouse IgG. A peroxidase substrate kit (Vector Laboratories Inc., Burlingame, California, USA) was used for visualization. Slides were stained simultaneously, and reactions were allowed to proceed for equivalent times.

Endpoint PCR. PCR detection of MGMT(P140K) was done on proteinase K–digested CFUs using the following primer pair: sense, 5′-GTGAGCAGGGTCTGCACGAA-3′; antisense, 5′-TTCATGGGCCAGAAGCCATT-3′. The positive control was mass-culture MGMT(P140K)–transduced $CD34^+$ cells. The negative control was either water or methylcellulose obtained from areas of plates where CFUs were absent.

Real-time PCR. Real-time PCR was performed on a LightCycler instrument (Roche Diagnostics, Basel, Switzerland) using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics). A standard curve to quantify genomic copies in individual CFUs was prepared from primary umbilical cord blood mononuclear cells using human GAPDL4-specific primers (sense, 5′-CCCTTCATTGACCTCAACTACATGGT-3′; antisense, 5′-GAGGGGCCATCCACAGTCTTCTG-3′). DNA was quantified by Hoechst fluorescence as described for the AGT activity assay, and one diploid genome was assumed to contain 6.4 pg DNA. CFU DNA was diluted 1:50 to bring it within the range of standards of 300 to 10^4 copies. Genome copies in CFU samples were based on two GAPDL4 copies per diploid genome.

MGMT(P140K) copy number was determined using the MGMT(P140K) primers described above and a standard curve made from dilutions of genomic DNA from a K562 cell line containing a single integrated copy of MGMT(G156A). Single-copy control K562 was confirmed by comparison with a negative genomic DNA sample spiked with known amounts of MGMT(G156A) plasmid. K562 DNA was quantified by Hoechst fluorescence as described above. One genome of K562 was assumed to contain 9.6 pg DNA, based on near triploid DNA content (20). Equal volumes of diluted CFU samples were used for GAPDL4 and MGMT(P140K) copy determination, and 90% of samples fell within the range of the standards.

MGMT(P140K) copy number per genome was determined by division of the number of MGMT(P140K) copies by the number of genome copies. Separate duplicate runs of GAPDL4 and MGMT(P140K) were completed on all mouse-derived CFUs, and the values were averaged.

K562 clone generation. Two K562 clonal populations, each containing integrated lentiviral provirus, were generated by transduction of K562 cells with LV–MGMT(P140K). Transduced cells were plated in methylcellulose containing 20 ng/ml IL-3 and 100 ng/ml GM-CSF, and isolated CFUs were plucked after 14 days and expanded in Iscove's complete medium. Two clones positive for provirus by endpoint PCR were chosen for real-time PCR analysis. These clones were replated as a 1:1 mixture in methylcellulose, and the resulting CFUs were prepared and analyzed by real-time PCR to determine the numbers of GAPDL4 copies and MGMT proviral copies as described above.

Statistics. Error bars, *P* values, and linear regressions were generated using GraphPad Prism software (GraphPad Software for Science Inc., San Diego, California, USA). Error bars represent SEM.

Results

The following experimental design was used to investigate in vivo selection of human HSCs (Figure 1). $CD34^+$ cells derived from human umbilical cord blood were transduced with a MGMT(P140K)–containing lentiviral vector at low MOI following 1 day of stimulation with SCF, Flt-3L, and TPO. Transduction proceeded for 1 day, and then cells were transplanted via the tail vein into NOD/SCID mice that had been conditioned 1 day prior to transplant with 30 mg/kg BG and 10 mg/kg BCNU. Mice were subsequently treated zero times, once, or twice at 3-week intervals with 30 mg/kg BG and 7.5 mg/kg BCNU before being sacrificed at 8–10 weeks after transplant. Control mice conditioned with irradiation (IR) were transplanted and otherwise left untreated.

Comparison of BG/BCNU and IR conditioning of bone marrow. The standard method to prepare NOD/SCID mice for human HSC engraftment uses a 250- to 350-cGy IR dose within 1 day of transplant. Profound sensitivity of these animals to IR, due to defective repair of DNA double-strand breaks, results in acute radiation sickness and mortality, especially at higher doses. Transplantation without this conditioning has not resulted in significant engraftment unless huge numbers of cells have been transplanted repeatedly (21).

In these studies, we chose to transplant mice with human $CD34^+$ cells following conditioning with 30 mg/kg BG and 10 mg/kg BCNU, to more closely model the clinical situation of nonmyeloablative conditioning and to mimic the likely HSC gene therapy setting in which infused transduced HSCs represent a minor population of the total marrow. The myelosuppressive effect of BG/BCNU on NOD/SCID bone marrow 1 day after treatment was compared with the effect of a 250-cGy dose of IR. As shown in Figure 2a, BG/BCNU treatment led to a small, 18% decrease in bone marrow cellularity at 1 day — $5.3 \times 10^6 \pm 1.8 \times 10^6$ cells per femur, compared with $6.5 \times 10^6 \pm 0.4 \times 10^6$ cells per femur in untreated control mice — while IR induced a large, 63% loss of cellularity, with $2.4 \times 10^6 \pm 0.8 \times 10^6$ cells per femur.

A greater difference between BG/BCNU– and IR-treated mice was observed when the frequency of CFCs was analyzed. BG/BCNU treatment led to a 15% ± 16% decrease in CFCs, while IR dramatically reduced colony-forming ability, by 94% ± 2% (Figure 2b). Apoptosis was increased similarly in each group, with 4.3% ± 0.6% and 4.6% ± 1.1% apoptotic cells in BG/BCNU– and IR-treated mice, respectively, compared with 1.1% ± 0.1% in untreated mice (data not shown). Rapid clearance of cells likely prevented greater apoptosis from being observed.

These data show that IR has greater acute effects on the bone marrow than BG/BCNU, which may influence the ability of transplanted cells to engraft NOD/SCID mice efficiently.

BG/BCNU treatment results in the emergence of human cells in NOD/SCID bone marrow. BG/BCNU–conditioned NOD/SCID mice transplanted with LV–MGMT(P140K)–transduced human $CD34^+$ cells were treated with BG/BCNU zero, one, or two times after transplant. Mice were sacrificed 8–10 weeks after transplant, and bone marrow was analyzed for the presence of human cells. Engraftment in five untreated and three once-treated mice was extremely low (Table 1) — below our ability to detect it by CD45 flow cytometry, which we estimate to have a detection limit of 0.4%. Similarly, we could recover only zero to four human CFUs per 10^6 nucleated bone marrow cells per mouse in a CFC assay. Strikingly, in twice-treated mice, human $CD45^+$ cells and CFUs were readily detectible (Figure 3a). Flow cytometry revealed 1.9–20% human $CD45^+$ cells in four of five mice. Levels of recoverable CFCs followed $CD45^+$ percentages, with 4–774 human CFUs per 10^6 nucleated bone marrow cells obtained in five of five mice (Figure 3b).

These data suggest that very small numbers of SRCs engraft in BG/BCNU–conditioned NOD/SCID mice, remaining inactive until rounds of BG/BCNU damage surrounding murine and sensitive human cells, resulting in outgrowth of surviving, resistant cells. Cumulative toxicity of multiple rounds of BG/BCNU treatment is required to achieve this effect.

Treatment enriches for MGMT(P140K)–transduced NOD/SCID–derived human CFCs. Bone marrow from NOD/SCID mice was harvested 8–10 weeks after transplant, and a CFC assay was performed. Resulting CFUs were digested with proteinase K and subjected to PCR to determine the presence of MGMT(P140K) transgene. Since we could not obtain sufficient numbers of CFUs from untreated mice, we plated bone marrow from transplanted, IR-conditioned but untreated (IR/untreated) mice in order to analyze base-line transduction levels in SRCs. In addition, a CFC assay was done before transplants to determine a pretransplant (in vitro) transduction level.

Analysis of the proportion of LV–MGMT(P140K) that contained CFUs showed in vitro transduction levels to be 35% ± 6% (32/91; Figure 4). Transduction of SRCs in IR/untreated control mice was lower, with 11% ± 11% (3/27) of CFUs transduced. However, human CFUs derived from mice treated twice with BG/BCNU were 99% ± 1% (67/68) positive for MGMT(P140K). In fact, we could detect the presence of provirus in 67 of 68 CFUs from four mice, indicating complete or near complete repopulation with transduced CFCs. Selection was consistent in individual mice, with 95–100% of CFUs positive for MGMT(P140K). Substantial selection (9.0-fold) of LV–MGMT(P140K)–transduced SRC-derived CFCs thus occurred in the human-cell population of BG/BCNU–conditioned and –treated mice. When the 9.0-fold increase in transduced CFC is combined with the at-least 100-fold increase in human CFCs (Figure 3b), there was an estimated minimum 900-fold enrichment of human LV–MGMT(P140K)–transduced CFCs in vivo. This is similar to estimates of 940- and 1,000-fold enrichment that we have previously reported in other systems using MGMT for stem cell drug selection (13, 22).

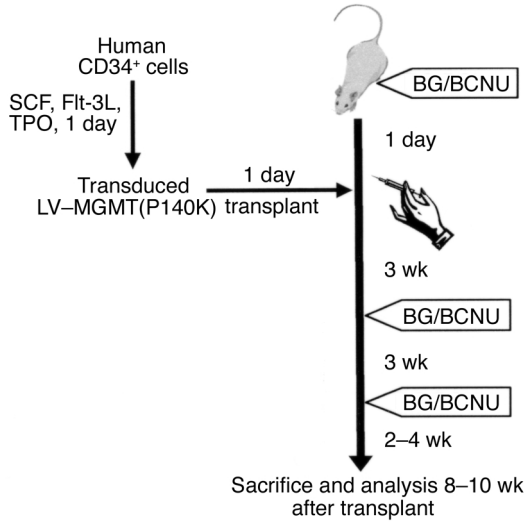

Figure 1
Experimental design. Human CD34+ cells were transduced by a lentiviral vector containing MGMT(P140K) as transgene after a 1-day stimulation with SCF, Flt-3L, and TPO. Following a 1-day transduction, cells were infused into NOD/SCID mice that had been given a conditioning dose of 30 mg/kg BG and 10 mg/kg BCNU the day before transplant. Mice were subsequently treated zero, one, or two times with 30 mg/kg BG and 7.5 mg/kg BCNU before sacrifice and analysis at 8–10 weeks after transplant.

Sustained expression of transgenic protein. BG/BCNU treatment should select for cells that express P140K AGT. Therefore, an increase in the number of human cells containing MGMT(P140K) should lead to an increase in BG-resistant AGT activity in the bone marrow. BG-resistant AGT activity can only be due to transgene-derived P140K AGT, since both mouse and human wild-type AGT is inactivated by BG. We tested the BG-resistant AGT activity in whole bone marrow extracts in mice that were untreated, treated once, or treated twice with BG/BCNU. There was no detectible BG-resistant AGT activity in untreated or once-treated mouse bone marrow extracts preincubated with 50 μM BG, but bone marrow extracts from twice-treated mice exhibited 0.44–5.7 fmol O^6-methylguanine/μg DNA activity (Table 2). Without inactivation of wild-type AGT by BG, activity in unselected mice ranged from 0.54 to 2.0 fmol O^6-methylguanine/μg DNA. These data show that BG/BCNU treatment selected for P140K AGT–expressing cells in the bone marrow, leading to increased ability to repair BCNU-mediated DNA damage especially after BG treatment.

Increased AGT expression was directly observed in human CFUs derived from NOD/SCID bone marrow. To accomplish this, we performed immunocytochemistry on individual CFUs after dispersing the cells and fixing them to slides. Immunostaining using the anti-AGT antibody mT3.1 and a peroxidase-conjugated secondary antibody revealed much more intense staining of CFUs from treated mice than from untransduced controls (Figure 5). Long-term AGT overexpression was thus maintained in committed progenitors derived from SRCs.

Taken together, these data show that lentiviral transduction of MGMT(P140K) to human SRCs leads to sustained expression and increased BG-resistant AGT activity in vivo following rounds of BG/BCNU treatment.

Repopulation is by SRCs containing multiple proviral integrations. The number of retroviral integrations per cell is an important theoretical consideration in gene therapy, since high numbers of integrations increase the chances of insertional oncogenesis. Circumstantial evidence has suggested that increased transgene expression in lentiviral vectors is a consequence of multiple vector insertions (18). We have found direct evidence of such a tendency (S.P. Zielske et al., unpublished observations). BG/BCNU selection could result in preferential survival of cells containing many viral insertions, or gene amplification in low-copy-number clones, as has been demonstrated in other drug-resistance gene systems such as that of the multidrug-resistance gene *MDR* (23). We thus analyzed individual human CFUs after in vitro transduction from IR/untreated and twice-treated mice for the number of proviral insertions, using real-time quantitative PCR.

Real-time PCR was used to quantify the number of MGMT(P140K) and GAPDL4 copies in CFU samples, and copy number was therefore expressed as MGMT(P140K) copies per genome, assuming two GAPDL4 copies per diploid genome. The number of GAPDL4 copies in unknown samples was determined from a standard curve constructed with genomic DNA prepared from primary human cells and quantified by a Hoechst fluorescence method as described in Methods.

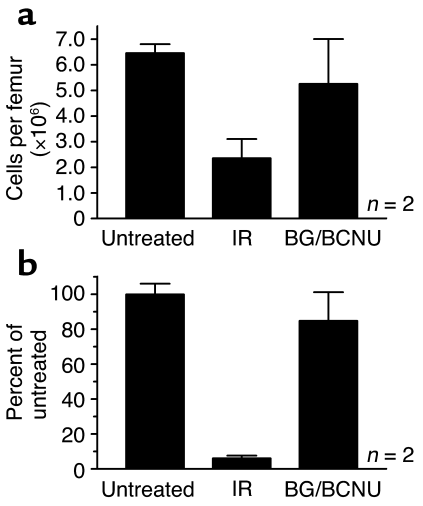

Figure 2
Effect of BG/BCNU and IR treatment on NOD/SCID bone marrow. Bone marrow aspirates were taken from two mice, each of which were treated 1 day prior either with 30 and 10 mg/kg BG and BCNU, respectively, or with 250 cGy IR. (**a**) The number of nucleated cells recovered from femurs was determined for each mouse. Femurs from IR-treated mice contained fewer cells than femurs from BG/BCNU–treated mice. (**b**) Following treatment, 3×10^5 nucleated bone marrow cells from each mouse were plated in 4 ml methylcellulose, and CFUs were counted after 10 days. Values are percent of untreated. The number of CFCs in IR-treated mice was substantially reduced compared with that in untreated or BG/BCNU–treated mice.

Table 1
Correlation of treatments and engraftment of NOD/SCID mice

Treatments	0	1	2
Number of mice engrafted	0/5	0/3	4/5
Average $CD34^+$ cell dose	5×10^5	6×10^5	5×10^5

Similarly, the number of MGMT proviral copies in unknown samples was determined using a standard curve constructed with genomic DNA prepared from a clonal K562 cell line known to contain a single integrated copy of MGMT.

Analysis of copy number in 29 CFUs derived from eight independent transductions with an average PCR transduction level of 35% revealed a median of three viral insertions per CFC (Figure 6). CFCs from IR/untreated control mice showed a similar copy-number profile, with a median of four copies per CFC. Compared with in vitro–derived CFCs, human CFCs from three of four twice-treated mice exhibited similar or slightly elevated median copy numbers, of three to five per cell. Examination of 19 CFUs from one mouse (no. 24), however, showed a very high median copy number of 22 insertions. These results show a low to medium vector-insertion rate (three to five) in a majority of SRCs following BG/BCNU–mediated selection, but SRCs with very high copy numbers that are originally present at low frequency in the transduction culture may selectively repopulate bone marrow.

Repopulation is by SRC clones containing similar numbers of proviral copies. The low initial levels of engraftment obtained with BG/BCNU conditioning (Figure 3) suggest that each mouse began with a small population of SRCs. If bone marrow repopulation following BG/BCNU treatment was by lentivirus-MGMT(P140K)–transduced SRCs containing the same number of proviral copies, then it follows that a plot of the number of MGMT(P140K) copies versus the number of genome copies, determined by real-time PCR of individual mouse-derived human CFUs, should result in a linear array of points with minimal scatter. Similarly, CFCs derived from two SRCs, each containing a different number of MGMT(P140K) proviral copies, should result in formation of points along two lines with different slopes. In addition, the slope of the line, as an indicator of proviral copies per genome, should tend toward integer values, because samples would be derived from cells with equal numbers of proviral copies. This analysis, while theoretically allowing determination of the minimum clonality of repopulation, cannot distinguish between CFUs derived from multiple SRCs with similar copy numbers.

To confirm the assumption that a plot of the number of MGMT copies versus the number of genome copies of CFUs derived from the same parental clone should be linear, we transduced K562 cells with lentivirus, isolated clones, and replated them as a 1:1 mix in methylcellulose. Real-time PCR was then performed on these CFUs, and the results are shown in Figure 7a. In affirmation of our assumption, analysis of CFUs showed an array of two linear sets of points. The slopes of each data set reveal that the two clones contained two and one proviral copies per genome, with r^2 values of 0.92 and 0.98, respectively. This experiment also shows that populations with similar copy numbers (one and two) can be distinguished in a mixture.

When we applied this analysis to CFUs from in vitro mass-transduced $CD34^+$ cells, we found that, as expected, a plot of the number of MGMT(P140K) copies versus the number of genome copies gave a wide scatter, since each CFU developed from an independent transduction event (Figure 7b). However, when data from NOD/SCID–derived CFUs were plotted, an obvious pattern of one or two lines emerged (Figure 7c). CFUs from mouse no. 21 gave a pattern of two easily distinguished and well-fit lines with slopes of 2 and 3, and r^2 values of 1.0 and 0.99, respectively. We conclude that this mouse was repopulated by two or more SRCs containing two and three proviral copies. SRCs containing two and three proviral copies contributed approximately equally to repopulation, as shown by the similar numbers of data points in each line. We also could delineate two sets of points with independent linear relationships in mouse no. 22. In this case, the slopes were 6 and 12, with r^2 values of 1.0 and 0.99, respectively. Again, approximately equal contribution was seen from SRCs containing 6 and 12 proviral copies. The few outlying points may indicate contribution from another minor population, but the data are insufficient to allow this determination.

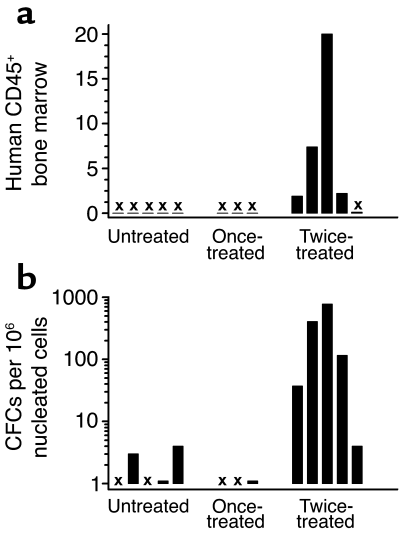

Figure 3
Analysis of the presence of human cells in NOD/SCID mice. (**a**) Eight to ten weeks after transplant, mouse bone marrow was stained for human CD45 and analyzed by flow cytometry. $CD45^+$ levels were below detection in untreated, in once-treated, and in one of five twice-treated mice. Human cells were detectable in four of five twice-treated mice. (**b**) Human CFUs recovered from NOD/SCID bone marrow were counted and are displayed as CFCs per 10^6 nucleated cells. Only twice-treated mice showed significant numbers of human CFCs. x, undetectable.

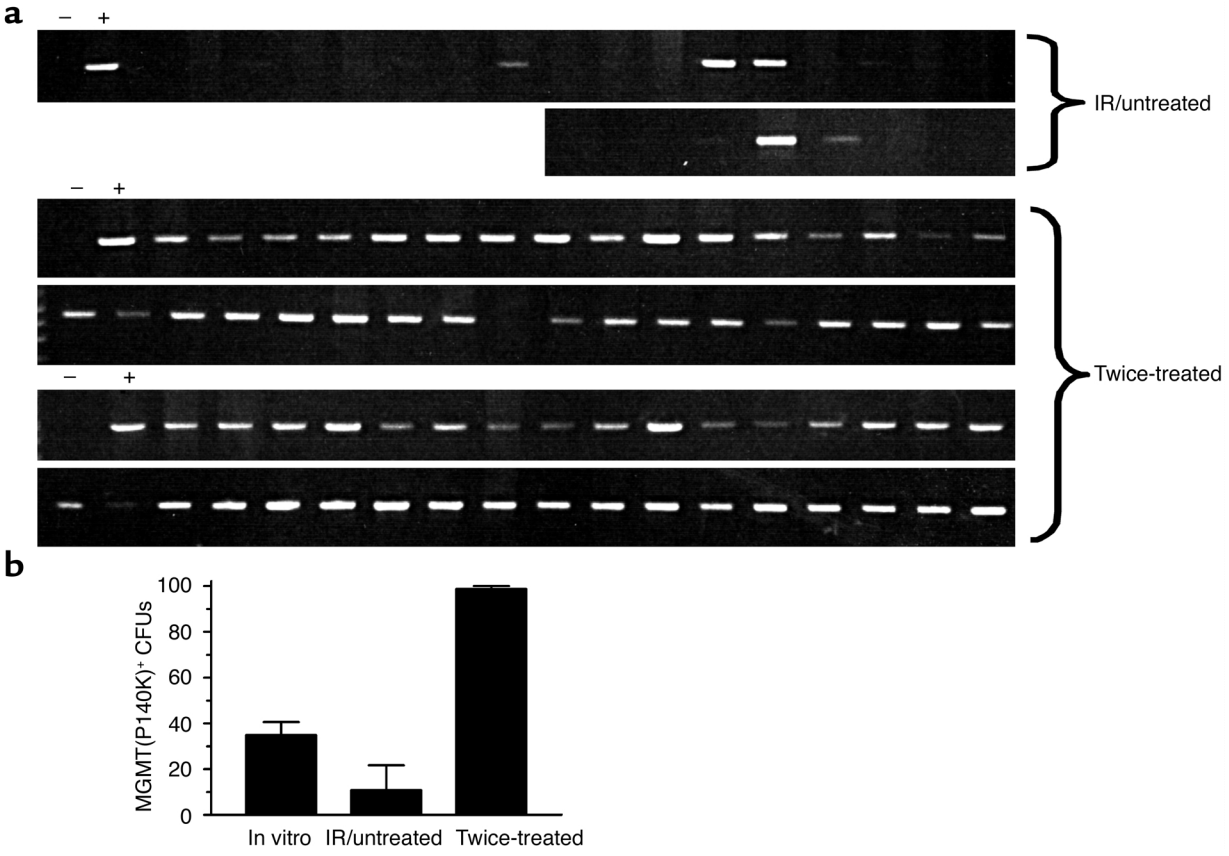

Figure 4
Analysis for the presence of MGMT(P140K) in CFUs. (**a**) CFU DNA from IR/untreated control mice or twice-treated mice was analyzed for the presence of MGMT(P140K) by PCR. Minus signs represent negative PCR control (water or blank methylcellulose), and plus signs represent positive PCR control (MGMT[P140K]–transduced mass culture); remaining lanes show representative CFUs as indicated. (**b**) Quantitation of MGMT(P140K)$^+$ CFUs obtained from in vitro and pretransplant culture (n = 8 independent transductions), IR/untreated control mice (n = 2 mice), and mice treated twice with BG/BCNU (n = 4 mice). BG/BCNU treatment resulted in selection for lentivirus-transduced CFCs.

A plot of data from mouse no. 24 showed that all points conformed to a line with an r^2 value of 0.98. These data imply that repopulation was monoclonal from a single SRC containing 22 proviral insertions (the median number from Figure 6). It is unlikely that these CFUs were derived from multiple SRC clones, because of the low probability of transducing multiple SRCs to the level of 22 copies.

Mouse no. 23 did not yield sufficient CFUs for confident analysis in this fashion. The slope of the linear regression resulted in an integer value (6.0), while the r^2 value was a reasonable 0.79.

Together these data show that repopulation of NOD/SCID mice by LV-P140K–transduced SRCs after BG/BCNU treatment exhibited clonal clustering. Real-time PCR of individual CFUs is a fast and simple method to distinguish CFU progeny derived from SRCs containing different numbers of proviral copies.

Discussion

We show here, for the first time to our knowledge, in vivo selection of human SRCs transduced with MGMT(P140K) by a lentiviral vector. Selection occurred in animals transplanted after BG/BCNU preconditioning, which could not effect high-level engraftment compared with IR. Although few or no human cells could be detected in bone marrow of untreated mice, two rounds of BG/BCNU treatment caused emergence of a lentivirus-transduced human-cell population. Nearly all human CFUs obtained from these mice were positive for MGMT(P140K) (99% ± 1%), indicating substantial selection of transduced human cells over both untransduced human and endogenous murine marrow. Transgene expression was sustained, as evidenced by detectible transgene-derived AGT activity in whole bone marrow and intense AGT staining of individual CFUs. Real-time PCR analysis of CFUs showed 3–22 proviral integrations per cell. Further analysis showed that repopulation was by a single SRC or a small number of SRCs, one of which was observed to contain a very high proviral-copy number of 22.

It has previously been shown that oncoretrovirus–MGMT(G156A)–transduced murine HSCs could be selected in vivo when transplanted without myeloablation (13). Similar to the current study with human cells in NOD/SCID mice, no donor-derived transduced

Table 2
Measurement of AGT activity in NOD/SCID bone marrow

Treatment	Mouse	– BG[A]	+ BG[A]
None	IR control	2.0	ND
None	1	0.82	<0.1
None	2	0.78	<0.1
1× BG/BCNU	11	0.54	<0.1
2× BG/BCNU	21	ND	5.7
2× BG/BCNU	23	ND	0.44
2× BG/BCNU	24	6.18	5.5

Samples from additional mice were not available for analysis. [A]Units are fmol O^6-methylguanine removed/μg DNA. ND, not determined.

CFUs could be detected until mice were subjected to rounds of BG/BCNU treatment. In fact, at low doses of transplanted cells, donor-derived CFUs were undetectable in two of three mice, and two or more rounds of BG/BCNU treatment were required for significant enrichment of transduced cells to occur. When we attempted to perform selection in mice given the standard IR conditioning, engraftment was similar to that in untreated mice but transduced human CFCs approached 95% after a single BG/BCNU treatment (data not shown). Interestingly, mice did not survive a second BG/BCNU dose. Recent work has suggested that the lack of recovery of donor cells from nonmyeloablated recipients is due to a proliferation defect, not a homing defect (24). Similarly, Benveniste et al. claim that engraftment of HSCs is highly efficient, but that full reconstitution can be limited by lack of expansion (25). In our studies, brief exponential expansion of transduced cells, following treatment, from very low numbers initially engrafted probably accounts for the inability to detect them until additional rounds of treatment have induced further exponential expansion. This finding will likely occur in humans as well, with several rounds of treatment required in order to detect significant numbers of transduced cells.

Lentiviral vectors, as retroviruses, integrate into the infected cell's genome. Ideally, transduction would be modulated such that a single integration event occurs, in order to minimize the chance of insertional mutagenesis. It has recently been reported that analysis of lentiviral vector insertions in SRCs by linear amplification–mediated PCR (LAM-PCR) showed one insertion in the BRCA1 tumor suppressor gene (26). Oncoretroviral insertion into the murine transcription factor Evi-1 together with expression of a dLNGFR transgene resulted in murine leukemia in mice given secondary bone marrow transplants (27). In humans, treatment of two children for SCID-X1 appears to have resulted in leukemia due to insertion into the oncogene LMO-2 (28). Our study of the number of vector copies in individual CFUs both in vitro and after transplantation into NOD/SCID mice shows that, on average, three to five integrations occur per cell. This is in agreement with Woods et al., who found five to six integrations per cell by LAM-PCR, under conditions resulting in transduction levels similar to what we report here (26). There are no data to suggest that BG/BCNU treatment selects for increased copy number or gene amplification, and we could find no evidence for gene amplification in this case (data not shown). However, the presence of a high-copy-number SRC with favorable expression properties may confer an advantage during selection over the presence of a number of very low-expressing cells. We do not have quantitative expression data from mouse no. 24, so it is unknown whether these cells exhibited higher expression than their low-copy-number counterparts. We have in vitro data showing, within limits, an association of high copy number with high expression (Zielske et al., unpublished observations). Nevertheless, the potent selection possible with BG/BCNU should allow design of transduction conditions that minimize insertion frequency without compromising selection. Advances in DNA sequence elements such as promoters, insulators, and enhancers may also obviate the need for high copy number to achieve expression goals (29).

The number of vector insertions per genome has usually been determined by Southern blot of mass cell populations. New techniques such as inverse PCR and LAM-PCR have allowed analysis of copy number in clonal populations or CFUs (30, 31). These techniques have also been used to determine clonal diversity in bone marrow following transplantation of mice or primates with retrovirus- or lentivirus-marked HSCs (32, 33). Inverse PCR and LAM-PCR are technically demanding but advantageous since the exact integration site can be determined by sequencing of genomic DNA that flanks the provirus, thus allowing tracking of clones. A disadvantage of these techniques is that distinguishing between different integration sites depends on the ability to resolve differently sized bands on a gel. In addition, inverse PCR appears to have a low success rate in CFUs, and LAM-PCR may be poorly reproducible for copy-number determinations (32, 33). We used quantitative real-time PCR to determine the copy number in NOD/SCID–derived human CFUs. This fast and simple method can also be used to derive the minimum number of clones contributing to

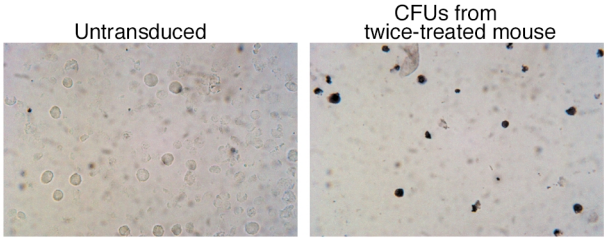

Figure 5
Immunocytochemistry of human CFUs obtained from treated mice. Untransduced CFUs or CFUs from a twice-treated mouse were dispersed into single cells and stained on slides with an anti-AGT primary antibody and an HRP-conjugated secondary antibody. Intense staining indicates high transgene expression.

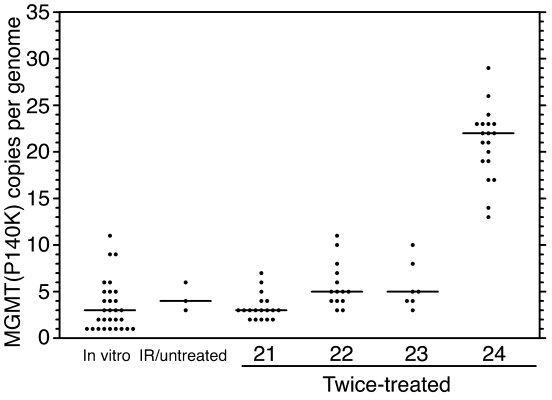

Figure 6
Analysis of the number of proviral copies per cell in individual CFUs. Individual CFUs were analyzed for frequency of proviral integration by real-time PCR. Genome copies were determined by quantitation of GAPDL4 copies, assuming two per diploid genome. Horizontal lines denote median copy number. Each point is the duplicate average of separate CFUs. Numbers below the horizontal axis indicate mouse numbers.

repopulation, when those clones contain different numbers of proviral copies. Since multiple CFUs are progeny of the same SRC, a plot of the number of MGMT copies versus the number of genome copies should be linear. This should also be the case when CFUs of similar copy number, but derived from multiple SRCs, are analyzed. Thus, this method cannot distinguish between clones of equal or very similar copy number, and therefore each linear plot could represent contribution from multiple SRCs.

Using real-time PCR, we were able to estimate that two mice displayed clonal clustering of repopulation by SRCs containing two different copy-number distributions, and one mouse showed what we believe is monoclonal repopulation by an SRC containing 22 proviral copies. These results were not unexpected given the very low initial engraftment. One caveat to the small-animal model is that there may be a skew toward repopulation by fewer HSCs than in large animals and humans. Previous studies in murine models have shown repopulation by only a few HSCs at best (14, 15, 34). A small bone marrow compartment may be the reason for this. Studies of humans and nonhuman primates have frequently shown polyclonal repopulation, even under conditions in which limited numbers of HSCs are infused (35, 36), further

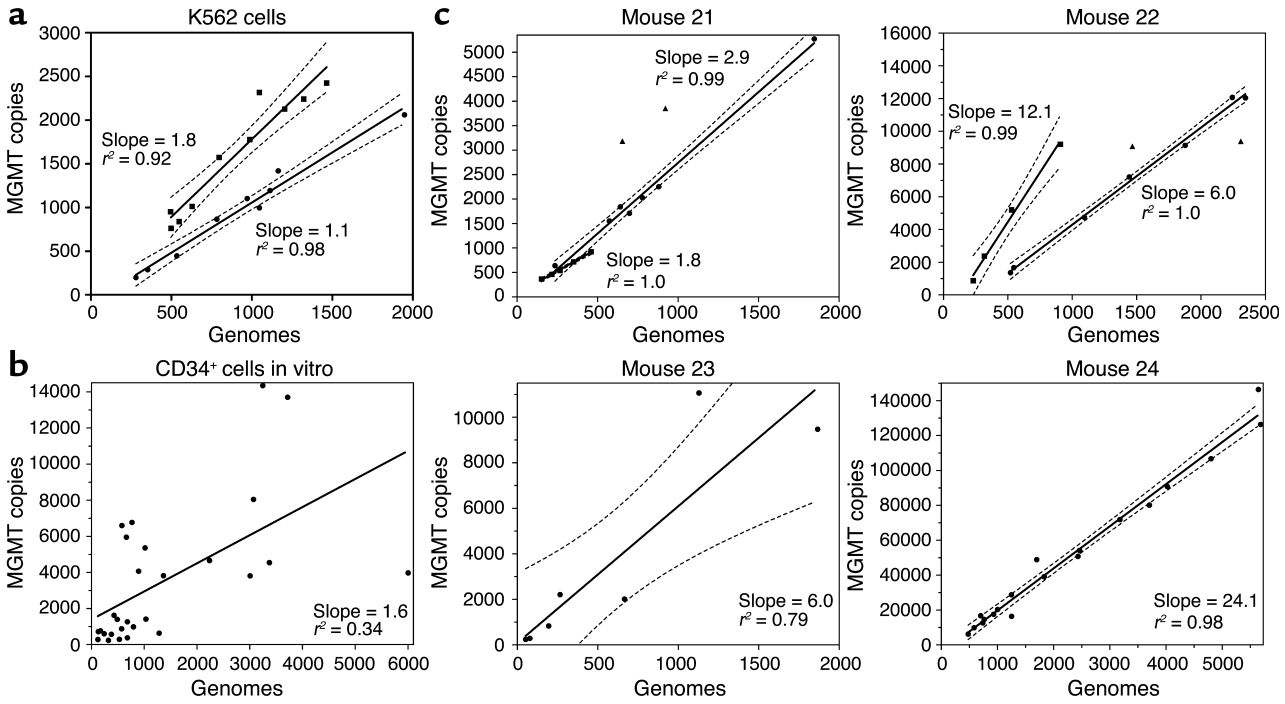

Figure 7
Repopulation analysis. Genome and MGMT copies were determined in individual CFUs as described in Methods and plotted as shown. (**a**) CFUs from a mixture of two K562 clones containing integrated provirus were analyzed by real-time PCR, and the numbers of MGMT copies versus genome copies were plotted for each sample. Solid lines indicate linear regression, and dashed lines are the 95% confidence intervals. (**b**) Data from CFUs obtained from CD34⁺ mass cultures and before transplant show scattered distribution of data points. (**c**) Data from human CFUs obtained from four twice-treated mice. Two lines were distinguishable in mice nos. 21 and 22, indicating CFU populations clustering around two different copy-number distributions. Data from mouse no. 23 were insufficient to discern multiple lines, and mouse no. 24 gave a single line, probably indicating a monoclonal population. Triangles represent points not included in the linear regressions. Regressions were obtained by separately combining data points represented using circles or squares. One to 4 points are off scale in the in vitro, mouse no. 21, and mouse no. 22 plots and were not included in the analysis.

implying that low clonality of murine bone marrow repopulation could be an artifact based on animal size.

In summary, we have shown potent selection of lentivirus-MGMT(P140K)–transduced human SRCs in vivo following BG/BCNU treatment. Transplants were done after a moderately myelosuppressive treatment of BG/BCNU. Although engraftment was very low, a transduced population of human cells emerged after rounds of BG/BCNU treatment, increasing BG-resistant AGT activity in the bone marrow. This population was enriched for lentivirus-transduced CFCs to 99%. Transgene expression was sustained in vivo with high BG-resistant AGT activity present in bone marrow and intense AGT immunostaining in CFUs. Real-time PCR analysis of CFUs showed that SRCs contained 3–22 proviral integrations per cell, and repopulation exhibited clonal clustering by one or more SRCs containing similar numbers of proviral copies. These studies thus, for the first time to our knowledge, define the power of BG/BCNU–mediated in vivo selection of human cells transduced with MGMT(P140K) by a lentiviral vector under nonmyeloablated transplant conditions.

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