Efficiency and Safety of O⁶-Methylguanine DNA Methyltransferase (MGMT^{P140K})-Mediated *In Vivo* Selection in a Humanized Mouse Model

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Abstract

Efficient O⁶-methylguanine DNA methyltransferase (MGMT^{P140K})-mediated myeloprotection and *in vivo* selection have been demonstrated in numerous animal models and most recently in a phase I clinical study in glioblastoma patients. However, this strategy may augment the genotoxic risk of integrating vectors because of chemotherapy-induced DNA damage and the proliferative stress exerted during the *in vivo* selection. Thus, to improve the safety of the procedure, we evaluated a self-inactivating lentiviral MGMT^{P140K} vector for transduction of human cord blood-derived CD34⁺ cells followed by transplantation of the cells into NOD/LtSz-scid/Il2r $\gamma^{-/-}$ mice. These experiments demonstrated significant and stable enrichment of MGMT^{P140K} transgenic human cells in the murine peripheral blood and bone marrow. Clonal inventory analysis utilizing linear amplification-mediated polymerase chain reaction and high-throughput sequencing revealed a characteristic lentiviral integration profile. Among the bone marrow insertions retrieved, we observed considerable overlap to previous MGMT^{P140K} preclinical models or the clinical study. However, no significant differences between our chemotherapy-treated and nontreated cohorts were observed. This also holds true when specific cancer gene databases and a functional annotation of hit genes by the Panther Database with respect to molecular function, biological process, or cellular component were assessed. Thus, in summary, our data demonstrate efficient and long-term *in vivo* selection without overt hematological abnormalities using the lentiviral MGMT^{P140K} vector. Furthermore, the study introduces humanized mouse models as a novel tool for the pre-clinical assessment of human gene therapy related toxicity.

Introduction

H EMATOPOIETIC STEM CELL (HSC) gene therapy has been applied with considerable success to a number of congenital, monogenic disorders, including X-linked severe combined immunodeficiency disorder (SCID-X1), adenosine deaminase deficiency, chronic granulomatous disease (CGD), Wiskott–Aldrich syndrome, and various leukodystrophies (Rivat *et al.*, 2012). However, this success has come at the expense of severe adverse events—most notably the occurrence of acute leukemia or myelodysplasias in several of these studies. Here in-depth analysis revealed retroviral insertions close to proto-oncogenes, leading to activation of these genes. As a consequence, major efforts have been undertaken to better define and predict the risks associated with HSC gene therapy. In this context, murine bone marrow (BM) transplantation models as well as surrogate *in vitro* assays were

developed to assess genotoxicity and have significantly contributed to improve vector safety (Modlich *et al.*, 2006; Montini *et al.*, 2006, 2009). As a result, recent clinical trials were conducted with safety-improved, self-inactivating (SIN) gammaretroviral and lentiviral vectors (Zhang *et al.*, 2013). Although this progress would not have been possible without meticulous studies in the various murine models, sole usage of mouse models for safety testing appears inappropriate given the inherent differences between murine and human biology (Hahn and Weinberg, 2002; Parekh and Crooks, 2012). Thus, relevant preclinical tools assessing genotoxicity directly in gene-modified human HSCs are needed. In this respect, “humanized” mouse models employing engraftment of human CD34⁺ cells in highly immunodeficient NOD/ShiLtSz-scid/Il2r $\gamma^{-/-}$ (NSG) or NOD/ShiJic-scid/Il2r $\gamma^{-/-}$ (NOG) mice are of great interest (Shultz *et al.*, 2007; McDermott *et al.*, 2010). In addition to the severe immunodeficiency that allows

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for the xeno-transplantation, it was recently demonstrated that the “scid” mutation also results in a stem cell defect in these mice even in the absence of selective pressure (Qing *et al.*, 2012).

Extensive genotoxicity analysis appears particularly warranted for gene therapy strategies exerting additional genotoxic stress on transduced cells such as *in vivo* enrichment or exposure to mutagenic agents. This applies specifically to myeloprotective gene therapy approaches employing the (over)expression of selectable drug-resistant genes in hematopoietic stem and progenitor cells (Lachmann *et al.*, 2013), as here the exposure to cytotoxic and mutagenic agents and the proliferative stress exerted during the *in vivo* selection process associated with this approach represent additional genotoxic risk factors. Besides mutants of the dehydrofolate reductase enzyme or the multidrug resistance 1 (*MDR1*) gene, mutants of the O⁶-methylguanine DNA methyltransferase (*MGMT*) gene have been investigated with considerable success in this context. *MGMT* encodes an evolutionarily conserved DNA repair protein that removes highly toxic O⁶-guanine adducts from the cellular DNA and confers resistance to chemotherapeutic drugs with a high O⁶-methylating or -chloroethylating potential such as temozolomide (TMZ) and other triazene derivatives or chloroethylnitrosoureas such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), respectively (Milsom and Williams, 2007). Transgenic overexpression of the O⁶-benzylguanine (BG)-resistant MGMT^{P140K} point mutant has been demonstrated to allow for efficient enrichment of transduced hematopoietic cells by the combined application of the BG and BCNU or TMZ. These studies have been conducted in murine models (Reese *et al.*, 1999; Ragg *et al.*, 2000; Sawai *et al.*, 2001; Jansen *et al.*, 2002; Davis *et al.*, 2003; Milsom *et al.*, 2008; Giordano *et al.*, 2011), including models of β -thalassemia (Persons *et al.*, 2003; Zhao *et al.*, 2009) and erythropoietic protoporphyria (Richard *et al.*, 2004), humanized mice (Pollok *et al.*, 2003; Zielske *et al.*, 2003; Cai *et al.*, 2006, 2008, 2010), as well as large animal models (Neff *et al.*, 2003, 2005; Beard *et al.*, 2009, 2010; Larochelle *et al.*, 2009; Gori *et al.*, 2012; Trobridge *et al.*, 2012). MGMT^{P140K} (henceforth referred to as MGMT) also has been utilized to select for primary lung epithelium (Reese *et al.*, 2008) and muscle cells (Lee *et al.*, 2009) *in vivo* or to enrich for transgenic and protected lymphocytes in the context of novel gene therapy approaches for AIDS (Trobridge *et al.*, 2009; Kiem *et al.*, 2010). Moreover, a recent clinical phase I study has demonstrated efficient myeloprotection and *in vivo* enrichment of genetically modified hematopoietic cells following MGMT gene therapy in a cohort of glioblastoma patients with extended survival demonstrated in individual patients (Adair *et al.*, 2012).

To screen for potential genotoxic side effects, the clonal repertoire of hematopoiesis after MGMT-mediated selection has been investigated for hematopoietic cells after exposure to alkylating agents *in vitro* (Grund *et al.*, 2010), in murine serial transplant (Giordano *et al.*, 2011), canine and primate models (Neff *et al.*, 2005; Beard *et al.*, 2009, 2010; Larochelle *et al.*, 2009), as well as in the recent clinical phase I study (Adair *et al.*, 2012). Reassuringly, these studies so far have failed to demonstrate any adverse effects of MGMT-mediated chemoselection on the clonal repertoire of MGMT-transduced hematopoietic cells. Nevertheless, a number of questions remain, as some of these studies were

compromised by relatively short observation periods (Pollok *et al.*, 2003; Zielske *et al.*, 2003; Cai *et al.*, 2006) or insufficient depth of clonal analysis (Neff *et al.*, 2003, 2005; Beard *et al.*, 2009, 2010; Larochelle *et al.*, 2009). Furthermore, the majority of these studies utilized gammaretroviral vectors (Pollok *et al.*, 2003; Zielske *et al.*, 2003; Cai *et al.*, 2006, 2008, 2010; Adair *et al.*, 2012), which inherently carry a relatively high risk for insertional mutagenesis (Modlich *et al.*, 2009). Therefore, to improve the safety of MGMT-mediated myeloprotection and *in vivo* chemoselection strategies, we here analyzed MGMT expression from a third-generation SIN lentiviral vector employing the truncated elongation factor 1 α (i.e., elongation factor-1 α short version; EFS) promoter for transgene expression. This promoter was chosen as it was previously reported to direct robust and stable MGMT expression while avoiding the very high expression levels associated with viral promoters such as the spleen focus forming virus promoter (SFFV) (Milsom *et al.*, 2008).

Materials and Methods

Generation of the SIN lentiviral MGMT vector construct

In our study a third-generation SIN lentiviral vector with an EFS as an internal promoter was used to coexpress MGMT^{P140K} mutant and enhanced green fluorescent (eGFP) protein coupled to internal ribosomal entry site. See Supplementary Materials and Methods (Supplementary Data are available online at www.liebertpub.com/hum).

Virus production

Viral supernatant was produced by transient transfection using calcium phosphate precipitation of 293T cells as previously described (Modlich *et al.*, 2009). See Supplementary Materials and Methods for a brief description.

Evaluation of peripheral blood for human cell engraftment in NSG mice and end analysis

Starting at 6–8 weeks post-transplantation, peripheral blood (PB) was collected from NSG mice and analyzed at regular intervals for human engraftment by flow cytometry. Mice were euthanized 24–28 weeks after transplantation and analyzed for human engraftment and the levels of GFP⁺ cells in the PB, spleen, thymus, and bone marrow (BM). See Supplementary Materials and Methods.

Isolation and transduction of CD34⁺ cells

Human umbilical cord blood (CB) was collected from the donors after their informed written consent and after approval by the Hannover Medical School institutional ethics committee and the Norddeutsches Knochenmark- und Stammzellspender Register (Hannover, Germany). The CB was processed within 4 hr of collection, and human CD34⁺ cells were isolated according to the Miltenyi human CD34⁺ isolation kit protocol (Bergisch Gladbach, Germany). Isolated CD34⁺ cells were prestimulated for 48 hr in StemSpan medium (StemCell Technologies, Cologne, Germany) supplemented with 2 mM glutamine and 1% penicillin/streptomycin (PAA, Coelbe, Germany) and 100 ng/ml rhSCF, 100 ng/ml rhFLT3L, and 50 ng/ml rhTHPO (all from PeproTech GmbH, Hamburg, Germany). Subsequently, the cells were transduced

(multiplicity of infection 20) with either EFS.MGMT.GFP or EFS.GFP viral supernatant on Retronectin-coated ($10 \mu\text{g}/\text{cm}^2$; Takara, Otsu, Japan) dishes and using spinoculation at 2,000 rpm for 45 min at 4°C . Cells were cultured for a day further before transplantation into NSG mice.

Transplantation of NSG mice

A breeding colony of NSG was established and maintained at Hannover Medical School. Mice were kept in IVC racks (Allentown Inc., Moebris, Germany) in specific pathogen-free conditions. All animal experiments were approved by the local animal welfare committee and performed according to their guidelines. For the *in vivo* experiments 8–10-week-old NSG mice received a sublethal dose of 300 cGy total body irradiation by the PRIMUS linear accelerator (Siemens, Berlin, Germany) and were transplanted within 24 hr. The drinking water was supplemented with Ciprobay (Bayer, Leverkusen, Germany) for the first 3 weeks after irradiation to protect the mice from potential infections.

Administration of chemotherapy

Nine weeks after transplantation, the mice infused with the EFS.MGMT.GFP-transduced CD34^+ cells were either treated with chemotherapy or left unexposed (non-treated group; NT). The treatment included weekly administration of 20 mg/kg BG (Sigma, Seelze bei Hannover, Germany) and 5–10 mg/kg of BCNU (Bristol Meyer Squibb GmbH & Co KGaA, Munich, Germany) 1 hr later for 2 consecutive weeks. BG and BCNU were prepared as described (Pollok *et al.*, 2003). The mice transplanted with the EFS.GFP-transduced CD34^+ cells ($n=3$), henceforth referred to as control, were not treated and monitored similar to the MGMT non-treated animals.

Linear amplification-mediated polymerase chain reaction

Vector insertion sites were amplified by linear amplification-mediated polymerase chain reaction (LAM-PCR) using 500 ng of DNA isolated from PB and BM samples using the QIAamp DNA mini Blood kit (Qiagen GmbH, Hilden, Germany). The LAM-PCR was performed as described previously (Schmidt *et al.*, 2007) with the following modifications. After the double-strand synthesis step, each sample was equally divided into three parts for restriction digestion with different enzymes, namely, *Tsp509I*, *HaeIII*, and *TaqI* (all from New England Biolabs, Ipswich, MA). Separate ligation reactions were carried out using enzyme-specific linkers (sequences in Supplementary Table S1) and were pooled for the denaturation step before the nested end-point PCRs. The PCR products were separated on a 2% agarose gel to visualize the bands and validate the PCR run. In total, we performed LAM-PCR on pretransplant (pre-TX; an aliquot of hCD34^+ cells before transplantation), PB (collected before and after chemotherapy), and BM samples for mice from all groups.

High-throughput sequencing and integration-site analysis

For the 454-pyrosequencing run, LAM-PCR samples were digested with the *SacI* restriction enzyme (New England Biolabs) after the first exponential PCR at 37°C for 2 hr, followed by heat inactivation at 65°C for 20 min. This step

removed most of the vector internal control bands. The digested DNA was diluted 1:30 with water, and $2 \mu\text{l}$ was used for the second exponential PCR along with the barcoded primers (Eurogentec, Seraing, Belgium). This final PCR product was purified via the QIAquick PCR Purification Kit (Qiagen GmbH), and the concentration was determined with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, Germany). Equal amounts of amplicons were used to create a library with a total of 500 ng DNA. The library was dispatched to GATC Biotech AG (Konstanz, Germany) for the 454 high-throughput sequencing with the Roche GS FLX+ Titanium platform. The resulting sequences were sorted for their DNA barcode, using a custom Bioperl script. A sequence was considered valid only if it contained the part of the SIN lentiviral LTR along with the nested LTR primer and linker sequences with >95% identity. This step was performed using *cross_match* (Phil Green, University of Washington; www.phrap.org). Subsequent to these preprocessing steps, the sequences were uploaded to the freely available High Throughput Insertion Site Analysis Pipeline (HISAP) (Arens *et al.*, 2012) for insertion-site analysis. The integration sites retrieved from HISAP analysis were ranked according to the read counts for each sample. The top integrations were selected for further validation using locus-specific PCR (ls-PCR). Insertions in various genes from our study were also compared with integration site databases retrieved from different MGMT studies (Beard *et al.*, 2009, 2010; Grund *et al.*, 2010; Giordano *et al.*, 2011; Adair *et al.*, 2012) and cancer databases [D'Antonio *et al.* (2012) and www.bushmanlab.org]. These post-HISAP processing steps were performed using in-house-developed scripts. The number of integrations for each BM sample along with the vector copy number (VCN) is given in Supplementary Table S2.

Locus-specific PCR and VCN determination

To validate individual vector integrations, reverse primers specific for the genomic region near the 3' vector boundary were designed. The LTR primer (lvLTRIII) from the nested PCR step during the LAM-PCR served as the forward primer for validation of all integration sites. At first, conventional PCR using the Taq polymerase (PEQLAB Biotechnologie GmbH, Erlangen, Germany) was performed specifically on the sample from which the particular integration site was retrieved to confirm its presence and also check the functionality of the primers. Subsequently, real-time TaqMan PCR (Life Technologies GmbH, Frankfurt, Germany) was carried out on all the PB samples collected at different time points using a StepOnePlus (Applied Biosystems, Carlsbad, CA). Additionally, the VCN was determined using primers detecting the woodchuck posttranscriptional element (PRE) and polypyrimidine tract-binding protein 2 (PTBP2) as an internal reference. All quantitative PCRs (qPCRs) were performed in triplicates with standard deviations in cycles ranging from 0.02 to 1.23 (with signals obtained in a range of 24–36 cycles). A plasmid standard harboring sequences for the residual part of the LTR, PRE, and PTPB2 was used for the quantification. The list of all primers is provided in Supplementary Table S1.

Statistical tests

Statistical significance was determined by nonparametric *t*-test, Fisher's exact test, chi-square test, and two-way

ANOVA test, and p -values ≤ 0.05 were considered statistically significant.

Results

In our xenograft model, human CB-derived CD34⁺ were transduced with a third-generation SIN lentiviral construct expressing MGMT from an internal EFS promoter (EFS.MGMT.GFP; Fig. 1A) and transplanted into immunodeficient NSG mice. For control, NSG mice were transplanted with (EFS.GFP)-transduced cells. PB and BM samples from these animals were procured for integration-site analysis. In all our experiments, the progenies of $1\text{--}2 \times 10^5$ CD34⁺ cells were transplanted per mouse with transduction efficiencies ranging between 10% and 35% ($n=4$ transplants). Subsequent to hematopoietic reconstitution, mice were subjected to combined BG/BCNU chemotherapy (Fig. 1B).

High-dose chemotherapy allows efficient enrichment of transgenic human cells but is toxic to NSG mice

In our initial experiment, four animals were treated with a BCNU dose of twice 10 mg/kg (a week apart). The pre-transplant transduction efficacy was 18.8%. This regimen resulted in a profound granulocytopenia and thrombocytopenia with nadir values of $<0.1 \times 10^3/\mu\text{l}$ and $112 \times 10^3 \pm 28 \times 10^3/\mu\text{l}$, respectively, measured 4 days after chemotherapy application (Fig. 2A and B). Within 8–21 days after treatment, three out of the four animals succumbed to severe pancytopenia. The only surviving animal was monitored for another 9 weeks (i.e., 17 weeks post-transplantation) with PB counts recovering to normal values 4–8 weeks after chemotherapy. Chemotherapy resulted in profound loss of human cells in the PB of all four NSG mice within 7 days of treatment

(Fig. 2C). However, a significant enrichment of transduced human cells from $2.7\% \pm 0.8\%$ before to $65\% \pm 8.8\%$ one week after treatment ($p=0.005$; $n=4$) was observed in the human CD45⁺ PB population, indicating effective selection (Fig. 2D). Animals were analyzed for the engraftment of human cells in the BM immediately after death (two animals) and at the end of the experiment for the only long-term survivor. At these time points, the human cells in the BM mainly were composed of myeloid and B-cells (Fig. 2E). High levels of GFP⁺ human cells were detected in all hematopoietic compartments of the BM (Fig. 2F). Thus, the high-dose BG/BCNU treatment schedule clearly resulted in selection of MGMT-transduced cells, but at the expense of lethal myelotoxicity in the majority of animals. To circumvent this fatality and protect the murine hematopoietic system, we applied the same chemotherapy dose schedule but combined it with the transplantation of 1×10^6 freshly isolated NSG whole BM cells 24 hr after the second dose of BCNU. While this approach substantially reduced the pancytopenia and led to survival of all animals until the end of the experiment, the percentage of human cells in the PB of these animals dropped to negligible levels ($<0.01\%$) within 4–5 weeks after the murine cell transplant (Supplementary Fig. S1). However, when these animals were treated for a second time with a reduced chemotherapy regimen of 2×20 mg/kg BG/7.5 mg/kg BCNU given a week apart, transduced human cells were efficiently recovered and even enriched within the hCD45⁺ PB and BM populations.

Efficient *in vivo* selection of MGMT-transduced human cells with dose-reduced chemotherapy

On the basis of these results, we reduced the dose of BCNU to 7.5 mg/kg followed by 5.0 mg/kg (intermediate

FIG. 1. Schematic representation of the lentiviral vectors and the xenotransplant model. **(A)** Third-generation SIN lentiviral vectors expressing the mutant MGMT^{P140K} coupled to enhanced green fluorescent protein (eGFP; EFS.MGMT.GFP) or eGFP (EFS.GFP) alone. **(B)** NSG-based xenotransplant model. Chemotherapy (Chemo; two doses of 20 mg/kg BG and 5–10 mg/kg of BCNU injected 1 hr later, given 1 week apart), specified by black arrows. Peripheral blood (PB) samples were collected at the indicated time points to analyze for chimerism (solid white arrows) or for integration analysis (broken arrows). Δ, U3 region with a deletion; Δ, R, U5 comprise the long terminal repeat (LTR) of the vectors; ψ, packaging signal; BCNU, 1,3-bis(2-chlorethyl)-1-nitrosourea; BG, O⁶-benzylguanine; EFS, human elongation factor 1α short promoter (intron-deleted form); IRES, internal ribosomal entry site; NSG, NOD/ShiLtSz-scid/Il2rγ^{-/-}; PPT, polypurine tract; pre*, woodchuck posttranscriptional regulatory element; RRE, Rev responsive element; SA, splice acceptor; SD, splice donor; SIN, self-inactivating.

FIG. 2. High-dose chemotherapy allows effective chemoselection of transduced human cells but also lethal myelosuppression. Mice were administered two doses of 20 mg/kg 6-BG/10 mg/kg BCNU 8 weeks after transplant (large arrows). PB values for (A) granulocytes and (B) platelets are shown. Three out of four animals succumbed to pancytopenia (small arrows) within 5 weeks of chemotherapy administration. Gray areas indicate mean $\pm$ SEM values of 16 nontransplanted wild-type NSG mice. PB was analyzed by flow cytometry for (C) human engraftment (%hCD45⁺ cells) and (D) the transduced population (GFP⁺) within the human cells before and after chemotherapy. BM was analyzed for the different hematopoietic sub-compartments either at the end of the experiment (open symbols) or when animals died (black symbols). (E) The contribution of B (hCD19⁺), T (hCD3⁺), immature myeloid (hCD33⁺), and mature myeloid (hCD11b⁺) cells to the human engraftment (hCD45⁺) and (F) the proportion of transduced (GFP⁺) cells in each lineage are depicted. Each animal is denoted by the same symbol throughout. BM, bone marrow; PB, peripheral blood.

dose) or 5.0 mg/kg twice (low dose). Mice tolerated these chemotherapy regimens relatively well, although severe granulocytopenia ($<0.1 \times 10^3/\mu\text{l}$) was observed for both groups 4 days after chemotherapy application. Thrombocytopenia, however, was only moderate ($652 \times 10^3 \pm 55 \times 10^3/\mu\text{l}$ for the intermediate and $580 \times 10^3 \pm 158 \times 10^3/\mu\text{l}$ for the low-dose regimen), and granulocyte as well as platelet values recovered to normal levels within 3 and 8 weeks of chemotherapy application, respectively (Fig. 3A and B). By the end of the experiment at 28 weeks, granulocyte counts dropped considerably in all animals (including non-treated controls;

data not shown), which could be caused by long-term chemotherapy toxicity as well as the prolonged observation period. In both treatment groups, two out of five animals were lost 5–12 weeks post-transplant, although the cause of death appeared unrelated to the chemotherapy. Contribution of human cells in the PB is depicted in Fig. 3C, showing significant decrease in both the treated groups ($p=0.008$, medium dose, and $p=0.0381$, low dose, compared with the non-treated group, NT), 2 weeks after chemotherapy application. Engraftment levels in both the treated groups improved over time but remained well below the values of the

FIG. 3. Reduced doses of BG/BCNU avoid myelosuppression and efficiently select MGMT^{P140K}-positive human cells. Mice were treated with two cycles of chemotherapy (black arrows) 8 weeks posttransplant. One group received 20 mg/kg BG/7.5 mg/kg BCNU and 20 mg/kg BG/5.0 mg/kg BCNU (intermediate dose; triangle), and the other cohort received two times 20 mg/kg BG/5.0 mg/kg BCNU (low dose; circle). The nontreated animals (NT; open square) served as controls. PB values for (A) granulocyte and (B) platelet are shown. Fluorescence-activated cell sorting analysis of PB for (C) human engraftment (% hCD45⁺ cells) and (D) transduced cells (GFP⁺) within the human cells before and after chemotherapy is depicted. At the end of the experiment, the BM was evaluated for the contribution of total GFP⁺ cells within the hCD45⁺ engraftment denoted as (E) percentages and (F) total BM absolute numbers. BM was analyzed for GFP⁺ cells within human hematopoietic lineages: B (hCD19⁺), T (hCD3⁺), myeloid (hCD11b⁺, hCD33⁺), progenitors (hCD34⁺ hCD38⁺), and primitive (hCD34⁺ hCD38⁻) cells for the NT (white column), 7.5--5.0 mg/kg (intermediate dose, checkered column), and 5.0--5.0 mg/kg (low dose, black column) animal cohorts. (G) Percentages and (H) absolute numbers for these GFP⁺ populations are shown. Values are plotted as mean $\pm$ SEM, $n=3-6$ animals. * $p \leq 0.05$ by nonparametric test.

NT cohort. For both the dose groups, chemotherapy resulted in considerable enrichment of the transduced human GFP⁺ population in the PB, peaking approximately 12 weeks after chemotherapy application (Fig. 3D). We detected an increase of GFP⁺ cells within hCD45⁺ PB cells from 2.3% ± 1.3% to 21.1% ± 7.2% ($p=0.02$; $n=3$) in the intermediate and from 0.89% ± 0.47% to 18.4% ± 10.4% ($p=0.26$; $n=3$) in the low-dose group. In the intermediate-dose group, these values were maintained until the end of the experiment (19.6% ± 2.3%), whereas selection was partially lost in the low-dose group (7.6% ± 4.7%; at week 26). In contrast, percentages of GFP⁺ cells remained low in non-chemotherapy-treated animals and even decreased throughout the experiment from 2.1% ± 0.42% to 0.5% ± 0.24% ($p=0.02$; $n=6$), a loss most likely reflecting the lower transduction rates that can be expected for primitive long-term SCID-repopulating cells (SRCs) in comparison to more short-term active stem cell clones.

Furthermore, effective *in vivo* enrichment of EFS.MGMT. GFP-transduced cells was confirmed in our model when BM cells were isolated from animals euthanized at the end of the experiment 18 weeks after BG/BCNU application (Fig. 3E). Here, the percentage of transduced GFP⁺ cells within the human cell population was significantly increased in the intermediate- and the low-dose treatment groups compared with NT animals (54.5% ± 13.7%, $p=0.02$, and 52.9% ± 15.6%, $p=0.02$ [$n=3$], respectively, vs. 1.8% ± 1.1% [$n=6$]). Substantially increased counts in comparison with NT controls ($2.3 \pm 1.2 \times 10^5$) were also detected regarding absolute numbers of transgenic human cells in the murine BM, with $7.7 \pm 2.7 \times 10^5$ cells ($p=0.09$; $n=3$) for the intermediate-dose group and $7.7 \pm 0.8 \times 10^5$ cells ($p=0.03$; $n=3$) for the low-dose group (Fig. 3F). Significant enrichment of transgenic B cells (hCD19⁺), mature myeloid (hCD11b⁺) and myeloid progenitor cells (hCD33⁺), as well as more differentiated (hCD34⁺ hCD38⁺) and primitive (hCD34⁺ hCD38⁻) stem/progenitor cells after BG/BCNU application also was observed when the data were broken down to distinct hematopoietic BM subcompartments (Fig. 3G). The expansion of transgenic cells was achieved primarily in the hCD33⁺ myeloid progenitor compartment (Fig. 3H; representative flow cytometry plots for a treated and NT animal are depicted in Supplementary Fig. S2). Although T (hCD3⁺) cell engraftment was negligible in the BM, T cells comprised 50–70% of human cells in the spleen and 70–75% in the thymus (see Supplementary Fig. S3A), and we observed higher percentages of transduced T cells in the chemotherapy-treated groups (21.8% ± 18.1% and 13.9% ± 5.6%) versus the non-treated (6.9% ± 5.4% and 0.24% ± 0.08%) cohort in the thymus and the spleen, respectively (data not shown). Thus, these results demonstrate that moderate doses of chemotherapy regimen can significantly reduce hematotoxicity while still allowing for the efficient and stable enrichment of transgenic GFP/MGMT-positive human cells in the major hematopoietic compartments for the entire observation period of up to 18 weeks after chemotherapy.

Characteristic lentiviral integration profile observed in all groups

As a next step we sought to assess the effect of chemoselection on the clonal repertoire of transduced human cells engrafted in these NSG employing LAM-PCR followed by high-throughput pyrosequencing for integration-site analy-

sis. After removal of the linker- and vector-specific sequences, 486 unique sequences were mapped to the human genome using the HISAP (Arens *et al.*, 2012) and assigned to the following samples: 231 in 2 pretransplant samples (pre-TX; from reduced chemotherapy and GFP control experiments), 144 in treated (10 BM and 20 PB; samples from all dose groups), 54 in NT (4 BM and 8 PB), and 57 in control (3 BM and 6 PB) samples. Irrespective of the vector or treatment, all groups exerted similar insertion-site profiles (Fig. 4). In accordance with previous studies employing lentiviral vectors (Mitchell *et al.*, 2004; Laufs *et al.*, 2006; Grund *et al.*, 2010), in our experiments we found most of the integrations within gene-coding regions; 76.5% in pre-TX samples, 67.4% in treated samples, 60.5% in NT samples, and 74% in control BM samples. Furthermore, among these insertions, 65.4% ± 4% occurred in introns. In line with a typical lentiviral insertion pattern, no preference for insertions in the vicinity of transcription start sites (TSS) was observed. Also, overall ~20% of hits were found ± 10 kb around CpG regions with no significant differences between the groups. These results indicate a representative insertion profile of SIN lentiviral vectors that was not significantly altered by chemotherapy application or MGMT expression. An important technical limitation regarding PB samples after xenotransplantation is the low absolute number of human cells in these samples. Combined with a low chimerism in some animals, these are suboptimal conditions for LAM-PCR and 454-sequencing. Consequently, we mapped either no or only very few (≤5) insertions in the PB samples of our animals. Thus, we mainly focused on the BM-derived insertions for further clonality assessment (Supplementary Table S2). We compared insertion sites retrieved from chemotherapy-treated, NT, and GFP control vector-transduced BM samples to the pre-TX samples using the retroviral insertion sites published in previous MGMT studies (Beard *et al.*, 2009, 2010; Grund *et al.*, 2010; Giordano *et al.*, 2011; Adair *et al.*, 2012). Moreover, we matched the genes closest to lentiviral integrations found in our model with the available cancer databases [D'Antonio *et al.* (2012) and www.bushmanlab.org/links/genelists] summarized in Supplementary Table S3. However, no significant

FIG. 4. Retroviral integration-site analyses. Insertion sites from different samples of control, treated, non-treated, and pre-transplant (pre-TX) were analyzed for their location with respect to the CpG islands (±10 kb distance; black column), transcription start sites (TSS; ±10 kb distance; checkered column), and hits within genes (white column). The chi-square test revealed no significant differences between the groups in any of the categories.

differences between the three *in vivo* groups and the pre-TX samples regarding overlaps with genes found in previous MGMT datasets or cancer datasets were detected. This argues against a selection for specific insertions as a result of chemotherapy treatment or expression of MGMT. We also compared integrations from these groups against annotated human genes in the Panther Database (Mi *et al.*, 2012) for any enrichment of specific gene classes related to the biological process, molecular function, or cellular component function, but no enrichment in any of the experimental groups was found (data not shown).

Oligoclonal repopulation in NSG recipients is not affected by chemotherapy application

The number of reads resulting from pyrosequencing for a particular insertion site is commonly used to estimate the abundance of a clone, thus providing an assessment of a sample's clonality (Biffi *et al.*, 2011). The pre-TX samples consisted of various clones harboring integrations without any obvious overrepresentation, as 99.4%±0.5% of the detected insertions were represented by low (≤ 5) reads (Fig. 5). In contrast, in the treated, NT, and GFP control BM samples, 32.7%±6.6%, 43.0%±6.0%, and 25.1%±1.6% of insertions with low read counts were detected ($p < 0.01$; two-way ANOVA). At the same time, high percentages of insertions with 101–1,000 (25.7%±6.3%, 12.9%±4.3%, and 35.4%±0.8%)

and >1,000 (21.6%±9.4%, 13.2%±4.8%, and 11.7%±0.5%) reads were retrieved for treated, NT, and control BM samples, respectively. Statistical analysis using the chi-square test, however, revealed these differences between the three experimental cohorts as not significant. As the presence of insertion sites with high read counts (e.g., >1,000 reads) can be an indication of clonal imbalance, it is important to stress that we did not observe any overt clonal expansion in our animals (with the potential exception of animal RP10; see below). This and the fact that insertions with high read counts were detected in all three experimental cohorts (treated, non-treated, and GFP controls) seem to suggest that the chemotherapy and the selection process as such did not affect the clonality in the treated cohort. The overall reduced clonality in the BM samples of all groups suggests that only a few clones contributed to the *in vivo* repopulation repertoire over a prolonged period.

Verification of top clones reveals discrepancies in read counts and quantification by qPCR

Among the many insertions detected by the high-throughput sequencing for each animal (see Supplementary Table S2), the 10 most abundant integrations (top 10 clones) were designated based on their contribution to the total read counts of a specific sample. Similar to quantitative reverse transcription-PCR in validation of microarray experiments, ls-qPCR can be used to verify 454-read counts and to measure the contribution of a particular integration among all insertions in the PB and BM samples (Fig. 6A–E). As depicted, the proportion of a particular insertion varied at different time points, supporting the theory that different clones may contribute to the hematopoietic clonal repertoire at a given time *in vivo*. In our data, specific clones tended to increase over time, which in some cases correlated with the higher number of transduced human cells in PB samples. These data do not, however, allow for differentiation between single clones with several insertions or several clones with a single integration. Along this line, we could validate insertions close to the genes *IFITM2*, *DNM2*, *JSRP1*, *FAM59A*, and *CCNB3*, for the treated cohort and *RAPGEF6*, *ARHGEF17*, *CAMSAP2*, and *TRAIP* in the NT group (representative graphs in Fig. 6A–C). For one animal of the high-dose-treated cohort, the 454-results implied the presence of only one insertion close to *TRGJP2* (Supplementary Table S2). As we observed >90% GFP expression in this animal, we cannot rule out that this represents a clonal amplification. However, we were unable to verify this insertion by ls-qPCR or to determine the VCN because of the very low amount of sample DNA available 7 days after chemotherapy when the animal died from myelosuppression. We here certainly cannot rule out clonal dominance, but given the discrepant results, the limited material available for analysis, the fact that the clone was not at all detected 3 weeks earlier (i.e., 1 week before chemotherapy), and also other explanations such as physiological clonal fluctuations in hematopoiesis have to be considered.

Apart from animal RP10, we did not detect overt clonal imbalance in our mice. However, insertions around +65.2 kb of the IKAROS family zinc finger 2 (*IKZF2*) gene and +95.2 kb of the *MDS1* and *EV11* gene complex (*MECOM*) were found in a control animal (RP49; Fig. 6D). *IKZF2* is known to

FIG. 5. Clonal repertoire reduces *in vivo* after repopulation. Every integration site within a sample was ranked according to the pyrosequencing reads recovered. Percentages of insertions with ≤ 5 reads (black area), 6–100 reads (dark-gray area), 101–1,000 reads (light-gray area), and $\geq 1,000$ reads (white area) are depicted for the pre-TX, GFP control, non-treated, and treated BM samples. The value above each column indicates the number of integration sites retrieved from the corresponding samples; *n* is the number of BM samples analyzed. Two-way ANOVA was used to compare pre-TX and BM samples ($***p < 0.01$).

FIG. 6. Validation of individual integration sites in the PB and BM. Locus-specific quantitative PCR (ls-qPCR) for the abundant integrations was performed on PB samples collected at different time points. Representative graphs indicate contribution of specific insertion to the total integrome (open symbols) along with the percentage of human cells (closed inverted triangle) and GFP⁺ human cells (asterisk) over time for individual insertion clones of (A, B) treated, (C) nontreated, and (D) control animals. Each graph represents one animal and with the gene symbol closest to the integration site detected in the BM of that animal. (E) Contribution of specific integrations to the total BM insertions retrieved by locus-specific qPCR (black column) and pyrosequencing (white column). (F) Engraftment levels of human cells (black column) and transgenic human cells in the BM (white column). RPXX codes indicate individual animals, and -X code denotes the rank of the particular insertion in this animal. Gene symbols are given over the respective bars.

encode for a hematopoietic-specific transcription factor essential for lymphocyte development and is mutated in leukemias and lymphomas (Huret *et al.*, 2000). MECOM, on the other hand, is a transcriptional regulator and is associated with esophageal and ovarian cancers, hematological malignancies, and so on (Metais and Dunbar, 2008). Furthermore, in the X-CGD clinical trial, insertions in this locus lead to clonal expansion and myelodysplasia in both the patients (Stein *et al.*, 2010). As seen in Fig. 6D, the animal harboring these integrations showed an increased proportion of the *IKZF2* insertion in the PB sample at 23 weeks, and at this point it also represented as the major insertion in the BM sample (Fig. 6E). In addition, in four of five methylcellulose colonies derived from BM cells of this animal, both insertions *IKZF2* and *MECOM* were detected by ls-qPCR, suggesting that a single clone harbored both the insertions. This example clearly demonstrates that with the help of integration-site analysis and deep-sequencing technology, clinically relevant insertions can be detected in the humanized xenograft model. Furthermore, when comparing the clonal contribution in BM samples either quantified by ls-qPCR or 454-sequence read counts, we frequently observed substantial discrepancies in the repre-

sentation by the two technologies (Fig. 6E). This incongruity was irrespective of the chimerism detected in the BM sample, arguing against insensitive detection as the basis of the observation (Fig. 6F). Furthermore, in few samples we observed discrepancies in the contribution of individual clones in BM and PB (see Supplementary Fig. S3B).

Discussion

We here report the use of a SIN lentiviral vector to express MGMT in human hematopoietic cells and successfully enriched these cells *in vivo* by the combined application of BG and BCNU. Recapitulating earlier studies by other groups in murine as well as xenotransplant models (Reese *et al.*, 1999; Ragg *et al.*, 2000; Sawai *et al.*, 2001; Jansen *et al.*, 2002; Pollok *et al.*, 2003; Zielske *et al.*, 2003; Cai *et al.*, 2006, 2010; Milsom *et al.*, 2008; Giordano *et al.*, 2011), our study documents robust and stable chemoselection of transgenic human cells over extended periods in a model employing the xenotransplantation of MGMT-transduced CB-derived CD34⁺ cells. Insertion-site analysis by LAM-PCR coupled with high-throughput sequencing revealed a typical lentiviral

integration pattern. An overall oligoclonal repertoire was detected in all experimental groups at the end of the 24–28-week observation period without enrichment of clones with integrations in or near-specific gene classes.

In the past, the majority of studies on MGMT-mediated *in vivo* selection, including the recent clinical trial, primarily used gammaretroviral vectors (Pollok *et al.*, 2003; Zielske *et al.*, 2003; Cai *et al.*, 2006, 2008, 2010; Adair *et al.*, 2012). As these vectors have a preference to integrate near the TSS of genes and carry a substantial risk of gene activation, we here evaluated a SIN lentiviral vector in order to reduce this risk. To direct MGMT expression, we specifically chose the EFS promoter, which in the context of the SIN gammaretroviral vector has been shown to allow for stable and functional MGMT expression while avoiding the toxicity associated with very high expression levels (Milsom *et al.*, 2008). To assess the efficacy and safety of our lentiviral MGMT vector in a relevant *in vivo* model, we exploited the xenotransplantation of human CB-derived CD34⁺ cells into NSG mice (McDermott *et al.*, 2010; Parekh and Crooks, 2012). Initial experiments with two cycles of 20 mg/kg BG/10 mg/kg BCNU resulted in effective selection of gene-marked human cells but also fatal myelotoxicity in the non-protected murine hematopoietic system. Infusion of 1×10^6 fresh murine BM cells 24 hr after high-dose BCNU application prevented this toxicity, but also resulted in a loss of the vast majority of engrafted human cells. Similar observations have been described by other investigators and in part may be related to the excess of murine hematopoietic cells competing with their human counterparts for the murine BM niche (Cai *et al.*, 2006). In contrast, reduced chemotherapy regimens employing 5–7.5 mg/kg BCNU allowed for effective selection of MGMT-transduced human cells without fatal myelotoxicity and resulted in significant enrichment of transgenic myeloid, B, and primitive CD34⁺ cells in the BM as well as selection of T cells in the spleen and thymus. These results are in accordance with previous findings stressing the importance of repetitive chemotherapy application to recruit dormant HSCs into an active cell cycle for effective MGMT-mediated chemoselection in murine transplant models (Jansen *et al.*, 2002; Lin *et al.*, 2011). In comparison to former xenotransplant studies (Cai *et al.*, 2006), however, we now have considerably prolonged the observation period in the NSG recipients, enabling us to gauge the effectiveness of our selection strategy on the level of bona fide SRCs (McDermott *et al.*, 2010). In this model, EFS-driven MGMT expression from our SIN lentiviral vector allowed for sustained *in vivo* selection over the entire observation period of up to 18 weeks after chemotherapy.

Insertion-site analysis by LAM-PCR demonstrated the well-known lentiviral integration pattern (Mitchell *et al.*, 2004), characterized by a majority of hits (55–75%) in intronic sequences of genes and less hits in the vicinity of the TSS or CpG islands. This profile was detected in all experimental groups, ruling out a major influence of the experimental conditions associated with the *in vivo* selection process. However, when pre-TX and BM samples were compared, a substantial decrease in clonal diversity became obvious in all experimental groups. This is not surprising as only a minority of the originally transduced CD34⁺ cells have long-term engraftment capacity. Given that we transplanted progeny of $1\text{--}2 \times 10^5$ hCD34⁺ cells and based on a frequency of 1–5 SRCs per 10^4 CB CD34⁺ cells (Wang *et al.*, 1997;

McDermott *et al.*, 2010) with a transduction efficiency of approximately 35–65%, we expect roughly 8–60 MGMT-positive SRCs in individual NSG mice. This correlates well with the results obtained from the BM samples of our BG/BCNU-treated animals (median 8 insertions, range 1–22).

In almost all animals, high read integrations were detected by end-point and quantitative PCR. When we analyzed the contribution of “top clones” to PB samples, we observed high variations with a strong trend for increased contributions to final samples (Fig. 6A–D). This observation clearly reflects substantial fluctuation in the contribution of individual insertion clones to the PB over time. The high-level contribution observed at the end of the experiment does not necessarily result from progressive clonal dominance but merely may reflect the fact that these clones initially were selected as “top clones” in the BM at even this time point. Furthermore, despite the successful validation of various insertions over time, we encountered substantial problems to quantify the contribution of a specific clone in a sample based on the pyrosequencing read counts. This was especially true for the PB samples and could not be overcome by reanalysis of the same sample. This technical limitation reflects the current concerns associated with clonality assessment based on sequential PCR-based techniques (Giordano *et al.*, 2011; Brugman *et al.*, 2013; Rittelmeyer *et al.*, 2013).

We did not detect significant differences with regard to gene classes hit by insertions in the different cohorts, utilizing the Panther database as a reference (Mi *et al.*, 2012). Importantly, this was also true for genes listed in cancer gene databases [D’Antonio *et al.* (2012) and www.bushmanlab.org/links/genelists]. Thus, in our humanized mouse model we could demonstrate successful MGMT-mediated *in vivo* selection without evidence for clonal imbalances generated by high-throughput sequencing. These results correlated well to the integration-site analysis reported for the *in vitro* BG/BCNU-selected human CD34⁺ cells transduced with an MGMT lentiviral vector employing the SFFV promoter (Grund *et al.*, 2010). Similarly, insertional analysis in a number of large animal models employing gammaretro and lentiviral vectors revealed polyclonal profiles without enrichment of specific clones after chemotherapy. However, in these *in vivo* studies the integration sites were not monitored by pyrosequencing but by sub-cloning of LAM-PCR products (Neff *et al.*, 2005; Beard *et al.*, 2009, 2010; Larochelle *et al.*, 2009). In a recent MGMT clinical trial, distinct prominent clones with integrations in *PRDM16* and *HMGA2* loci were described; however, no associated adverse events were observed (Adair *et al.*, 2012). In contrast, enrichment of genes associated with cancer, DNA repair, or cell-fate decisions in response to chemotherapy exposure recently was reported from a high-dose chemotherapy murine serial transplant model employing MGMT-based chemoselection in up to tertiary recipients (Giordano *et al.*, 2011). While some of these differences may arise from basic species disparity (Hahn and Weinberg, 2002), also profound differences among the chemotherapy regimen administered have to be taken into consideration.

Thus, in summary, our study convincingly demonstrates the potential of SIN lentiviral vectors in MGMT-based chemoprotection and *in vivo* selection strategies while at the same time avoiding HSC toxicity. Our xenotransplantation model combined with integration analysis allowed to detect and to monitor long-term the clonal fate of clinically relevant

insertions. Nevertheless, the limited size of our experimental cohorts clearly calls for additional studies utilizing more animals and potentially for further evaluation of the construct specifically in the context of TMZ application, given the relevance of this agent in the treatment of glioblastomas.

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Author Disclosure Statement

No competing financial interests exist.

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