Sleeping Beauty-Mediated Drug Resistance Gene Transfer in Human Hematopoietic Progenitor Cells

Kendra A. Hyland,^{1,*} Erik R. Olson,¹ and R. Scott McIvor^{1,2}

¹Discovery Genomics, Inc., Minneapolis, Minnesota; and ²Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, Minnesota.

The *Sleeping Beauty* (SB) transposon system can insert sequences into mammalian chromosomes, supporting long-term expression of both reporter and therapeutic genes. Hematopoietic progenitor cells (HPCs) are an ideal therapeutic gene transfer target as they are used in therapy for a variety of hematologic and metabolic conditions. As successful SB-mediated gene transfer into human CD34⁺ HPCs has been reported by several laboratories, we sought to extend these studies to the introduction of a therapeutic gene conferring resistance to methotrexate (MTX), potentially providing a chemoprotective effect after engraftment. SB-mediated transposition of hematopoietic progenitors, using a transposon encoding an L22Y variant dihydrofolate reductase fused to green fluorescent protein, conferred resistance to methotrexate and dipyridamole, a nucleoside transport inhibitor that tightens MTX selection conditions, as assessed by *in vitro* hematopoietic colony formation. Transposition of individual transgenes was confirmed by sequence analysis of transposon–chromosome junctions recovered by linear amplification-mediated PCR. These studies demonstrate the potential of SB-mediated transposition of HPCs for expression of drug resistance genes for selective and chemoprotective applications.

INTRODUCTION

THE SLEEPING BEAUTY system (SB) was molecularly reconstructed from teleost fish¹ and has the ability to integrate new sequences into the chromosomes of vertebrate cells and tissues. The SB system consists of two components: (1) a transposon, made up of a gene of interest flanked by SB inverted repeats (IRs), and (2) a source of SB transposase. During SB-mediated transposition, the SB transposase recognizes the ends of the IRs, excises the transposon from the delivered plasmid DNA, and then inserts the transposon into chromosomal DNA, referred to as a “cut-and-paste” mechanism.¹ Since its discovery, SB has been shown to mediate transposition in cultured cells from different species: human cells² and in mouse cells and tissues.^{3–5} The advantages of nonviral gene transfer compared with viral vectors include less expensive vector manufacturing, reduced risk of contamination and toxicity, reduced immunogenicity, and a more stable shelf life (reviewed by Hackett et al.⁶).

In human cord blood-derived CD34⁺ progenitor cells, the SB system has been shown to mediate

successful transposition and expression of reporter genes encoding enhanced green fluorescent protein (eGFP)^{7,8} and *DiscoSoma* species red fluorescent protein (DsRed).⁹ A hyperactive variant of SB transposase (SB100x), engineered by *in vitro* evolution of the transposase gene with targeted amino acid substitutions, was required to mediate transposition into hematopoietic stem and progenitor cells capable of multilineage, long-term engraftment.^{7,9} The SB system was subsequently shown to mediate integration of the human β -globin gene into CD34⁺ human hematopoietic progenitor cells (HPCs).^{10,11} SB-transposed CD34⁺ cells can be differentiated *in vitro* to T cells, B cells, natural killer (NK) cells, and myeloid cells,⁹ and enucleated red blood cells.¹⁰ Here we sought to extend previous studies with CD34⁺ cells to introduce a drug-selectable gene, conferring resistance to methotrexate (MTX).

Dihydrofolate reductase (DHFR) catalyzes the NADPH-dependent reduction of folate to dihydrofolate, and of dihydrofolate to tetrahydrofolate in mammalian cells.¹² Rapidly dividing cells re-

*Correspondence: Dr. Kendra A. Hyland, 614 McKinley Place NE, Minneapolis, MN 55413. E-mail: kendrah@discoverygenomics.net

quire reduced folates as cofactors for enzymes involved in thymidylate synthesis, *de novo* purine biosynthesis, and glycine synthesis. MTX, a commonly used and clinically effective chemotherapeutic agent, is a potent competitive inhibitor of DHFR.^{12,13} MTX has been successfully used to treat a number of malignancies such as acute lymphoblastic leukemia, non-Hodgkin's lymphoma, and osteosarcoma.¹⁴ However, the therapeutic dose that can be administered is limited by toxicity in rapidly dividing cells of the bone marrow and gastrointestinal tissues.^{15,16} Several amino acid substitutions in the DHFR enzyme have been described that confer resistance to MTX toxicity in normal drug-sensitive cells and tissues.^{17–19} For example, expression of murine DHFR with wild-type leucine substituted by tyrosine at position 22 (Tyr-22) results in a high level of resistance to MTX.^{19,20} Expression of drug-resistant DHFR can mediate protection from antifolate toxicity after transplantation in mice using either transgenic hematopoietic stem cells (HSCs)²⁰ or HSCs transduced with DHFR-encoding retroviral or lentiviral vectors.^{21,22} *In vivo* expansion of DHFR(Tyr-22)-transduced hematopoietic stem cells has been achieved after antifolate treatment, allowing for selective outgrowth.^{23,24} These studies demonstrate the potential for protection of normal cells during administration of antifolate chemotherapy after DHFR gene transfer.

Here, we demonstrate that the nonviral SB transposon system can additionally modify human HPCs for expression of DHFR(Tyr-22), thus conferring resistance of hematopoietic colony-forming progenitors to high concentrations of methotrexate. These results have implications for the application of the nonviral SB system to drug resistance gene transfer for the purpose of protecting normal cells from the toxic side effects of chemotherapy as well achieving selective outgrowth of genetically engineered hematopoietic cells either *in vitro* or *in vivo*.

MATERIALS AND METHODS

Construction of transposons and transposase-encoding plasmids

Plasmid pCMV-SB100x, encoding hyperactive SB100x transposase expressed from the cytomegalovirus (CMV) immediate-early promoter,⁷ was kindly provided by Z. Izsvak (Max Delbrück Center for Molecular Medicine, Berlin, Germany). The pKT2/mCAGGs-DHFR-GFP transposon plasmid was constructed from the previously described pDL2G plasmid,²² containing the GGGGSGGGS glycine-serine linker between the mouse DHFR(Tyr-22) and eGFP

domains of the fusion protein.²⁵ A fragment encoding DHFR(Tyr-22) and the [Gly₄Ser]₂ linker was amplified from the pDL2G template by PCR using sense oligonucleotide (5'-TAGAATTCACCATGGTTTCGACCGTTGAATTGC-3'; *Eco*RI and *Nco*I restriction sites underlined) and antisense oligonucleotide (5'-CTCGCGGCCGCTTACTTGTACAGCTCGTCCATG-3'; *Not*I restriction site underlined) at the 5' and 3' fragment ends, respectively, and cloned into HL602, a pBluescript variant (Agilent, Santa Clara, CA). The HL602-DHFR(Tyr-22)-eGFP plasmid was verified by nucleotide sequencing and then the *Eco*RI–*Not*I fragment was cloned into transposon plasmid pKT2-mCAGGs containing the CMV early enhancer element, β -actin promoter, noncoding exon, mini-intron, and rabbit β -globin 3'-untranslated region and poly(A) signal.²⁶ All restriction enzymes were from New England BioLabs (Beverly, MA). Transposon pKT2/mCAGGs-eGFP was constructed from pKT2/EF1 α -GFP²⁷ by PCR using sense oligonucleotide 5'-TAGGAATTCATGGTGAGCAAGGG-3' and antisense oligonucleotide 5'-CAGGCGGCCGCTTTACTTGTACAG-3', ligating the GFP-encoding DNA fragment into pKT2/mCAGGs, between the *Eco*RI and *Not*I restriction sites (underlined).

Nucleofection of CD34⁺ cells

Umbilical cord blood (UCB) was obtained either from the New York Blood Center (New York, NY) under a protocol approved by the University of Minnesota (Minneapolis, MN) Institutional Review Board, or was purchased from the National Disease Research Interchange (Philadelphia, PA). CD34⁺ cells were purified by positive selection and cultured overnight in X-VIVO 10 serum-free medium with gentamicin (Lonza, Walkersville, MD), containing stem cell factor (SCF), thrombopoietin (TPO), interleukin (IL)-3, and Flt3 ligand (Flt3L) (30 ng/ml each; Bio-technie, Minneapolis, MN), as described previously.²⁸ CD34⁺ cells pooled from multiple cords were mixed with CD34[−] cells, supplemented with 10 μ g of plasmid DNA consisting of a 3:1 ratio of transposon to transposase-encoding plasmid and/or filler DNA, and nucleofected at a concentration of 1×10^6 cells/0.1 ml of human CD34⁺ cell Nucleofector solution (Lonza), using program U-08 and a Nucleofector I device. The transfected cells were immediately transferred to 24-well plates containing 37°C prewarmed medium.

In vitro culture of CD34⁺ cells

After 2–3 days in liquid culture, CD34⁺ cells were plated in methylcellulose medium containing human cytokines (HSC005; Bio-technie) in the

Figure 1. Transposons used in this study. Maps of pKT2/mCAGGs-DHFR(Tyr-22)-eGFP, pKT2/mCAGGs-eGFP, and hyperactive pCMV-SB100x are shown. eGFP, enhanced green fluorescent protein gene; DHFR, dihydrofolate reductase; mCAGGs, mini-CAGGs; pA, poly(A); CMV, cytomegalovirus promoter. Color images available online at www.liebertpub.com/hum

presence or absence of 100 nM MTX (Hospira, Lake Forest, IL) and 5 μ M diipyridamole (Sigma-Aldrich, St. Louis, MO). After 12–16 days at 37°C, 5% CO₂, progenitor colonies containing >50 cells were counted with an inverted fluorescence microscope (Olympus IX71) (excitation filter, 485 nm; emission filter, 525 nm). Colonies were categorized as either erythroid (burst-forming unit-erythroid [BFU-E] or colony-forming unit-erythroid [CFU-E]) or granulocyte/monocyte morphology (CFU-G, CFU-M, or CFU-GM)²⁹ and scored as GFP positive or negative.

Linear amplification-mediated PCR

Individual progenitor-derived colonies were harvested from methylcellulose cultures and genomic DNA was purified with a Quick-gDNA MicroPrep kit (Zymo Research, Irvine, CA). Ten nanograms of genomic DNA (gDNA) was whole-genome amplified with an illustra GenomiPhi V2 DNA amplification kit (GE Healthcare, Piscataway, NJ), and 1 μ g of amplified material was evaluated for

transposon integrants by linear amplification-mediated PCR (LAM-PCR) as previously described,³⁰ using primers designed for recovery of SB transposon–chromosome junction sequences.³¹ The recovered sequences were evaluated by nucleotide–nucleotide Basic Local Alignment Search Tool analysis³² against the human genome, using the National Center for Biotechnology Information (NCBI, Bethesda, MD) database.

RESULTS

Sleeping Beauty transposons were constructed to express murine DHFR(Tyr-22) using the mini-CAGGs promoter,²⁶ containing the CMV early enhancer, chicken β -actin promoter, and a truncated β -globin intron sequence (Fig. 1). Because a minimum of 1 million cells is ideal for the nucleofection procedure, we routinely mixed purified CD34⁺ cells with CD34[−] cells (25–50% CD34⁺) and nucleofected at a 3:1 ratio of transposon/transposase-encoding plasmids in *trans*.⁹ After 3 days of liquid culture with human cytokines, CD34⁺ cells exhibited selective uptake of nucleofected DNA, expansion, and expression of eGFP, without any difference in eGFP levels when transposase was either present or absent (Fig. 2). eGFP was expressed by the CD34⁺ CD38^{low/−} population (Fig. 2B), containing less mature, pluripotent hematopoietic progenitors that can reconstitute a functional human immune system in immunodeficient mice.^{33,34} Cells were plated in semisolid methylcellulose medium containing human cytokines and methotrexate for selective outgrowth of DHFR-transposed, drug-resistant progenitors into erythroid (BFU-E, CFU-E) and myeloid (CFU-GM, CFU-G, CFU-M) colonies. Diipyridamole (DP), a nucleoside transport inhibitor,

Figure 2. CD34⁺ cells selectively express GFP 2 or 3 days after nucleofection of a mixed population of CD34-positive and -negative cells. **(A)** Representative flow cytometric analysis of CD34⁺ GFP⁺ cells. Cells were gated on forward scatter (FSC) and side scatter (SSC) and stained with antibodies to CD34 and CD38. **(B)** Enhanced GFP expression by both CD34⁺ CD38^{high} cells (open column portions) and CD34⁺ CD38^{low} cells (gray column portions) was similar in the presence and absence of SB100x, 2–3 days post nucleofection. A mixture of CD34⁺ and CD34[−] cells was nucleofected with plasmids encoding SB transposon with or without CMV-SB100x plasmid. DHFR-GFP, pKT2/mCAGGs-DHFR-eGFP; GFP, pKT2/mCAGGs-eGFP. *n*=4. Color images available online at www.liebertpub.com/hum

Figure 3. Long-term expression of GFP and methotrexate (MTX) resistance by hematopoietic progenitor colonies after transposition with transposon encoding GFP or DHFR(Tyr-22)-eGFP fusion gene and SB100x. **(A)** eGFP expression by both types of progenitor colonies after nucleofection of CD34⁺ cells followed by culture in semisolid methylcellulose medium without selection. *Open column portions*, CFU-GM colonies; *solid column portions*, erythroid colonies. **(B)** Half of drug-resistant CFU-GM (51%) and erythroid colonies (56%) were bright enough to be scored GFP⁺ by fluorescence microscopy when plated under selective conditions. *Gray column portions*, GFP⁺ MTX^r-resistant colonies; *open column portions*, GFP⁻ MTX^r-resistant colonies. CFU-GM, granulocyte and/or monocyte colonies; DG, pKT2/mCAGGs-DHFR-eGFP; GFP, pKT2/mCAGGs-eGFP. *n* = 3 or 4.

was included in the medium to prevent uptake of exogenous purine nucleosides and thymidine that can compromise MTX selection.^{35–37} Nucleofection in the presence or absence of pCMV-SB100x plasmid did not alter the morphology of colony-forming cells (CFCs); approximately 300 erythroid and 135 granulocyte/monocyte colonies per 1000 CD34⁺ cells were enumerated. Colonies scoring GFP positive by fluorescence microscopy were observed only when pCMV-SB100x plasmid was conucleofected with either DHFR-GFP fusion or GFP transposon DNA before plating (Fig. 3A). Only half of the drug-

resistant colonies scored positive for GFP (Fig. 3B), likely due to the high background fluorescence of methylcellulose. Growth of drug-resistant progenitor colonies was observed only when cells were nucleofected with transposon encoding DHFR(Tyr-22)-eGFP along with pCMV-SB100x plasmid (Figs. 3B and 4). CFC colonies that were eGFP⁺ ranged from uniform bright expression of eGFP to dim eGFP expression (Fig. 4). This variability in expression has been previously reported in CD34⁺ cell-derived colonies,³⁸ and likely is due to position effect variegation.

Figure 4. Enhanced GFP expression by drug-resistant hematopoietic progenitor colonies ranged from bright **(A)** to dim **(C)** after 12–14 days of drug selection with MTX and dipyrindamole. CD34⁺ cells were nucleofected in the presence of pKT2/mCAGGs-DHFR-eGFP transposon (Tn) with and without pCMV-SB100x plasmid as described in MATERIALS AND METHODS.

For molecular analysis of SB-mediated transposition, chromosome–transposon junction fragments were recovered from individual colonies by linear amplification-mediated PCR (LAM-PCR) after culture for 12–14 days, when episomal plasmid was no longer likely to be present. Eleven transposon integrants were identified in the genome and contained the signature TA dinucleotide positioned between chromosomal and SB inverted repeat sequences (Table 1). In this small sample set, 5 of the 11 integrants were intergenic, and 6 of the 11 were found within the introns of genes. These data verify that the formation of MTX-resistant, eGFP⁺ hematopoietic colonies was molecularly associated with SB-mediated transposition and transgene expression.

DISCUSSION

We demonstrate that the nonviral SB transposon system can effectively deliver a pharmacologically relevant therapeutic drug resistance gene to human hematopoietic progenitor cells. Drug-resistant variants of DHFR have been expressed in human umbilical cord blood CD34⁺ cells after gammaretroviral vector transduction.^{39,40} However, in clinical trials to treat X-linked severe combined immunodeficiency by transduction of hematopoietic cells with gammaretroviral vectors, genotoxicity was observed.⁴¹ Although deep sequencing of SB integrants in CD34⁺ cells has not been reported, analysis of other human cells (HeLa cells, primary T cells, etc.) has shown that SB-mediated integration in human cells is closer to random integration than that mediated by lentiviral vectors. One of the advantages of SB for gene therapy is the lack of preferential integration at transcriptional start

sites.^{42–44} Lentiviral (HIV-1-derived) vectors tend to integrate within gene sequences of CD34⁺ cells, whereas gammaretroviral vectors (based on Moloney murine leukemia virus) tend to integrate near areas of actively transcribed regulatory elements in CD34⁺ cells.⁴⁵ This supports the use of nonviral gene therapy for genetic modification of hematopoietic progenitor cells as it is less prone to insertional mutagenesis.

SB has been the most extensively tested transposon system for preclinical gene therapy studies and is currently being used in clinical trials to engineer T cells for treatment of patients with B-lineage lymphoid malignancies.⁴⁶ Since it was initially described, several variants of SB transposase exhibiting increased activity have been generated, first by targeted mutagenesis to create SB11,⁴⁷ and later a hyperactive variant, SB100x, was engineered and has been a key component in achieving stable reporter gene expression in human CD34⁺ cells.^{7,9}

There are two possible applications for drug-resistant DHFR gene transfer in HSCs. First, DHFR gene transfer in HSCs followed by transplantation and engraftment would permit administration of normally toxic doses of antifolate chemotherapy.^{48–50} Expression of drug-resistant DHFR in hematopoietic cells has also been found to confer a protective effect to normally drug-sensitive gastrointestinal tissues.²⁰ Second, drug-resistant DHFR could be coexpressed along with a nonselectable gene, followed by exposure to MTX, allowing enrichment of human hematopoietic progenitors or lymphoid cells expressing both genes, thereby permitting an *in vivo* selective growth advantage.^{51,52} Such an approach would overcome the lower efficiency of SB-mediated gene transfer compared with viral vectors.

Table 1. Molecular Analysis of Chromosomal Integrants in Colony-Forming Cells

Colony	Morphology ^a	IR/DR ^b	TA	Sequence	Homology	Gene location	Gene strand	Gene symbol
CFC 3 ^c	Erythroid	TCAACTG	TA	GGTATCTACCTAAGGCT...	Chr22q11.1	Intergenic	N/A	N/A
	Erythroid	TCAACTG	TA	GATACTGGGAAACACA...	Chr20q13.12	Intergenic	N/A	N/A
CFC 8 ^c	CFU-GM	TCAACTG	TA	CATACACATAATTATGT...	Chr10q22.2	Intergenic	N/A	N/A
	CFU-GM	TCAACTG	TA	GATTCTTAGATGTAAGA...	Chr13q33.3	Intronic	(+), (–)	PKIG, ADA
CFC 10 ^c	CFU-GM	TCAACTG	TA	TGTCCTTTGATCACCCT...	Chr4q22.3	Intergenic	N/A	N/A
CFC 13 ^c	CFU-GM	TCAACTG	TA	TATAAAACAACATCGAT...	Chr4q28.2	Intergenic	N/A	N/A
CFC 14 ^c	CFU-GM	TCAACTG	TA	CATAGATTTCAGTTACT...	Chr9q31.3	Intronic	(–)	PPP3CB
CFC 19 ^c	CFU-GM	TCAACTG	TA	TATATTTTGATCATCT...	Chr7p22.1	Intronic	(–)	FAM155A
CFC 27 ^d	CFU-GM	TCAACTG	TA	TATTCTGTATAATAACA...	Chr9q34.2	Intronic	(–)	PTPN3
CFC 36 ^d	CFU-GM	TCAACTG	TA	CAATCTGAATTATTAA...	Chr12p13.31	Intronic	(+)	RAC1
	CFU-GM	TCAACTG	TA	ACTCGTCTTCTCTGC...	Chr6q13	Intronic	(–)	VAV2

CFC, colony-forming cell; CFU-GM, colony-forming unit-granulocyte/monocyte; IR/DR, indirect repeat/direct repeat; N/A, not applicable.

^aErythroid (BFU-E or CFU-E) or granulocyte/monocyte morphology (CFU-GM).

^bThe sequences from the right indirect repeat/direct repeat (IR/DR).

^cpKT2/mCAGGs-DHFR-eGFP transposon, CFCs cultured under selection conditions.

^dpKT2/mCAGGs-eGFP transposon, CFCs cultured without selection.

Our data demonstrate that the SB transposon system can be used to confer methotrexate resistance after drug-resistant DHFR gene transfer in CD34⁺ human myeloid progenitors. The next challenge is to use the SB transposon system to integrate therapeutic genes into hematopoietic stem cells with multilineage differentiation potential, capable of engraftment and long-term reconstitution in recipient animals. Others have demonstrated detectable expression of fluorescent reporter genes after transplantation of SB-engineered CD34⁺ cells into immunodeficient mice.⁹ However, the frequency of stable SB-mediated gene transfer, reported as a mean of 7% in the bone marrow of immunodeficient mice,⁹ is lower than that achieved using retroviral or lentiviral vectors, reported as 20–25% of peripheral blood cells in human patients.⁵³ One possible reason for the low efficiency of gene transfer using the SB system is the toxicity of nucleofection, with only 50% of cells surviving the procedure.¹⁰ This could be overcome by using mRNA encoding SB transposase along with DNA encoding the transposon⁵⁴ or by

modification of nucleofection conditions. Coexpression of drug-resistant DHFR along with a therapeutic gene would allow for selective outgrowth of transposed cells and enrichment of cells expressing the nonselectable therapeutic gene either *in vitro* or *in vivo*.

ACKNOWLEDGMENTS

The authors thank Cindy Eide (University of Minnesota, Pediatric Department) for technical assistance and Colin Sweeney (NIAID, NIH) for his protocol, adapting the LAM-PCR method to SB transposons.

AUTHOR DISCLOSURE

This work was supported by grant R44 HL076908 from the National Institutes of Health (NHLBI) to Discovery Genomics, Inc. (DGI) (R.S.M.). R.S.M. is a founder (with stock) of DGI. K.A.H., E.R.O., and R.S.M. are current employees of DGI, including stock options.

REFERENCES

- Ivics Z, Hackett PB, Plasterk RH, et al. Molecular reconstruction of *Sleeping Beauty*, a Tc1-like transposon from fish, and its transposition in human cells. *Cell* 1997;91:501–510.
- Izsvák Z, Ivics Z, and Plasterk RH. *Sleeping Beauty*, a wide host-range transposon vector for genetic transformation in vertebrates. *J Mol Biol* 2000;302:93–102.
- Luo G, Ivics Z, Izsvák Z, et al. Chromosomal transposition of a Tc1/mariner-like element in mouse embryonic stem cells. *Proc Natl Acad Sci U S A* 1998;95:10769–10773.
- Montini E, Held PK, Noll M, et al. *In vivo* correction of murine tyrosinemia type I by DNA-mediated transposition. *Mol Ther* 2002;6:759–769.
- Belur LR, Frandsen JL, Dupuy AJ, et al. Gene insertion and long-term expression in lung mediated by the *Sleeping Beauty* transposon system. *Mol Ther* 2003;8:501–507.
- Hackett PB, Largaespada DA, and Cooper LJN. A transposon and transposase system for human application. *Mol Ther* 2010;18:674–683.
- Mátés L, Chuah MKL, Belay E, et al. Molecular evolution of a novel hyperactive *Sleeping Beauty* transposase enables robust stable gene transfer in vertebrates. *Nat Genet* 2009;41:753–761.
- Sumiyoshi T, Holt NG, Hollis RP, et al. Stable transgene expression in primitive human CD34⁺ hematopoietic stem/progenitor cells, using the *Sleeping Beauty* transposon system. *Hum Gene Ther* 2009;20:1607–1626.
- Xue X, Huang X, Nodland SE, et al. Stable gene transfer and expression in cord blood-derived CD34⁺ hematopoietic stem and progenitor cells by a hyperactive *Sleeping Beauty* transposon system. *Blood* 2009;114:1319–1330.
- Sjeklocha LM, Wong PY, Belcher JD, Vercellotti GM, Steer CJ. β -Globin *Sleeping Beauty* transposon reduces red blood cell sickling in a patient-derived CD34(+) based in vitro model. *PLoS One* 2013;8(11):e80403.
- Sjeklocha LM, Park C-W, Wong PY, et al. Erythroid-specific expression of β -globin from *Sleeping Beauty*-transduced human hematopoietic progenitor cells. *PLoS One* 2011;6:e29110.
- Blakely R, and Benkovic S. *Folates and Pterins*. John Wiley & Sons, New York. 1984.
- Schornagel JH, and McVie JG. The clinical pharmacology of methotrexate. *Cancer Treat Rev* 1983;10:53–75.
- Bertino JR. Karnofsky Memorial Lecture: ode to methotrexate. *J Clin Oncol* 1993;11:5–14.
- Sirotnak FM, and Moccio DM. Pharmacokinetic basis for differences in methotrexate sensitivity of normal proliferative tissues in the mouse. *Cancer Res* 1980;40:1230–1234.
- Rivera GK, Evans WE, Kalwinsky DK, et al. Unexpectedly severe toxicity from intensive early treatment of childhood lymphoblastic leukemia. *J Clin Oncol* 1985;3:201–206.
- Belur LR, James RI, May C, et al. Methotrexate preconditioning allows sufficient engraftment to confer drug resistance in mice transplanted with marrow expressing drug-resistant dihydrofolate reductase activity. *J Pharmacol Exp Ther* 2005;314:668–674.
- Mclvor RS. Drug-resistant dihydrofolate reductases: generation, expression and therapeutic application. *Bone Marrow Transpl* 1996;18(Suppl 3):S50–S54.
- Morris JA, and Mclvor RS. Saturation mutagenesis at dihydrofolate reductase codons 22 and 31: a variety of amino acid substitutions conferring methotrexate resistance. *Biochem Pharmacol* 1994;47:1207–1220.
- James RI, May C, Vagt MD, et al. Transgenic mice expressing the Tyr22 variant of murine DHFR: protection of transgenic marrow transplant recipients from lethal doses of methotrexate. *Exp Hematol* 1997;25:1286–1295.
- Li MX, Banerjee D, Zhao SC, et al. Development of a retroviral construct containing a human mutated dihydrofolate reductase cDNA for hematopoietic stem cell transduction. *Blood* 1994;83:3403–3408.
- Gori JL, Podetz-Pedersen K, Swanson D, et al. Protection of mice from methotrexate toxicity by *ex vivo* transduction using lentivirus vectors expressing drug-resistant dihydrofolate reductase. *J Pharmacol Exp Ther* 2007;322:989–997.
- Persons DA, Allay JA, Bonifacino A, et al. Transient *in vivo* selection of transduced peripheral blood cells using antifolate drug selection in

- rhesus macaques that received transplants with hematopoietic stem cells expressing dihydrofolate reductase vectors. *Blood* 2004;103:796–803.
24. Allay JA, Persons DA, Galipeau J, et al. *In vivo* selection of retrovirally transduced hematopoietic stem cells. *Nat Med* 1998;4:1136–1143.
 25. Lewis VA, Basso L, Blake N, et al. Human nerve growth factor receptor and cytosine deaminase fusion genes. *Hum Gene Ther* 2003;14:1009–1016.
 26. Ohlfest JR, Frandsen JL, Fritz S, et al. Phenotypic correction and long-term expression of factor VIII in hemophilic mice by immunotolerization and nonviral gene transfer using the *Sleeping Beauty* transposon system. *Blood* 2005;105:2691–2698.
 27. Clark KJ, Carlson DF, Foster LK, et al. Enzymatic engineering of the porcine genome with transposons and recombinases. *BMC Biotechnol* 2007;7:42.
 28. Moriarty BS, Rahrmann EP, Beckmann DA, et al. Simple and efficient methods for enrichment and isolation of endonuclease modified cells. *PLoS One* 2014;9:e96114.
 29. Eaves CJ, and Lambie K. Atlas of Human Hematopoietic Colonies. Stem Cell Technologies, Inc., Vancouver, BC, Canada. 1995.
 30. Schmidt M, Schwarzwaelder K, Bartholomae C, et al. High-resolution insertion-site analysis by linear amplification-mediated PCR (LAM-PCR). *Nat Methods* 2007;4:1051–1057.
 31. Hyland KA, Olson ER, Clark KJ, et al. *Sleeping Beauty*-mediated correction of Fanconi anemia type C. *J Gene Med* 2011;13:462–469.
 32. Altschul SF, Madden TL, Schäffer AA, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;25:3389–3402.
 33. Terstappen LW, Huang S, Safford M, et al. Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34⁺CD38[−] progenitor cells. *Blood* 1991;77:1218–1227.
 34. Ishikawa F, Yasukawa M, Lyons B, et al. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor γ chain(null) mice. *Blood* 2005;106:1565–1573.
 35. Warlick CA, Sweeney CL, and McIvor RS. Maintenance of differential methotrexate toxicity between cells expressing drug-resistant and wild-type dihydrofolate reductase activities in the presence of nucleosides through nucleoside transport inhibition. *Biochem Pharmacol* 2000;59:141–151.
 36. Sur P, Matsuo Y, Otani T, et al. Reversal of methotrexate-induced folate pool depletion by thymidine in a human leukemia cell line *in vitro*. *Tumori* 1993;79:433–438.
 37. Nelson JA, and Drake S. Potentiation of methotrexate toxicity by dipyrindamole. *Cancer Res* 1984;44:2493–2496.
 38. Sjeklocha L, Chen Y, Daly MC, et al. β -Globin matrix attachment region improves stable genomic expression of the *Sleeping Beauty* transposon. *J Cell Biochem* 2011;112:2361–2375.
 39. Flasshove M, Banerjee D, Leonard JP, et al. Retroviral transduction of human CD34⁺ umbilical cord blood progenitor cells with a mutated dihydrofolate reductase cDNA. *Hum Gene Ther* 1998;9:63–71.
 40. Takebe N, Xu L-C, MacKenzie KL, et al. Methotrexate selection of long-term culture initiating cells following transduction of CD34⁺ cells with a retrovirus containing a mutated human dihydrofolate reductase gene. *Cancer Gene Ther* 2002;9:308–320.
 41. Nienhuis AW, Dunbar CE, and Sorrentino BP. Genotoxicity of retroviral integration in hematopoietic cells. *Mol Ther* 2006;13:1031–1049.
 42. Yant SR, Wu X, Huang Y, et al. High-resolution genome-wide mapping of transposon integration in mammals. *Mol Cell Biol* 2005;25:2085–2094.
 43. Field A-C, Vink C, Gabriel R, et al. Comparison of lentiviral and Sleeping Beauty mediated $\alpha\beta$ T cell receptor gene transfer. *PLoS One* 2013;8:e68201.
 44. Turchiano G, Latella MC, Gogol-Döring A, et al. Genomic analysis of *Sleeping Beauty* transposon integration in human somatic cells. *PLoS One* 2014;9:e112712.
 45. Ambrosi A, Glad IK, Pellin D, et al. Estimated comparative integration hotspots identify different behaviors of retroviral gene transfer vectors. *PLoS Comput Biol* 2011;7:e1002292.
 46. Singh H, Huls H, Kebriaei P, et al. A new approach to gene therapy using *Sleeping Beauty* to genetically modify clinical-grade T cells to target CD19. *Immunol Rev* 2014;257:181–190.
 47. Geurts AM, Yang Y, Clark KJ, et al. Gene transfer into genomes of human cells by the *Sleeping Beauty* transposon system. *Mol Ther* 2003;8:108–117.
 48. Sweeney CL, Frandsen JL, Verfaillie CM, et al. Trimetrexate inhibits progression of the murine 32Dp210 model of chronic myeloid leukemia in animals expressing drug-resistant dihydrofolate reductase. *Cancer Res* 2003;63:1304–1310.
 49. Budak-Alpdogan T, Alpdogan O, Banerjee D, et al. Methotrexate and cytarabine inhibit progression of human lymphoma in NOD/SCID mice carrying a mutant dihydrofolate reductase and cytidine deaminase fusion gene. *Mol Ther* 2004;10:574–584.
 50. Zhao RC, McIvor RS, Griffin JD, et al. Gene therapy for chronic myelogenous leukemia (CML): a retroviral vector that renders hematopoietic progenitors methotrexate-resistant and CML progenitors functionally normal and nontumorigenic *in vivo*. *Blood* 1997;90:4687–4698.
 51. Allay JA, Galipeau J, Blakley RL, et al. Retroviral vectors containing a variant dihydrofolate reductase gene for drug protection and *in vivo* selection of hematopoietic cells. *Stem Cells* 1998;16(Suppl 1):223–233.
 52. Jonnalagadda M, Brown CE, Chang W-C, et al. Engineering human T cells for resistance to methotrexate and mycophenolate mofetil as an *in vivo* cell selection strategy. *PLoS One* 2013;8:e65519.
 53. Cartier N, Hacein-Bey-Abina S, Bartholomae CC, et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* 2009;326:818–823.
 54. Wilber A, Frandsen JL, Largaespada DA, et al. RNA as a source of transposase for *Sleeping Beauty*-mediated gene insertion and expression in somatic cells and tissues. *Mol Ther* 2006;13:625–630.

Received for publication May 5, 2015;
accepted after revision July 10, 2015.

Published online: July 14, 2015.