Correction of DNA Protein Kinase Deficiency by Spliceosome-mediated RNA *Trans*-splicing and *Sleeping Beauty* Transposon Delivery

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Spliceosome-mediated RNA *trans*-splicing (SMaRT) is an emerging technology for the repair of defective pre-messenger RNA (pre-mRNA) molecules. It is especially useful in the treatment of genetic disorders involving large genes. Although viral vectors have been used for achieving long-lasting expression of *trans*-splicing molecules, the immunogenicity and suboptimal safety profiles associated with viral-based components could limit the widespread application of SMaRT in the repair of genetic defects. Here, we tested whether the non-viral *Sleeping Beauty* (SB) transposon system could mediate stable delivery of *trans*-splicing molecules designed to correct the genetic defect responsible for severe combined immune deficiency (SCID). This immunological disorder is caused by a point mutation within the 12.4 kilobase (kb) gene encoding the DNA protein kinase catalytic subunit (DNA-PKcs) and is associated with aberrant DNA repair, defective T- and B-cell production, and hypersensitivity to radiation-induced injury. Using a novel SB-based *trans*-splicing vector, we demonstrate stable mRNA correction, proper DNA-PKcs protein production, and conference of a radiation-resistant phenotype in a T-cell thymoma cell line and SCID multipotent adult progenitor cells (MAPCs). These results suggest that SB-based *trans*-splicing vectors should prove useful in facilitating the correction of endogenous mutated mRNA transcripts, including the DNA-PKcs defect present in SCID cells.

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INTRODUCTION

Spliceosome-mediated RNA *trans*-splicing (SMaRT) is an emerging technology used for repairing defective pre-messenger RNA (pre-mRNA) molecules by *trans*-splicing between two individual RNA molecules.[1] Three main components are necessary for this process: (i) an endogenous precursor messenger RNA target (*i.e.*, pre-mRNA transcripts), (ii) an engineered pre-*trans*-splicing molecule (PTM) that carries the wild-type sequence, and (iii) factors that comprise the cellular splicing machinery. A PTM is comprised of a binding domain (BD) that hybridizes to an intron of the target pre-mRNA molecule, a *trans*-splicing domain and a coding sequence corresponding to the new 3′ end of the targeted pre-mRNA. SMaRT can mediate simultaneous repair, replacement and removal of RNA sequences from defective cellular pre-mRNA targets by exon replacement.[2] *In vitro* studies have demonstrated that SMaRT can mediate RNA repair in primary cells and in cell lines.[1,3,4] Functional gene correction using SMaRT has been reported in several preclinical models of human diseases, including cystic fibrosis,[5] hemophilia A,[6] and X-linked CD40 ligand immunodeficiency.[7]

SMaRT technology can repair defective mRNA molecules only as long as the plasmid persists in target cells. Although virus-mediated SMaRT delivery can be effective in achieving long-term correction, there are technical issues involved in production, including the cost of producing the virus. In contrast, non-viral vectors are typically much more easily produced. One such non-viral system, the *Sleeping Beauty* (SB) transposon system,[8] consists of a transposon that can carry a therapeutic gene between terminal inverted repeat/direct repeat (IR/DR) sequences, and a transposase enzyme that binds to those IR/DR sequences. Although SB-mediated gene delivery has shown promise in several animal models of human disease,[9,10] the efficiency of SB-mediated gene transfer is compromised by an increase in the size of the transposon.[11,12] The currently available SB transposon plasmid vectors are not of practical use in delivering large genes (*e.g.*, >10 kilobase (kb) in size), such as the gene mutated in severe combined immune deficiency (SCID).

SCID is a heterogeneous group of genetic disorders that leads to severe defects in T- and B-lymphocyte development.[13] The spontaneously arising murine SCID phenotype is caused by a nonsense mutation at tyrosine 4046 near the C-terminus of

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the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs).[14] Because DNA-PKcs plays a central role in the mammalian non-homologous end-joining pathway,[15] cells harboring a DNA-PKcs defect exhibit a markedly increased sensitivity to ionizing radiation[16] because of their inability to properly repair damaged DNA. The size of the DNA-PKcs gene makes it difficult to treat the SCID defect with SB-based technology. Therefore an ideal approach for the repair of such a large gene could consist of RNA *trans*-splicing of the mRNA encoded by the terminal 2 exons released by SB/SMaRT integrants, without the need to deliver the entire 86 exon-containing DNA-PKcs gene.

Multipotent adult progenitor cells (MAPCs) are bone marrow-derived, non-hematopoietic stem cells that possess the capacity to differentiate into cells derived from ectoderm, endoderm, and mesoderm.[17] In contrast to hematopoietic stem cells, MAPCs can be propagated *in vitro* without obvious signs of senescence and are highly susceptible to gene transfer techniques, including SB-based gene delivery.[18,19]

In this study, we used the SB transposon system and SMaRT to achieve integration and repair of the mutated mRNA endogenous transcripts in a T-cell line and in MAPCs derived from SCID mice.

RESULTS

Screening for optimal *trans*-splicer

In order to select the preferred episomal *trans*-splicer for correction of the DNA-PKcs gene (**Figure 1a**), we designed six PTMs, each containing a different 87–88 nucleotide (nt) BD capable of targeting a distinct region within intron 84 (**Figure 1b**). These six BDs span the region from nucleotides 228 to 903 of the 932 bp intron 84. Nucleofection was performed with each of the six plasmid *trans*-splicers together with the wild-type fusion of exon 85 and exon 86, PTMEx85-6BD1-6 (**Figure 1**). As plasmid controls, a PTM containing an unrelated BD and unrelated DNA were tested.

In order to measure radiation resistance as a general indicator of the level of DNA-PKcs mutation correction, initial studies were performed using irradiated scid.adh cells, with a wild-type lymphoid cell line as a positive control. Scid.adh cells were nucleofected with PTM plasmids or an unrelated DNA plasmid. After 48 hours, cells were first exposed to a 3 gray (Gy) radiation dose, which eliminates ~85% of non-treated scid.adh cells, and then cultured for 11 days prior to functional analysis. After exposure to radiation, scid.adh cells nucleofected with PTMEx85-6BD2 showed highest expansion in numbers, with approximately three-fold more radiation-resistant cells than those nucleofected with either an unrelated BD or an irrelevant plasmid control (**Figure 2**). On this basis, PTMEx85-6BD2, a plasmid *trans*-splicer that contains an 87 nt hybridization domain targeting the region of intron 84 from nucleotide 318 to 405, was chosen for further analysis.

SB/SMaRT dual vector repairs the mutated pre-mRNA and restores the DNA-PKcs function in scid.adh cells

In order to make use of the integrating ability of the SB transposon system, and simultaneously to benefit from SMaRT technology to repair DNA-PKcs (with a significant size reduction of the cargo from over 13 kb to ~600 nt), we cloned the PTMEx85-6BD2 expression cassette between the IR/DRs of a pT2-based SB

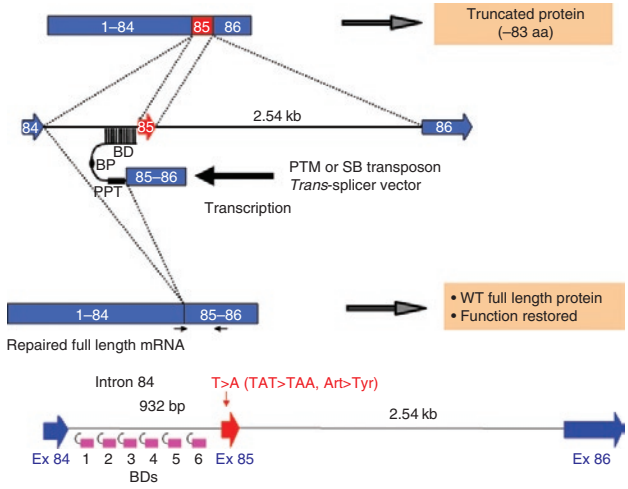

Figure 1 Schema of the *trans*-splicing strategy used for correction of the mutated pre-messenger RNA (pre-mRNA) DNA-PKcs transcripts with PTMEx85-6BD1-6 vectors. (**a**) Pre-*trans*-splicing molecule (PTM) vector binds and inserts wild type exon 85–86 into target pre-mRNA, resulting in the replacement of the two terminal exons, including the mutation (exon 85), with the wild-type exons, leading to the production of full-length wild-type protein and restoring protein function. In contrast, natural splicing of the pre-mRNA target leads to a mutated mRNA transcript which produces a truncated protein with the last 83 amino acids missing. Arrows indicate the locations of the primers used for real time-polymerase chain reaction (RT-PCR). (**b**) Approximate positions of binding domains (BDs) with respect to the target pre-mRNA are indicated. BDs were approximately 90-nt in length. They were designed to be complementary to intron 84, spanning its sequence from 5′ to 3′ end. WT, wild type; Ex, exon, aa, amino acid; kb, kilobase; bp, base pair; PPT, polypyrimidine tract; BP, branch point; Arg, arginine; Tyr, tyrosine; SB, Sleeping Beauty.

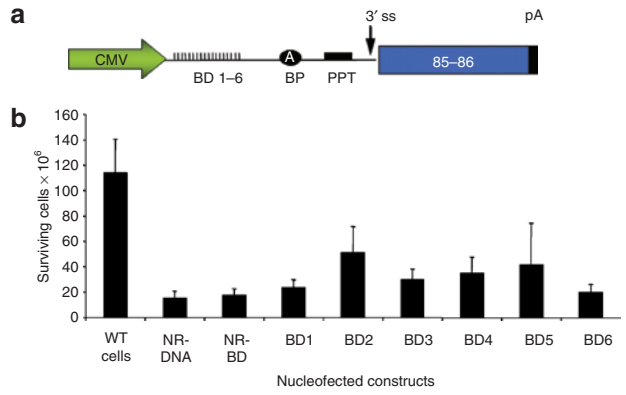

Figure 2 Selection of the optimal pre-*trans*-splicing molecule (PTM) episomal *trans*-splicer. (**a**) Schematic illustration of the PTM episomal *trans*-splicer used with six individual binding domains (BDs) designed to hybridize at different locations in intron 84. (**b**) The results of the radiation toxicity assay indicating the survival fraction of cells nucleofected with the six PTMs are shown. Controls were either cells nucleofected with non-related DNA (NR-DNA) or PTM with a non-related BD (NR-BD). CMV, cytomegalovirus; BP, branch point; PPT, polypyrimidine tract; pA, poly A sequence; WT, wild type (a non-malignant wild type B-cell line); ss, splice site.

transposon vector (**Figure 3a**). Scid.adh cells were nucleofected with the SB *trans*-splicer vector in the presence or absence of a transposase-expression vector that stimulates vector integration by DNA transposition. For assessing the degree of protection from radiation sensitivity conferred to scid.adh cells by the

various treatments, non-manipulated wild-type lymphoid cells and untreated scid.adh cells were included as controls. After 7 days post-nucleofection, scid.adh cells were irradiated at 3 Gy and cultured, as described earlier, for an additional 11 days.

When compared with wild-type cells, scid.adh cells exhibited a radiation-sensitive phenotype as assessed by quantifying the number of surviving cells (**Figure 3b**). The inclusion of a source of transposase along with the SB *trans*-splicer vector resulted in a 4.3-fold increase of surviving cells over irradiated, untreated scid.adh cells (P = 0.029; **Figure 3b**). SB-*trans*-splicer/transposase treated cells showed a 72% recovery rate as compared to the 100% rate exhibited by irradiated wild-type control cells. This suggests that stable integration of the SB *trans*-splicer vector can support mRNA correction at levels sufficient to restore a DNA-PKcs-dependent response to ionizing radiation in scid.adh cells.

In order to formally demonstrate molecular correction of the mutated DNA-PKcs mRNA, total RNA was isolated from scid.adh cells (after nucleofection and selection for resistance to radiation) and analyzed by Quantitative real time-polymerase chain reaction (Quantitative RT-PCR) using primers that target the exon–exon junctions between exons 84 and 85 and between 85 and 86 (**Figure 1a**). In this way, amplification of exon 85 (where the mutation resides) is achieved while avoiding the amplification of genomic DNA. Total RNA was extensively treated with DNase I to eliminate both exogenously administered wild-type plasmid carrying exons (85 and 86) in the SB *trans*-splicer vector, and genomic DNA. Direct sequencing of the complementary DNA (cDNA) products revealed that the mutation was maintained in scid.adh cells treated with an unrelated DNA, and also in scid.adh cells treated with SB *trans*-splicer in the absence of a transposase source (**Figure 4**). In contrast, correction of underlying A to T SCID mutation was found in samples obtained from scid.adh cells treated with SB *trans*-splicer along with a transposase (**Figure 4**).

Since the primers used for RT-PCR (**Figure 1a**) do not discriminate between the mutated and the corrected mRNA transcripts, the amplified cDNA represents a heterogeneous population of the corrected (T%) versus the non-corrected (A%) spliced mRNA molecules. To quantify the degree of mutation correction in this pool, we performed quantitative sequence analysis on the cDNA products using pyrosequencing, by which the relative amounts of wild-type and mutant alleles can be directly measured.[20] Sequence analysis indicated 100% A at position 12,138 in samples from both controls, whereas there was 79.1% T (corrected) and 20.9% A (non-corrected, mutation) in cells that were treated with SB *trans*-splicer plus transposase. The 79.1% correction level correlates with the 72% radiation resistance (in comparison with wild-type cells) that was previously observed (**Figure 3**). This suggests that the SB *trans*-splicer integrants mediate RNA repair by continuous production of the therapeutic mRNA molecules.

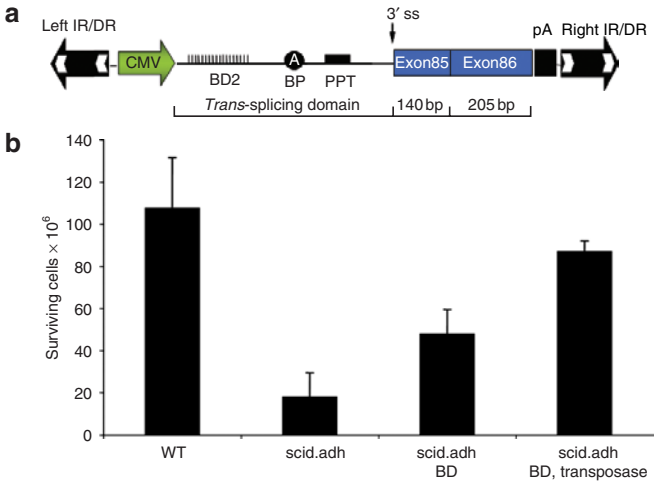

Figure 3 *Sleeping Beauty* (SB) transposon *trans*-splicer restores radiation resistance in scid.adh cells. (**a**) Schematic representation of the SB transposon *trans*-splicer vector carrying BD2. (**b**) Cells were nucleofected with the SB *trans*-splicer with and without transposase, allowed to grow for 7 days, exposed to 3 gray (Gy), then left to grow for another 11 days and analyzed for radiation resistance. IR/DR, inverted repeat/direct repeat; PPT, polypyrimidine tract; BP, branch point; CMV, cytomegalovirus; BD, binding domain; pA, poly A sequence.

SB/SMaRT dual vector mediates mRNA repair and DNA-PKcs protein production in MAPC-SCID cells

In order to determine whether SB/SMaRT vectors can be used for correcting non-hematopoietic stem cells (which may be useful in the correction of the immune defect in SCID), MAPC-SCID cells were nucleofected with the SB transposon *trans*-splicer vector along with pT2/CAGGS DsRed2 transposon in the presence and in the absence of pCMV-HSB5 as a source of transposase. After

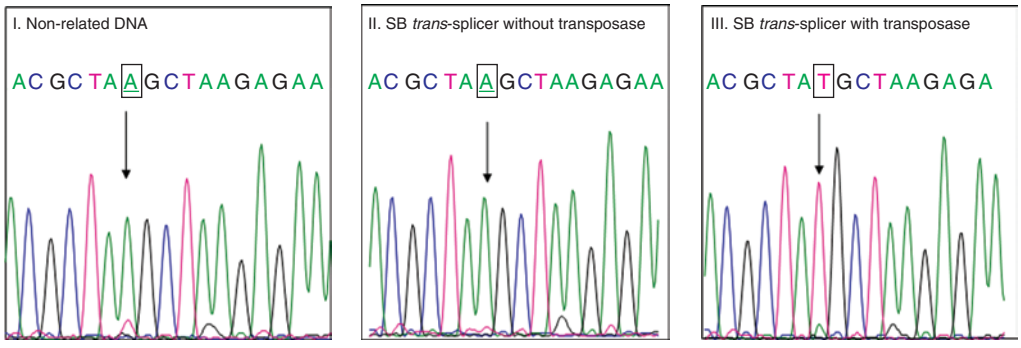

Figure 4 *Sleeping Beauty* (SB) *trans*-splicers repair the messenger RNA (mRNA) defect in scid.adh cells. Elution profiles are shown below the assessed DNA sequence. Cells treated with either non-related DNA or SB/*trans*-splicer without the transposase retained the mutant genotype (boxed A, arrows). In contrast, SB *trans*-splicer/transposase-treated cells showed correction of the SCID mutation at position 12,138 from A to T (boxed T, arrow).

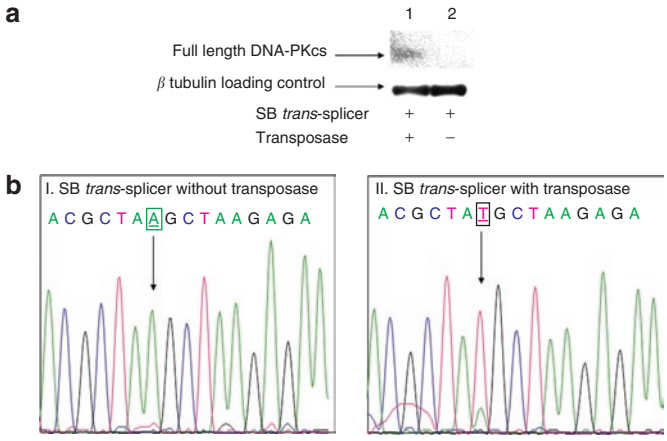

Figure 5 *Sleeping Beauty* **(SB) transposon/*trans*-splicer dual vector repairs DNA protein kinase catalytic subunit (DNA-PKcs)-mutated messenger RNA (mRNA) and produces full length protein in multipotent adult progenitor cell-severe combined immune deficiency (MAPC-SCID) cells.** (**a**) Total protein from 10^6 cells of SB *trans*-splicer ± transposase-treated, were prepared and analyzed by Western blot for DNA-PKcs and β tubulin-loading control. Lane 1, MAPC-SCID cells treated with SB *trans*-splicer plus transposase. Lane 2, MAPC-SCID cells treated with the transposon *trans*-splicer vector without transposase. (**b**) Elution profiles are shown below the assessed DNA sequence. SB/*trans*-splicer without the transposase-treated cells retained the mutant genotype (boxed A, arrow). In contrast, SB/*trans*-splicer/transposase-treated cells exhibited correction of the SCID mutation at position 12,138 (boxed T, arrow).

48 hours, MAPC-SCID cells were sorted for DsRed2 expression. One week after nucleofection, MAPC-SCID cells and wild-type control cells were exposed to 7 Gy irradiation dose and then cultured for an additional 2 weeks to assess mRNA expression and its therapeutic potential.

Nucleofection with the SB *trans*-splicer/transposase resulted in a radiation resistance phenotype: when compared with MAPC wild-type cells (100%), ~50% of the treated MAPC-SCID cells survived, which is approximately five-fold higher than for untreated cells and for cells not exposed to SB transposase. Cultures of untreated cells and of MAPC-SCID cells treated with only SB-*trans*-splicer did not expand (data not shown), thereby suggesting that continued expression of the repairing molecules produced from SB *trans*-splicer integrants is necessary for correction to be achieved.

In order to confirm that the acquired radiation resistance was due to proper production of DNA-PKcs protein, we performed Western blot analysis (**Figure 5a**) of total protein isolated from MAPC-SCID cells treated with the SB *trans*-splicer (with or without a transposase), selected on the basis of their resistance to radiation. Controls were either untreated MAPC-SCID or MAPC wild-type cells. Cells treated with the SB *trans*-splicer without a source of transposase showed no detectable levels of the full-length 456 kd protein (lane 2). In contrast, cells nucleofected with the SB *trans*-splicer along with a source of transposase exhibited substantial levels of the full-length protein (lane 1). These results confirm the effectiveness of the dual transposon *trans*-splicer vector in achieving significant *trans*-splicing levels of DNA-PKcs pre-mRNA and protein synthesis.

Molecular correction was investigated using direct sequencing and pyrosequencing. Direct sequencing showed correction of the mutation when a source of transposase was included, but not in the absence of transposase (**Figure 5b**). In order to assess the degree of correction in a quantitative manner, pyrosequencing was performed: in the presence of transposase, 64.3% correction was achieved compared with wild type (T%), while the remaining 35.7% cDNA molecules were SCID mutant (A%). These data are in agreement with the finding described earlier, namely that there is ~50% MAPC-SCID cell recovery after radiation in comparison with irradiated MAPC wild-type cells.

Table 1 SB *trans*-splicer integration by transposition

ID	Flanking sequence[a]	Chromosome number
Plasmid	CGTAATACGACTCACTATAGGGCGAAT TGGAGCTCGGATCCCTA**TACAGT**	
scid.adh	**Clone 1**: TGAGCAGATTTCCTTCCTTTCATCAA GAAGTAATAACAGCTAAGA**TACAGT**	**8**
	Clone 2: GTGAAGTCGGAAGAGCTTAACACTTA ATGTGAGGATATGCAACTA**TACAGT**	**6**
MAPC-SCID	**Clone 1:** ATTTATATGTACTGTGACACACACATAC CTATATTTATATATACATA**TACAGT**	**12**
	Clone 2: GGTGATATTAACATTAGCGAGGCTATA TAACCATATGGCTTTATTA**TACAGT**	**19**

[a]The last 4-nucleotide from the left inverted repeat/direct repeat (IR/DR) of pT2 transposon vector is shown in bold and underlined. TA nucleotides (duplicated as a result of transposase mediated integration) are shown in bold. The remaining sequence is a portion of adjacent genomic DNA. Sites of integrations were mapped using the National Center for Biotechnology Information Mouse Genome Database and the University of California Santa Cruz Mouse Blat Search Database.

In order to determine whether the correction observed both in the scid.adh and in MAPC-SCID cells was indeed attributable to *trans*-splicing and not to the homologous recombination of the DNA-PKcs encoding gene, genomic DNA was extensively treated with RNase A and used for PCR amplification of exon 85. Direct sequencing of the PCR product showed that the mutation was present both in scid.adh and in MAPC-SCID cells (data not shown), thereby suggesting that the correction was mediated by SB *trans*-splicer at the level of the mRNA.

In order to link repairing RNA machinery to specific transposon integrants, we performed splinkerette PCR on the left IR/DR(L)-genomic DNA junctions both in the scid.adh and in the MAPC-SCID cells nucleofected with SB *trans*-splicer and transposase source, after recovering them from the irradiation selection experiment earlier described. We found several chromosomal integration sites (chromosomes 6, 8, 12, and 19; **Table 1**).

These results suggest that the repair was due to SB integration events that led to continuous expression of therapeutic mRNA molecules.

DISCUSSION

We demonstrate for the first time that a non-viral dual vector, that combines the integration ability of SB with the mRNA repair capabilities of SMaRT, can efficiently repair endogenous mutated pre-mRNA DNA-PKcs transcripts in two different cell types: scid.adh and MAPC-SCID cells. The correction was accompanied by

production of wild-type DNA-PKcs protein and by restoration of its functional activity (radiation resistance).

The main advantage of this hybrid vector is that each of its individual components (SB transposon and SMaRT) overcomes the limitations of the other: (i) SMaRT sequence is no longer episomal, since SB mediates PTM integration by transposition, thereby permitting stable mRNA correction; and (ii) the cargo size limitations associated with the use of currently available SB vectors are overcome by the ability of SMaRT technology to correct large genes (*e.g.,* DNA-PKcs, ~12.4 kb) using dramatically smaller *trans*-splicing cassettes.

We have designed the dual non-viral vector with several innovations for continuous mRNA production from SMaRT. By using an improved IR/DR (pT2 transposon vector), a hyperactive transposase enzyme (HSB5), and strong splice elements[21] along with an optimized program for nuclear gene delivery (nucleofection technology), we were able to deliver DNA components directly to the nucleus and achieve DNA-PKcs correction, despite the fact that DNA-PK has been found to be a host factor that is critical for efficient SB transposition.[22–24]

We wish to point out, however, that there are risks associated with any gene correction technique. For SMaRT repair, for example, overexpression of a *trans*-splicing construct containing a 3′ signal splicing region has been shown to result in promiscuous *trans*-splicing.[25] This limitation is not absolute, however, because it can be overcome, in theory, by including optimal BD sequence for the target gene along with a tissue-specific promoter.

As integrating vectors, SB *trans*-splicers were able to restore the function and reverse the radiation hypersensitive phenotype in both scid.adh and MAPC-SCID cells. In populations of cells that survived the radiation selection, 79.1% scid.adh cells and 64% MAPC-SCID cells were corrected to wild-type genotype. The frequency of mRNA correction reported here is at least comparable with *in vivo* and *in vitro* studies reported by others. In cultured cells transfected with two RNA molecules designed to splice each other, RNA repair was achieved with 75% efficiency.[26] Using an adenoviral delivery system, Liu *et al.*[5] were able to restore up to 22% of normal protein function *in vivo* in a cystic fibrosis xenograft model, a correction which reached a therapeutic level. Using a modified adenoviral vector and Factor VIII (F8)-PTM sequence, Chao *et al.*[6] were able to achieve F8 expression at 12% of the wild-type level in F8-deficient (hemophilia A) mice. Tahara *et al.*[7] used lentivirus encoding a PTM to correct a murine CD40 ligand deletional model of human X-linked immunodeficiency. In addition to DNA-PKcs (~12.4 kb) (current manuscript), hybrid *trans*-splicing-SB technology to deliver PTMs could serve as an efficient strategy for monogenic disorders affecting large genes, for example muscular dystrophy (dystrophin, 11 kb),[27] and various forms of epidermolysis bullosa (type VII collagen, 9.2 kb; plectin, 14.8 kb).[28]

In the current work, some episomal SMaRT constructs too were capable of inducing radiation resistance in scid.adh cells, thus permitting a substantially higher level of cell outgrowth than with untreated scid.adh cells. It is noteworthy that radiation selection favors the outgrowth of cells expressing functional DNA-PKcs, and even low levels of corrected mRNA may have been sufficient to rescue irradiated scid.adh cells. Although expression is most likely transient (because of the use of an episomal vector) some level of random integration could have occurred. Alternatively, persistence of DNA-PKcs protein produced by non-integrating SMaRT constructs may have been sufficient to mediate DNA repair after radiation exposure.

Because the study used radiation exposure to allow preferential outgrowth of corrected cells, the true frequency of correction in the absence of selection cannot be easily derived from the data presented. However, since the selection pressure by radiation exposure resulted in 5.5-fold (scid.adh) to 9-fold (MAPC-SCID) fewer surviving cells than wild-type cells (**Figure 3b** and data not shown), we estimate that the frequencies of correction in the absence of selection are ~15 and ~7% for scid.adh and MAPC-SCID cells, respectively.

This level of correction should be sufficient to permit the *in vivo* use of SB/SMaRT corrected cells, which may be subject to selective outgrowth *in vivo.* This has been observed in a patient with SCID who acquired somatic mosaicism due to spontaneous reversion,[29] and in selective repopulation by gene-corrected bone marrow cells in murine SCID caused by to Jak3 kinase deficiency.[30,31]

For this strategy, MAPCs are the ideal cellular targets. MAPCs have been shown to give rise to hematopoietic cells in irradiated nonobese diabetic-SCID mice, and this is critical to SCID therapy.[17] When MAPCs generated under improved culture conditions were transplanted into irradiated nonobese diabetic-SCID mice, lymphohematopoietic reconstitution occurred, resulting in T- and B-cell production and restoration of immune function.[32]

Human cells with MAPC properties have been isolated,[33] and therefore it is conceivable that human MAPCs isolated from SCID patients and corrected *in vitro* using the SB/SMaRT approach could be used for autologous therapy. Under these circumstances the only fully functional lymphocytes would be derived from corrected MAPCs. Since these lymphocytes would expand in a T- and B-cell deficient patient, correction levels that we have already achieved may be sufficient to treat such patients, even in the absence of radiation selection.

In summary, these results show that SB *trans*-splicer is able to repair endogenous mRNA and restore phenotypic function in non-hematopoietic stem cells. This approach, therefore, offers a potential alternative to viral gene therapy for the treatment of genetic diseases and demonstrates the use of a non-viral vector for delivery of SMaRT to correct mutations, especially those involving large genes such as the DNA-PKcs mutation in SCID.

MATERIALS AND METHODS

Vectors. The pcDNA3.1-PTM24 plasmid, which contains the cytomegalovirus promoter to drive PTM expression[3] was cut with *Eco*RV/*Afl*II. Exon 85 and exon 86 fusion were PCR amplified using the forward primer: 5′-GATATCCTGCAGAGTTTTGAGCAGACAAT GCTGAGAAAAG-3′ and the reverse primer: 5′-CTTAAGCTTTACA TCCAGGGCTCCCATCCTTCCC-3′. The two exons replaced the 3′ exon in the pcDNA3.1-PTM24 plasmid to form the plasmid PTMEX85-6 (the template DNA, pMEPK7, which contained the full-length open reading frame of the DNA-PKcs, was kindly provided by Masumi Abe, National Institute of Radiological Sciences, Japan[34]). Six different BDs with an average of ~90 bp length, were subcloned in the *Nhe*I/*Sac*II sites (bold, below) to replace the existing BD which covers most of intron 84 or a non-related DNA plasmid containing the two fused wild-type 85–86 exons with

irrelevant sequence (non-related BD). As an additional control, a non-related DNA, pDRIVE-mB29 plasmid (InvivoGen, San Diego, CA) was used. Sequences of the BDs used in the current study were as follows:

BD1: (**GCTAGC**GGAAAGTTCTAGACTAGCCCAGACTAGAGCAAGA CCCTGTTTATAATTAAAACAACAATCAGAGCAACAAATTGC GCATGAAAACAG**CCGCG**),
BD2: (**GCTAGC**GTTACAGGTTCGGAGAAGGATTGTTATAATTTTA GAAATCAAGAGACTGAGACGAGATTCTTTTGAGCTCGAGG ACAGCCTTGGCTA**CCGCGG**),
BD3: (**GCTAGC**CAGAAACATTGACTACATCTTCTGCAATTCTAATT CAGACGCTCCCTCTTCTGGACTTTGCGGGCACTGCATGCA TATGATGCACTTA**CCGCGG**),
BD4: (**GCTAGC**CTGTTCTTAGATAAGCAAATTAAGGAAAAATTCA CATGGAATGATTTTTAAATCAAAGACTGTTACTGGCTGGAG AGATGGCTCAAGG**CCGCG**),
BD5: (**GCTAGC**GACACTGACATGGCACATATCACCATGATCATGC GATGCAGAGAGACGAGAAGAAGTCCACATAAAAAGATGAG CTTAATGCTTTCGG**CCGCG**),
BD6: (**GCTAGC**CAAAACTCTAAGGGAAAAAACACATTTTTTTCTA CTTATTATAATAGCATATACAAATTACATTAAAAAACATTAA ACCAAGTAATGA**CCGCGG**.

Oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Each complementary pair of oligonucleotides was heated to 94 °C for 2 minute, and annealed by cooling to room temperature. The resulting double stranded-oligonucleotides were digested with *Nhe*I and *Sac*II restriction endonucleases and then individually ligated to the PTMEX85-6 vector. All clones were sequence verified.

PTMEX85-6 containing BD2 was amplified using the forward primer: 5′-GCGCGAAGATCTGCGTTGACATTGATTATTGAC-3′ and the reverse primer 5′-ATAAGAATGCGGCCGCGAAGCCATAGAGCCC ACC-3′ with *Bgl*II/*Not*I overhangs, and was cloned into the multiple cloning site of the pT2-BH plasmid (kindly provided by Perry Hackett, University of Minnesota, Minneapolis, MN) (sequence available at: http://www.cbs.umn.edu/labs/perry). pT2/CAGGS DsRed2 cloning has been previously described.[19] pCMV-HSB5 is a modified hyperactive version of the wild-type SB10 transposase enzyme. Genomic DNA was prepared using the DNeasy tissue kit (Qiagen, Valencia, CA) with RNase A treatment for 20 minutes at room temperature.

Cell lines. Scid.adh, a radiosensitive thymic lymphoma cell line, carries a premature stop codon in the DNA-PKcs gene (SCID mutation).[35] As a control for radiation sensitivity, a non-malignant B cell line LDN-1[36] was used (kindly provided by David Rawlings, University of Washington, Seattle, WA). SCID.adh and B cells were maintained in RPMI 1640 with L-glutamine supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin, 1 mM sodium pyruvate, 1× non-essential amino acids, and 50 mM β-mercapto-ethanol. MAPCs were isolated from wild-type BALB/c and BALB/c-DNA-PKcs-deficient (termed MAPC-SCID) mice purchased from The Jackson Laboratories (Bar Harbor, ME). MAPCs were cultured at low density and induced to differentiate *in vitro* into neurons, hepatocytes and endothelium.[17] All protocols involving mice were approved by the Institutional Animal Care and Use Committee.

Nucleofection and radiation resistance assay. For testing transient SMaRT-mediated mRNA correction of scid.adh cells, 4 µg of each of SMaRT plasmid construct containing a unique DNA-PKcs-intron 84-specific BD, an unrelated BD, or non-related DNA plasmid was nucleofected into 10^6 scid.adh cells using an Amaxa Biosystems Nucleofector device (Gaithersburg, MD). For scid.adh cells, program G-016 and Cell Line Nucleofector kit V was used according to the manufacturer's recommendations. Forty-eight hours after nucleofection, scid.adh cells (2×10^6 cells) were exposed to 3 Gy by ^{137}Cesium irradiator and then immediately plated in T-175 flasks (Corning, Minneapolis, MN). Scid.adh or wild-type cells were cultured for an additional 11 days and counted. Cell viability was determined using the trypan blue exclusion method. Under these conditions, 3 Gy was sufficient to eliminate ~85% of scid.adh cells.

In order to determine the effects of SB *trans*-splicing vectors with the potential for permanent integration by transposition, scid.adh cells were nucleofected with 4 µg of SB *trans*-splicer construct and 200 ng of pCMV-HSB5 transposase. Two controls were used: scid.adh cells nucleofected with SB *trans*-splicers and pDRIVE-mB29 plasmid (a filler DNA used instead of the transposase vector to keep the amount of DNA constant), and non-manipulated scid.adh cells. Cells were irradiated 7 days after nucleofection.

For MAPC-SCID studies, cells were nucleofected (Amaxa Nucleofector, Gaithersburg, MD Mouse ES Cell Nucleofector Kit, T-20 program) with the SB *trans*-splicer with and without transposase, together with pT2/CAGGS.DsRed2 plasmid (DNA dose per 10^6 cells, SB *trans*-splicer: transposase: pT2/CAGGS.DsRed2 = 20 mg:1 mg:10 mg), and sorted for DsRed2 fluorescence after 48–72 hours. Cells were expanded for 7 days and then irradiated with a dose of 7 Gy (cell number: 2×10^6). The dose of 7 Gy was chosen since it resulted in the elimination of ~75% of MAPC-SCID cells as compared to wild-type MAPCs (data not shown). Two weeks after radiation exposure, surviving cells were counted. Negative controls included untreated MAPC-SCID and cells that did not receive the transposase. Positive controls were wild-type MAPCs.

RT-PCR and pyrosequencing. In order to determine whether the mutated DNA-PKcs mRNA had been corrected by the SB *trans*-splicing vector, total RNA was prepared from scid.adh cells 18 days after nucleofection using the SV total RNA isolation system kit (Promega, Madison, WI). Reverse transcription and PCR were carried out with the access RT-PCR system (Promega, Madison, WI) using the following primers for reverse transcription and RT-PCR: forward primer: 5′-TGATTGGAAGAGTTTTGAGC-3′; reverse primer: 5′-GCTCATCACAAGTTATAACAG-3′. For these studies, total RNA was treated with DNase I at 37 °C for 30 minutes to eliminate both the exogenously administered wild-type plasmid carrying exons 85–86 in the SB *trans*-splicer vector and the genomic DNA. In other studies designed to establish that the correction was caused by *trans*-splicing and not by homologous recombination at the DNA-PKcs locus, genomic DNA was extensively treated with RNase A, exon 85 was PCR amplified, and the products were sequenced.

For pyrosequencing, cDNA obtained by PCR was re-amplified using the same forward primer and the reverse primer: 5′-biotin-CTCATCA CAAGTTATAACAGC-3′. Isolation of the biotin-tagged reverse strand, which served as the template for sequencing, was performed according to the manufacturer's instructions (Biotage AB, Uppsala, Sweden). Pyrosequencing was performed using the PSQ primer (5′-TGGTATCCACAA CATAAAA-3′), SQ96MA instrument and the SQA software (Biotage AB, Uppsala, Sweden) in accordance with the manufacturer's instructions. The cDNA was also automatically sequenced using the forward primer.

Western blot. Scid.adh and MAPC-SCID (1×10^6 cells) were stored in Trizol (Invitrogen, Carlsbad, CA). Total protein concentration was determined (PQ02-10, Dojindo Inc., Gaithersburg, MD) and 20 µg of total protein was added to NuPage LDS sample buffer and separated using gel electrophoresis (Nupage Novex 3–8% Tris acetate 1.5 mm 10 well precast gels, EA0378BOX, Invitrogen, Carlsbad, CA). DNA-PKcs protein was detected using rabbit polyclonal DNA-PKcs antibody (AB 13852, Abcam Inc, Cambridge, MA). Blots were also probed with rabbit polyclonal β tubulin-loading control antibody (AB 6046; Abcam Inc, Cambridge, MA) and developed using WesternBreeze Chemiluminescent kit for rabbit (WB7106, Invitrogen, Carlsbad, CA).

Splinkerette PCR. For determining the genomic sites of SB *trans*-splicer integration, splinkerette PCR was performed as described.[37] Briefly, *Hin*dIII-digested genomic DNA was extracted from MAPC-SCID and scid.adh cells nucleofected with SB *trans*-splicers and transposase. The following primers were used: primary PCR forward: 5′-ATCGTAACCGTTCGTACGAGAAT

TC-3′, primary PCR reverse: 5′-GTAGATGTCCTAACTGACTTG-3′; nested PCR forward: 5′-GTACGAGAATCGCTGTCCTCTCC-3, and nested PCR reverse for the IR/DR(L) 5′-CTGACTTGCCAAAACTA TTG-3′. The product was directly sequenced and sites of integration were mapped using the National Center for Biotechnology Information blast service Mouse Genome Database and the University of California Santa Cruz Mouse Blat Search Database.

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