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To cite this version:

Myriam Fenina, Dominique Simon-Chazottes, Sandrine Vandormael-Pournin, Jihane Soueid, Francina Langa, et al.. I-SceI-mediated double-strand break does not increase the frequency of homologous recombination at the Dct locus in mouse embryonic stem cells.: Homologous recombination at the Dct locus. PLoS ONE, Public Library of Science, 2012, 7 (6), pp.e39895. 10.1371/journal.pone.0039895 . pasteur-01325896
I-SceI-Mediated Double-Strand Break Does Not Increase the Frequency of Homologous Recombination at the Dct Locus in Mouse Embryonic Stem Cells

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Abstract

Targeted induction of double-strand breaks (DSBs) at natural endogenous loci was shown to increase the rate of gene replacement by homologous recombination in mouse embryonic stem cells. The gene encoding dopachrome tautomerase (Dct) is specifically expressed in melanocytes and their precursors. To construct a genetic tool allowing the replacement of Dct gene by any gene of interest, we generated an embryonic stem cell line carrying the recognition site for the yeast I-SceI meganuclease embedded in the Dct genomic segment. The embryonic stem cell line was electroporated with an I-SceI expression plasmid, and a template for the DSB-repair process that carried sequence homologies to the Dct target. The I-SceI meganuclease was indeed able to introduce a DSB at the Dct locus in live embryonic stem cells. However, the level of gene targeting was not improved by the DSB induction, indicating a limited capacity of I-SceI to mediate homologous recombination at the Dct locus. These data suggest that homologous recombination by meganuclease-induced DSB may be locus dependent in mammalian cells.

Introduction

The natural efficiency of the introduction of defined sequences at specific locations of the mouse genome in embryonic stem (ES) cells by homologous recombination (HR) varies between 10⁻⁵ and 10⁻⁸ events per treated cell. Such a frequency is too low to consider the iterative introduction of a number of genes of interest at a given locus in standard practice. This problem can be overcome by enhancing recombination reactions at the target site through the induction of a double-strand break (DSB) [1]. Such DSBs can be induced with the yeast mitochondrial I-SceI meganuclease which has an 18-bp recognition site, absent normally in the mammalian genome but that may be added to the genome of recipient cells. In previous studies performed with I-SceI in Chinese hamster ovary (CHO) cells, mouse 3T3 fibroblasts, PCCG7-S multipotent cells, and also in several ES cell lines, specific DSBs were shown to stimulate the repair of a tandem duplication by intrachromosomal HR or gene targeting by plasmid-to-chromosome HR [2-8]. More recently, plasmid-mediated gene targeting was achieved in CHO cells after lentiviral delivery of the I-SceI protein [9]. Expression of I-SceI was also shown to be relevant to improve the efficiency of gene targeting in other organisms, including flies and plants [10,11]. Based on these data, it is generally admitted that a system based on the introduction of an I-SceI recognition site close to the locus to be targeted in the genome of recipient ES cells, combined with transient expression of the I-SceI meganuclease to create a DSB, should enhance the introduction of donor sequences at this site.

The Dct gene encodes the dopachrome tautomerase, a melanogenic enzyme. In the embryo, Dct is expressed in pigment cell precursors, i.e. melanoblasts, derived from the neural crest, in cells of the retinal pigment epithelium and in the developing forebrain [12,13]. In the adult epidermis, Dct is expressed in pigment cells at all differentiation states: in stem cells that reside in the bulge region of the hair follicle, in progenitors of the outer root sheath and in melanocytes of the hair matrix [14]. Studies performed in the mouse embryo with a LacZ reporter gene expressed under the control of 3.4 kb of the Dct promoter (Dct-LacZ gene) allowed to monitor cells of the melanocyte lineage in adult mice [16]. Altogether, Dct promoter-driven expression was shown to mimic largely the endogenous expression pattern of the gene. The Dct promoter has thus been used to drive the expression of genes in melanocytes and their precursors in transgenic mice [17–21]. However the use of combination of regulatory region from Dct and the coding regions of exogenous genes, either reporter genes or genes whose function is to be evaluated, can also
have large drawbacks. First, several independent lines are required to distinguish the specific expression of the transgene from ectopic expression. Second, the transgene may be expressed in tissues that do not normally express endogenous Dct. This has been repeatedly observed with the Dct promoter [15,17,22]. It is worth noting that Dct knockout mice are viable and fertile, and exhibit no defects, with the exception of a dilated coat colour [23], making Dct an interesting driver to monitor the effects of expression of genes of interest, such as genes that may be involved in melanoma progression [24,25]. Indeed, either homozygous knockin mice or double heterozygotes for a reporter gene and the gene of interest may be studied. We thus became interested by constructing a genetic tool that would allow to insert with a high efficiency any gene of interest in place of the Dct gene.

Our approach relied on the combination of three components: an ES cell line carrying a Dct allele with the I-SceI recognition site, an I-SceI expressing plasmid and a template for the DSB-repair process carrying sequence homologies to the Dct locus. The targeted integration at the Dct locus was tested after transfection of both the I-SceI expressing plasmid and the repair construct in the modified ES cell line. We report here that an I-SceI recognition site embedded within the Dct gene sequence can be cleaved by transiently expressed I-SceI meganuclease in ES cells. We further show, contrary to expectations, and using two different repair vectors, that I-SceI-mediated DSB did not increase the frequency of HR at the Dct locus compared to conventional gene targeting experiments.

Materials and Methods

Ethics Statement

Animals were housed in animal facilities accredited by the French Ministry of Agriculture to perform experiments on live mice, in accordance with the French and European regulations on care and protection of the Laboratory Animals (accreditation number B 75 15-01 and B 75 15-07). The veterinary staff of the Institut Pasteur animal facility approved protocols. Protocols were performed in compliance with the NIH Animal Welfare Insurance #A5476-01 issued on 02/07/2007.

Plasmids and Cells

Plasmid pPBSK2.B#4 was given by J.I. Jackson (MRC, Edinburgh, UK). Plasmids pl253, pLA42, pSW23 were provided by N. Copeland and N. Jenkins (Frederick, MD, USA). To produce pCAG-I-SceI, the enhancer of the major immediate-early enhancer of the human cytomegalovirus (CMV) contained in pCMV-I-SceI [26] was replaced by the chicken β-actin promoter and cytomegalovirus enhancer [27]. CK35 ES cells [28] were grown on mitomycin C-treated NeoR primary fibroblasts in Dulbecco’s modified Eagle’s Medium + GlutaMAX (Invitrogen) supplemented with 15% fetal bovine serum, 0.1 mM β-mercaptoethanol (Sigma), 10 U/ml murine LIF (PAA Laboratories) as previously described [29].

Design of Repair Vectors

To insert an I-SceI recognition site at the Dct locus, a replacement vector was constructed. A 6.5 kb SmaI-HincII fragment from pPBSK2.B#4 that contains 18 kb of Dct gene (MGI:102563; [30]) was inserted into the pNeo site of pL253 to produce pL253-Dct plasmid. To introduce an I-SceI recognition site and a NeoR cassette into the 6.5 kb Dct fragment near the first exon, we took advantage of a unique NheI site located within intron 1, 112 bp downstream of the first exon. A 5’ Dct fragment containing the NheI site was amplified and flanked with KpnI and EcoRI sites using the following primers: 5’-ATAGTGACCTCCTAAATTTAAGAAGGATGG-3’ and 5’-EcoRI reverse 5’-GGCAATTCCGCTTTCCTGAGTGAA-3’ and 5’-EcoRI forward 5’-GTAGGAATCCATTTGTGTGGTTTG-3’ and 3’-SacII reverse 5’-ATACCCGGGAGACATGAACCCGAG-3’. The amplicon was inserted into a pcR2.1 plasmid. pSW23 plasmid was digested by KpnI and SacII, and filled in with the three fragments: the 5’ I-SceI-EcoRI fragment containing the I-SceI site, an EcoRI-BamHI NeoR cassette from pL452 plasmid and the 3’ BamHI-SacII fragment. The replacement vector was produced by a recombineering reaction between the modified pSW23 and pL253-Dct plasmid. The replacement vector carries a herpes simplex virus-thymidine kinase (HSV-TK) negative selection cassette downstream of the 6.5 kb Dct fragment.

The construction of HR repair vectors HR1 and HR2 relied on the Gateway® technology (Invitrogen). Entry and destination vectors were produced, pENTR1A entry vector (Invitrogen) contains ccdB flanked with multi-cloning sites (MCS). The SV40 polyadenylation sequence (pA) was inserted at the EcoRV site to give pENTR1ApA. The Lago gene, a synthetic CpG-free LacZnls reporter gene that contains a SV40 nuclear localization signal (Invivogen), with its start codon was inserted in pENTR1ApA in place of ccdB (Fig. 1). The first destination vector (DV1) was constructed as follows. Starting from pL253-Dct plasmid, a SexAI-AvrII 400 bp fragment containing the ATG start codon was removed from Dct sequence. A linker made of the following primers was used to fill the gap in Dct sequences: HpaPm forward 5’-CTAGGTTAACGTCTAA-3’ and HpaPm reverse 5’-CCTGTGTTAAAACTTAC-3’. The linker allowed the insertion of a unique HpaI recognition site into pL253-Dct, giving pL253-Dct-HpaI plasmid. To insert the NeoR cassette into pL253-Dct-HpaI, a recombineering reaction was performed and gave pL253-Dct-Dct-HpaI-NeoR plasmid. Finally, a reading frame cassette A (RIA) (Gateway® technology) that contains the CmR-ccdB cassette flanked by attR1 and attR2 sites, was inserted at the HpaI site into pL253-Dct-Dct-HpaI-NeoR to obtain DV1. In DV1, the negative selection cassette HSV-TK from pL253 is downstream of the 3’ sequence homology of Dct (Fig. 1). To produce the first repair vector (HR1), the CmR-ccdB cassette in DV1 was replaced by Lago gene using LR reaction (Gateway® technology) (Fig. 1).

To remove the short regions of homology between the HR1 repair vector and the first intron of Dct gene, a second destination vector (DV2) was produced. The H2B-mCherry reporter gene with its start codon [31] was inserted in place of ccdB in pENTR1ApA entry vector (Fig. 2). To obtain DV2, a 2.8 kb AvrII-BsaI61 fragment was synthesized. It contains a NeoR cassette framed with FRT sites and 1 kb of Dct genomic sequence. A 1 kb AvrII-BsaI61 fragment was removed from the pl253-Dct-HpaI plasmid and replaced by the 2.8 kb synthesized AvrII-BsaI61 fragment. Then RIA was ligated at the HpaI site. Figure 2 shows the map of DV2. To produce the second repair vector (HR2), the CmR-ccdB cassette of DV2 was replaced by the H2B-mCherry sequences using LR reaction (Fig. 2). The integrity of the repair vectors was verified by sequence analysis.

Homologous Recombination Assay

To insert a unique I-SceI site at the Dct locus, approximately 1.6 × 105 CK35 ES cells were electroporated with the Nol-linearized replacement vector. G418 (300 μg/mL) was added 48 h after plating for 12 days and gancyclovir (2 μM) was
added 96 h after plating for 4 days. The NeoR cassette was removed using pIC-Cre plasmid [32] in which the transcription of Cre recombinase is driven by a synthetic HSV-TK promoter and enhancer. Fifteen micrograms of pIC-Cre plasmid were electroporated into approximately 1.6 × 10⁷ ES clone 4 cells and the cells were cultured without G418. For the gene targeting with HR1 repair vector, approximately 1.3 × 10⁷ MF1 ES cells were electroporated with 20 mg of supercoiled HR1 alone or with 33 μg pCMV-I-SceI [26] or 37.5 μg pCAG-I-SceI. The ratio of expression plasmid to repair vector was 5 to 1. For the experiment using HR2, approximately 1.6 × 10⁷ MF1 ES cells were electroporated with 30 mg of supercoiled HR2 plasmid alone or with either 11 μg pCMV-I-SceI or 33 μg pCAG-I-SceI expression plasmids. The ratio of expression plasmid to repair vector was 1 to 1. Approximately 1.6 × 10⁷ MF1 ES cells were independently electroporated with 30 mg of NotI-linearized HR2 plasmid, as a control.

**DNA Analysis in Selected Clones**

Genomic DNAs of ES clones obtained after selection with G418 and gancyclovir were digested with BamHI. Correct gene targeting was analyzed by Southern blot using a 1 kb 5'-external probe produced by PCR amplification with the following primers: 5DCTF forward 5'-TTGGGTCAGGGAGATACAG-3' and 5DCTR reverse 5'-TGAGCAGCAGTGGAATTGG-3'.

**Generation of Det^Sed/−/+ Mice.** Two independent Det^Sed/−/+ ES clones, named MF1 and MF2, were introduced into C57BL/6N blastocysts, which were transferred to pseudo-pregnant C57BL/6N females. Male chimeras (129/Sv Det^+/−/+ × C57BL/6N Det^+/−/) were mated with C57BL/6N females. The progeny was genotyped at the Det locus to evaluate the percentage of Det^Sed/+ mice.

**Ligation-Mediated PCR (LM-PCR) Analysis**

Approximately 1.6 × 10⁷ MF1 ES cells were electroporated with 50 μg pCMV-I-SceI, pCAG-I-SceI or mock plasmid. Four hours later, genomic DNA was extracted. Two micrograms of genomic DNA from MF1 cells transfected with the mock plasmid were digested with PstI and I-SceI and precipitated. LM-PCRs were performed with these PstI- and I-SceI-digested DNAs, and with undigested DNA from MF1 cells transfected with mock plasmid, pCMV-I-SceI or pCAG-I-SceI. The specific LM-C1 primer 5'-AATTCTTACCGGACAT-3' was used for the first extension. The asymmetrical synthetic double-stranded linker was prepared by hybridization of two oligonucleotides: linkerF forward 5'-GCGGTGACCCGGGAGATCTGAATTC-3' and reverse linkerR 5'-GAATTCAGATC-3'. The specific LM-C2 primer 5'-CGGACATGCAAATGCACAGGTGAGG-3' was used for a first PCR amplification. The PCR product was subjected to nested PCR with the specific LM-C3 primer 5'-CCCTTGGGCAAGGCGACATGTCACT-3') and linkerF. After agarose gel electrophoresis and alkaline transfer to a nylon membrane, the DNA was hybridized to the specific 36-mer radioactive probe LM 5'-CTTTCTGAAGGAGAGGCACACTGGTGAGGGACACTGTTA-3'.
Results

Our experiments aimed at testing the efficiency of a ready-to-use tool to produce ES cells, and eventually mice, carrying any sequence of interest inserted in place of the Dct gene. Our strategy relied on the reported stimulation of gene targeting frequency at a natural locus associated with a DSB induced by the yeast meganuclease I-SceI in ES cells [3]. We performed a two-step experiment. In a first step, the I-SceI restriction site was inserted to the Dct gene in ES cells using conventional gene targeting procedures. The Dct gene carrying a unique I-SceI restriction site was thus considered as a preferential target for HR. In a second step, an I-SceI-expression plasmid was introduced together with a repair vector sharing a 5.9-kb of Dct isogenic DNA by electroporation in the engineered ES cells, and the efficiency of gene targeting at the Dct locus was assayed.

Production of a New Target Allele at the Dct Locus in ES Cells

As a first step, the I-SceI restriction site was inserted within Del intron 1 in ES cells. A replacement vector containing a unique I-SceI restriction site, a positive selection (Neo⁺) cassette flanked with FRT sites, and 1.4 and 4.5 kb in length, depicted as grey rectangles. The black circle denotes 109 bp of Dct intron absent in DV1 destination vector that were inserted in DV2 destination vector. DV2 also contains a Neo⁺ cassette flanked with FRT sites depicted as white diamond symbols. The repair vector (HR2) is produced by LR reaction, allowing the replacement of Cm⁺-ccdB cassette by the H₂B-mCherry gene.

doi:10.1371/journal.pone.0039895.g002

Figure 2. Construction of the HR2 repair vector. The pENTR1ApA entry vector is represented in the upper left. The entry vector containing the H₂B-mCherry gene is represented in the upper right. The DV2 destination vector contains the Dct homologous arms, 1.4 and 4.5 kb in length, depicted as grey rectangles. The black circle denotes 109 bp of Dct intron absent in DV1 destination vector that were inserted in DV2 destination vector. DV2 also contains a Neo⁺ cassette flanked with FRT sites depicted as white diamond symbols. The repair vector (HR2) is produced by LR reaction, allowing the replacement of Cm⁺-ccdB cassette by the H₂B-mCherry gene.

Homologous Recombination at the Dct Locus

To delete the Neo⁺ cassette, a Cre recombinase-expressing plasmid (pIG-Cre) was electroporated into clone 4 Del⁻Sod⁻Neo⁺/⁺ ES cells. A preliminary experiment indicated that more than 30% of the cells transfected with pIG-Cre plasmid died in presence of G418, presumably because they had lost the Neo⁺ cassette. pIG-Cre plasmid was electroporated into Del⁻Sod⁻Neo⁺/⁺ ES cells and the cells were cultured without G418. Twenty-four clones were picked up and their sensitivity was assessed by adding G418 on a duplicate plate: 8 clones were Neo⁻. All eight clones had lost the Neo⁺ cassette as shown by PCR (data not shown), and later confirmed by Southern blot analysis (Fig. 3B). To test whether the meganuclease I-SceI is able to specifically cleave the new Del⁻Sod⁻Neo⁺ allele, genomic DNA of the ES clone 4 was treated with both BamHI and I-SceI restriction enzymes. Southern blot analysis using an external 5’ probe revealed the 4.5 kb BamHI-I-SceI distinctive fragment, indicating that the I-SceI site inserted at the Del locus was indeed cut in vitro by the meganuclease (Fig. 3C).

To test whether Del⁻Sod⁺ MF1 and MF2 clones are able to colonize the germ line, we injected MF1 and MF2 cells into C57BL/6N blastocysts, and thereafter transferred the embryos to pseudo-pregnant females. Twelve and ten chimeras were produced from MF1 and MF2 cells, respectively. Altogether 18 chimeras were more than 95% chimeric, based on their coat colour pattern. Several male chimeras were mated to C57BL/6N females. Half of their progeny was Del⁻Sod⁺, indicating that the
genome of MF1 and MF2 ES cells was transmitted via the germ line.

**Insertion of Lago1 at the Dct Locus**

We wished to repeatedly introduce gene of interest at the Dct locus. As a first attempt, we used the Lago1 gene. The HR1 repair vector contained Lago1, a NeoR cassette framed with loxP sites, two regions of homology with the DctI-SceI allele, 1.4 and 4.5 kb in length, and a HSV-TK negative selection cassette (Fig. 4A). The construction of the HR1 repair vector relied on the Gateway® technology (see Materials and Methods, and Fig. 1). We assessed the rate of insertion of Lago1 gene at the Dct locus following DSB-induced HR. MF1 ES cells were electroporated with supercoiled HR1 either with or without an I-SceI expressing plasmid. Two different I-SceI-expressing plasmids were tested: (i) pCMV-I-SceI, in which I-SceI expression is driven by the cytomegalovirus promoter [3,26], and (ii) pCAG-I-SceI, where I-SceI is expressed under the control of the CAG composite promoter (see Materials and Methods). The cells were exposed to G418 and gancyclovir. A total of 215, 235 and 252 colonies resistant to both antibiotics were obtained when MF1 ES cells were transfected with HR1 alone, and in combination with pCMV-I-SceI or pCAG-I-SceI respec-

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*Figure 3. Production of a new target allele at the Dct locus. (A) Introduction of an I-SceI site at the Dct locus. From top to bottom are represented the Dct wild-type allele (Dct*), the replacement vector, the DctI-Sce-Neo targeted allele, and the DctI-Sce allele produced after deletion of the NeoR cassette. The grey boxes represent exons 1 and 2 of the Dct gene. The black circle represents 109 bp of Dct intron 1 sequence that are lost during an homologous recombination event. The horizontal black bar represents the external 5' probe used for the Southern blots. The NeoR and HSV-TK cassettes are depicted as white rectangles. loxP sites are represented by white triangles. The Dct homologous arms, 1.4 and 4.5 kb in length, are denoted as grey rectangles. I-SceI and BamHI restriction sites are indicated. (B) Southern blot analysis of Dct* ES cells and targeted ES cells (clone 4). Genomic DNAs of ES cells were digested with BamHI. The 11.7 and 6.4 kb fragments are distinctive of the Dct* and DctI-Sce-Neo alleles, respectively. (C) Test of the ability of I-SceI meganuclease to specifically cleave DctI-Sce-Neo/+ ES cells. Southern blot analysis of Dct*/+ ES cells and clone 4. Genomic DNAs were digested with I-SceI and BamHI. The 4.5 kb fragment is distinctive of the DctI-Sce-Neo allele. (D) Southern blot analysis of Dct* ES cells, clone 4, MF1 and MF2 clones. Genomic DNAs were digested with BamHI. The 4.5 kb fragment is distinctive of the DctI-Sce allele.*

doi:10.1371/journal.pone.0039895.g003
tively (Table 1). For each experiment, 136 colonies were individually picked up and PCR tested. Transfection with either HR1 alone or with HR1 and pCMV-I-SceI gave no targeted colonies. Transfection with HR1 and pCAG-I-SceI gave a positive PCR signal (data not shown), which was confirmed by Southern blot analysis (Fig. 4B and data not shown). Thus gene targeting using the HR1 repair vector and pCAG-I-SceI led to a frequency of HR that could be estimated at $1.4 \times 10^{-7}$ events per treated cell. This frequency is not higher than that obtained with the conventional gene targeting procedures (generally range between $10^{-5}$ and $10^{-6}$ events per treated cell).

Elliott and colleagues (1998) reported previously that, in I-SceI-induced gene targeting with a transfected circular plasmid, the majority of recombination events occurred within 100 bp from the cleavage site [33]. Actually, the HR1 repair vector contains two short regions of homology with the targeted DctI-SceI allele next to the I-SceI site. These regions are shown in Figure 4C. In the DctI-SceI allele, the first short region of homology is located between exon 1 of the Dct gene and the I-SceI site. It encompasses 58 bp of Dct intron 1 sequence. Still in the DctI-SceI allele, a second region of homology is located between I-SceI site and the end of loxP site. It encompasses 125 bp of Dct intron 1 sequence and loxP sequence.

In HR1 repair vector, both 58 bp and 125 bp regions are located between the attB2 site and the NeoR cassette. We hypothesized that these homology regions, 183 bp in total length, could be used as an efficient repair template and would produce by HR a recombinant allele harbouring neither a Lago1 gene nor a NeoR cassette (Fig. 4C). Hence, clones that have undergone HR would die in the presence of G418.

Insertion of H2B-mcherry at the Dct Locus

We thus decided to remove the two short regions of homology (including the loxP site) and to construct a novel repair vector. Therefore, a second destination vector (DV2) was produced using the H2B-mCherry reporter gene [31]. The HR2 repair vector was constructed (Fig. 2). It carries H2B-mCherry, a NeoR cassette flanked with FRT sites, two regions of homology with the DctI-SceI allele, 1.4 and 4.5 kb in length, and a HSV-TK negative selection cassette (Fig. 5A). By contrast with HR1, HR2 displays neither a short region of homology with the Dct gene next to the I-SceI site nor a loxP site.

We assessed the rate of targeted insertion of H2B-mCherry at the Dct locus by I-SceI-induced HR. MF1 ES cells were electroporated with supercoiled HR2 plasmid alone or with either pCMV-I-SceI

figure 4. Lago1 gene targeting by HR at the Dct locus. (A) Insertion of Lago1 gene at the Dct locus. The DctI-SceI allele, the HR1 repair vector and the DctI-SceI targeted allele are represented from top to bottom. A lightning denotes I-SceI expression from pCMV-I-SceI or pCAG-I-SceI plasmid. The 1.4 and 4.5 kb of Dct isogenic DNA are depicted by grey rectangles. (B) Southern blot analysis of DctI-SceI/+, DctI-SceI/+ and DctLago1-I-SceI/+. Genomic DNAs were digested with BamHI. The probe used for the hybridization is the external 5' probe depicted by a black bar. The 11.7, 4.5, and 10.1 kb fragments are distinctive of the DctI-SceI, DctI-SceI and DctLago1-I-SceI alleles, respectively. (C) Diagram of DSB-induced homologous recombination with no insertion of the Lago1 gene. The DctI-SceI allele, and the HR1 repair vector are represented from top to bottom. The short regions of homology, 58 bp and 125 bp in length, between the HR1 repair vector and the genomic DNA at the Dct locus in DctI-SceI/ cells, are depicted by grey rectangles. An HR between these two short regions of homology would lead to loss of the I-SceI site with no integration of the NeoR cassette. The resulting cells would die in the presence of G418.

doi:10.1371/journal.pone.0039895.g004
or pCAG-I-SceI expression plasmids (Fig. 5A). As an additional control, MF1 ES cells were electroporated with linearized HR2 plasmid. The cells were cultured in the presence of G418 and gancyclovir. Colony counting revealed 397 and 370 colonies in presence of pCMV-I-SceI and pCAG-I-SceI plasmids respectively, and 653 colonies in the absence of the meganuclease (Table 1). Electroporation with linear HR2 plasmid, representative of a conventional gene targeting experiment, revealed 635 resistant colonies to both antibiotics. For each experiment, 144 colonies were individually picked up, amplified and PCR tested. Electroporation with linear HR2 plasmid resulted in one targeted clone (clone 7E). No targeted clones were seen in the supercoiled HR2-electroporated MF1 ES cells. The same results were obtained in the MF1 ES cells electroporated with both supercoiled HR2 and pCMV-I-SceI plasmids. Electroporation with HR2 and pCAG-I-SceI led to one targeted clone (clone 11E). Both conventional (linear HR2) and I-SceI-mediated (supercoiled HR2 and pCAG-I-SceI) gene targeting HR were confirmed by Southern blot analysis (Fig. 5B). Thus gene targeting using the HR2 repair vector and pCAG-I-SceI led to a frequency of HR that could be estimated at $1.6 \times 10^{-7}$ events per treated cell whereas conventional gene targeting led to a frequency of $2.7 \times 10^{-7}$ events per treated cell. Therefore, the I-SceI-induced DSB strategy does not seem to improve the frequency of HR at the Dct locus.

### Table 1. Frequency of homologous recombination at the Dct locus.

| Repair vector and conformation | I-SceI-expressing plasmid | Total electroporated cells | Total G418' gancyclovir | Analyzed G418' gancyclovir | Targeted integration | Gene targeting frequency |
|--------------------------------|---------------------------|-----------------------------|-------------------------|---------------------------|----------------------|--------------------------|
| HR1 circular                   | None                      | $13 \times 10^6$            | 215                     | 136                       | 0                    | 0                        |
|                               | CMV-I-SceI                | $13 \times 10^6$            | 235                     | 136                       | 0                    | 0                        |
|                               | CAG-I-SceI                | $13 \times 10^6$            | 252                     | 136                       | 1                    | $1.4 \times 10^{-7}$     |
| HR2 circular                   | None                      | $16 \times 10^6$            | 653                     | 144                       | 0                    | 0                        |
|                               | CMV-I-SceI                | $16 \times 10^6$            | 397                     | 144                       | 0                    | 0                        |
|                               | CAG-I-SceI                | $16 \times 10^6$            | 370                     | 144                       | 1                    | $1.6 \times 10^{-7}$     |
| HR2 linear                     | None                      | $16 \times 10^6$            | 635                     | 144                       | 1                    | $2.7 \times 10^{-7}$     |

Transient Expression of I-SceI Triggers DSB in Dct<sup>I-SceI</sup> ES Cells

To test whether the meganuclease could indeed trigger DSB in vivo at the Dct locus in Dct<sup>I-SceI</sup> ES cells, we electroporated I-SceI-expressing plasmids into MF1 ES cells and assayed the DNA lesion at the I-SceI site using a sensitive technique, known as ligation-mediated PCR (LM-PCR), that allows the specific detection of

![Figure 5. H₂B-mCherry gene targeting by HR at the Dct locus.](https://doi.org/10.1371/journal.pone.0039895.g005)
Homologous Recombination at the Dct Locus

breaks in a defined region of genomic DNA [34]. pCMV-I-Sol, pCAG-I-Sol and a mock plasmid were independently electroporated into MF1 ES cells and four hours later the genomic DNAs were extracted.

In a first step, we tested the specificity and sensitivity of the LM-PCR on transfected ES cells. We used two sites recognized by restriction endonucleases: (i) the I-Sol site, whose cleavage was under evaluation; (ii) a Pst I site at position +52 relative to the I-Sol site. Approximately 2 μg of extracted genomic DNA from mock plasmid-transfected Def−Sol+/+ MF1 ES cells were digested with Pst I or I-Sol restriction endonuclease respectively. Then the digested DNA was heated to allow annealing with a first Det gene-specific primer (LM-C1) located at position −185 relative to the I-Sol site. This was followed by LM-C1 primer extension that terminated at the I-Sol restriction site.

This was followed by LM-C1 primer extension that terminated at the I-Sol restriction site. Approximately 2 μg of extracted genomic DNA from mock plasmid-transfected Def−Sol+/+ MF1 ES cells were digested with Pst I or I-Sol restriction endonuclease respectively. Then the digested DNA was heated to allow annealing with a first Det gene-specific primer (LM-C1) located at position −185 relative to the I-Sol site. This was followed by LM-C1 primer extension that terminated at the I-Sol restriction site.

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The newly synthesized DNA molecule was denatured to allow annealing with a second Det gene-specific primer (LM-C2) located at position −174 relative to the I-Sol site and amplification in a PCR reaction with linker primer. The PCR-amplified products were exponentially amplified by nested PCR using a third Det gene-specific primer (LM-C3) located at position −148 relative to the I-Sol site and linker primer, as shown in Fig. 6A. Finally, the PCR products were separated on an agarose gel, alkaline blotted to a nylon membrane, and hybridized with a radioactive probe which does not overlap the primer sequences. PCR products of the predicted sizes, 148 bp for I-Sol digestion and 200 bp for Pst I digestion, were seen (Fig. 6B). These data indicate that the LM-PCR technique allowed the specific detection of a cleavage generated in vitro on the genomic DNA from MF1 ES cells.

In a second step, we evaluated the ability of the meganuclease to trigger a DSB in vivo at the Det locus. Approximately 2 μg of extracted genomic DNA from mock plasmid-transfected MF1 ES cells, pCMV-I-Sol-transfected MF1 ES cells and pCAG-I-Sol-transfected MF1 ES cells were directly analyzed by LM-PCR. Figure 6C shows that no DNA lesions occurred at significant level at the I-Sol site in the absence of I-Sol expression. When the genomic DNA from pCMV-I-Sol-transfected MF1 ES cells was used as a template in the LM-PCR reaction, a 148 bp amplification product was detected, showing that expression of the meganuclease in MF1 ES cells triggered DSB at the I-Sol restriction site.

LM-PCR in which the genomic DNA from pCAG-I-Sol-transfected cells was used as a template produced similar results (Fig. 6C), suggesting that both I-Sol expression vectors were equally efficient in triggering DSB at the target locus.

Discussion

In this report, we provided evidence that I-Sol-induced DSB in ES cells does not improve the efficacy of the gene targeting methodology at the Det locus compared to the conventional approach. Electroporation was used to introduce the I-Sol expressing plasmids into ES cells and a low efficiency of transfection could explain these results. However, we were able to detect the expression of enhanced green fluorescent protein (GFP) from Aequora victoria by fluorescence-activated cell sorter (FACS) analysis in 65% of a population of CK35 ES cells electroporated with a plasmid containing the CMV promoter driving the expression of the GFP, indicating efficient electroporation (data not shown). These data agree with a previous report [35]. Both repair vectors (HR1 and HR2) contain 3.9 kb of Det homology. The same homology was used previously by Guyonnet et al. [23] to inactivate the Det gene in ES cells, indicating that such a length is efficient for a gene replacement event.

However, we cannot exclude that increasing the length of homology may improve I-Sol-mediated HR at the Det locus. Since the first repair vector (HR1) contained short regions of homology in the vicinity of the I-Sol site, we hypothesized that these regions were preferentially used to repair the DSB, thus generating homologous recombinant clones that did not integrate the Neo+ cassette and died eventually in the presence of G418. Therefore, a second repair vector (HR2) with no homology to the sequence surrounding the I-Sol site was generated, but we still failed to demonstrate improvement of the frequency of gene targeting.

Previous experiments suggested that non-homologous recombination may be more efficient than plasmid-to-chromosome HR to repair a chromosomal DSB introduced by I-Sol. Indeed, when mouse Ltk− fibroblasts carrying a selectable herpes simplex virus thymidine kinase (tk) gene mutated by the 18-bp I-Sol site were, electroporated with I-Sol meganuclease and a repair plasmid with the functional tk gene, tk+ clones were recovered. However, all analyzed tk+ cells contained deletions that restored the reading frame of the tk gene, indicating that the recovery of a functional tk gene did not occur through HR of the integrated tk gene with a transfected tk fragment, but rather via resection and ligation [36]. These data were obtained in mouse Ltk− fibroblasts, not in ES cells. This deserves mention since distinct differences in frequencies of targeted integration driven by a DSB among cell types have been reported. HR after cleavage by a zinc-finger nuclease (ZFN) at the CCR5 locus in presence of cognate donor linear and circular episomes was more efficient in a panel of immortalized cell lines from human leukemia than in human stem cells, such as cord blood CD34+ hematopoietic cells and human ES cells [37]. It has also been reported that the rate of ZFN-mediated gene targeting at the Rosa26 locus was higher in primary fibroblasts from adult mice than in murine ES cells [38]. These results suggest that DSB-induced gene targeting may be lower in ES cells than in somatic cells. This contention seems inconsistent with reports showing that HR is the predominant pathway to repair DSBs in ES cells, whereas somatic cells utilize non-homologous end joining (NHEJ) [39,40]. It has also been reported that ES cells that had been allowed to differentiate preferred the error-prone NHEJ pathway to the high-fidelity HR to repair DNA DSBs [39]. Because ES cells and somatic cells are intrinsically different in the extent to which they preserve their genomic integrity [41], it was important to assess that our experiments were made with genuine ES culture rather than differentiated ES culture. We confirmed that the Det−Sol+/+ CK35 cells are truly pluripotent ES cells, able to colonize the germ line. Furthermore, CK35 ES cells have been previously used to demonstrate highly efficient gene targeting after DSB [3].

We observed that the repair vector was not inserted at the Det locus in the majority of neomycin- and gancyclovir-resistant clones. One possible explanation was that I-Sol did not cleave its recognition sequence in Det−Sol+/+ ES cells. However, we showed here the existence of I-Sol-induced DSBs at the Det locus using LM-PCR. This result does not rule out the possibility that the efficiency of cleavage by I-Sol is an important factor to favor HR at a given individual locus. Recent findings by Daboussi et al. [42] support this conclusion. They found that chromatin accessibility modulates the ability of meganucleases to induce targeted gene modification in human 293-H cells.

Seminal experiments of gene targeting in ES cells based on an I-Sol-induced gene replacement system were first performed with mutated resistance genes integrated in chromosomal sequences [8], and later extended to natural endogenous genes, Hprt, Villin and Dlx1 genes [3,4,43]. Importantly, random integrations could
Homologous Recombination at the Dct Locus

A

Denaturation and LM-C1 annealing

-185  -167
LM-C1

Extension from LM-C1

LM-C1

Ligation with Linker (F+R)

LM-C1  Linker F
LM-C1  Linker R

Denaturation and LM-C2 annealing

-174  -149  Linker F
LM-C2

PCR amplification

LM-C2  Linker F
LM-C2  Linker F

Nested PCR amplification

-148  -123  Linker F
LM-C3

148 bp

B

Mock

+PstI + I-SceI

Mock

CMV

CAG

I-SceI

200 bp

148 bp

148 bp
not be detected in the previous studies performed with I-SceI in mouse ES cells when selection strategies were specifically designed to single out the gene targeting events and eliminate the nonrecombinant recombination events [3, 4, 8]. Altogether, the number of actual genes that have been efficiently targeted following I-SceI-mediated HR in ES cells is still limited. It is widely accepted that the efficiency of conventional HR depends on the target locus. Our results suggest that, similarly, efficiency of gene correction by DSB-induced HR may be highly dependent on the targeted locus. We anticipate that deeper analysis of the meganuclease, repair vector, target locus and cells that do not show enhanced HR by DSB may also shed light on the nature of the factors that contribute to gene targeting in mammalian cells.

Acknowledgments

We thank Neal Copeland, Ian Jackson, Nancy Jenkins, and Anna-Katerina Hadjantonakis for the gift of plasmids. We thank Gaëlle Chauveau-Le Frere, Anne Carbon, and Abokona Zago, for technical help in the generation of chimeric and heterozygous mice. We are grateful to Stéphane Commo, Olivier Danos, Marie Le Boutelleur, Jean-François Nicolas and Jean-Stéphane Joly for helpful discussions.

Author Contributions

Conceived and designed the experiments: MF SVP MCT BAB JJP. Performed the experiments: MF DSC JS FL. Analyzed the data: MF DSC SVP MCT BAB JJP. Contributed reagents/materials/analysis tools: DSC SVP JS FL. Wrote the paper: MF MCT BAB JJP.

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