Biochemical and Biophysical Research Communications 441 (2013) 815–819

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

CRISPR/Cas-induced double-strand breaks boost the frequency of gene replacements for humanizing the mouse *Cnr2* gene

Benjamin Gennequin, David-Marian Otte, Andreas Zimmer *

Institute of Molecular Psychiatry, University of Bonn, Bonn, Germany

ARTICLE INFO

Article history:
Received 18 October 2013
Available online 6 November 2013

Keywords:
Cannabinoid receptor
ES cells
Knockout
Gene replacement
Surveyor assay

ABSTRACT

The CRISPR/Cas technology has been successfully used to stimulate the integration of small DNA sequences in a target locus to produce gene mutations. However, many applications require homologous recombination using large gene-targeting constructs. Here we address the potential of CRISPR/Cas-mediated double-strand breaks to enhance the genetic engineering of large target sequences using a construct for "humanizing" the mouse Cnr2 gene locus. We designed a small-guide RNA that directs the induction of double strand breaks by Cas9 in the Cnr2 coding exon. By co-transfection of the CRISPR/Cas system with the 10 kb targeting construct we were able to boost the recombination frequency more than 200-fold from 0.27% to 67%. This simple technology can thus be used for the homologous integration of large gene fragments and should greatly enhance our ability to generate any kind of genetically altered mouse models.

1. Introduction

Genomic engineering techniques based on homologous recombination are widely used for the generation of mutant mouse models, which are essential tools for biomedical research to analyze gene functions in disease processes. In some instances these techniques have also been used to generate mice in which a human disease-associated gene variant replaced the cognate mouse gene. Such "humanized" mice can provide important information about the role of specific protein variants and they may be useful for drug development [1]. Despite their widespread use, these techniques are still time consuming and costly.

Novel technologies for the generation of mutant alleles have therefore been developed that are based on the introduction of double-strand breaks into the target gene locus by sequence specific nucleases [2]. For this purpose, either zinc-finger nucleases or transcription activator-like effector nucleases (TALENs) have been engineered to recognize and cleave specific DNA sequences (Zinc finger: [3], TALEN: [4–6]). The repair of these double-strand breaks often results in insertions or deletions due to non-homologous end joining processes, which can disrupt gene functions. In additions, these double-strand breaks also increase the frequency of homologous recombination with exogenously delivered small DNAs and thus allowed specific gene modifications [6,7]. Nevertheless, cloning of these nucleases still requires a considerable effort, which offsets their advantages to some extent. Recently, the very powerful and much simpler CRISPR/Cas system for the introduction of targeted double-strand breaks has been developed [8,9]. It is based on the discovery that many eu- and archea-bacteria detect and destroy invading phages using ribonucleoprotein complexes composed of "clustered regularly interspaced short palindrome repeat" CRISPR-RNAs (crRNAs), *trans*-activating crRNAs (tracrRNA), and CRISPR-associated (Cas) proteins. In particular, Cas9 from *Streptococcus pyogenes* has been directed by single-guide RNAs (sgRNA), a fusion of crRNA and tracrRNA, to produce double-strand breaks at specific genomic locations [10]. Thus, the system can be adapted to target different genomic locations simply by modifying the sequence-specific motive in the sgRNA [7,11]. This system has been used to enhance the frequency of gene targeting by homologous repair in *Caenorhabditis elegans* [12,13], as well as protoplasts from *Arabidopsis thaliana* and *Nicotiana benthamiana* [14].

In this study, we have addressed the potential of the CRISPR/Cas system to enhance the efficiency of "humanizing" the mouse Cnr2 gene, which encodes the cannabinoid CB2 receptor, in mouse embryonic stem cells. We and others have previously demonstrated that a common polymorphism in the human gene leading to an amino acid exchange (Q63R) in the first intracellular loop of this G-protein coupled receptor is associated with low bone density and osteoporosis [15,16], liver disease [17], as well as psychiatric disorders [18]. Cell and mouse models carrying the two

* Corresponding author. Address: Institute for Molecular Psychiatry, University of Bonn, Sigmund-Freud-Str. 25, 53125 Bonn, Germany. Fax: +49 228 688 5301.
E-mail address: neuro@uni-bonn.de (A. Zimmer).

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http://dx.doi.org/10.1016/j.bbrc.2013.10.138

different CB2 variants would greatly enhance functional studies and drug development.

2. Materials and methods

2.1. Generation of targeting construct

The targeting vector hCB2-neo was cloned using the Red®/ET® recombination technology (Genebridges) and conventional cloning strategy based on restriction digestion/ligation. Two kits were used to clone the targeting vectors. First, the BAC subcloning kit was used in order to subclone, into a plasmid vector, a fragment of 9 kb from the genomic clone RPCIB731B063Q (ImaGenes) containing the mouse CB2 open reading frame of the coding exon 2 plus a left arm of 5 kb and a right arm of 1.8 kb for the homologous recombination in embryonic stem (ES) cells to occur. The mouse open reading frame was replaced by the human sequence using conventional cloning strategy (digestion/ligation) with *Bam*HI and *Aat*II restriction sites surrounding the coding exon 2. The mouse sequences surrounding the open reading frame containing the two restriction sites were added to the human by two consecutive PCR using 75 bp primers. In a last step a cassette containing a kanamycin and neomycin resistance (neo) genes flanked by FRT sites was inserted 933 bp after the open reading frame using the Quick & Easy Conditional Knock out Kit (FRT/FLPe). The hCB2-neo targeting construct was linearized with *Eco*RV. All oligonucleotide sequences are provided in the Supplementary materials.

2.2. CRISPR generation

The pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid was obtained from Addgene (Addgene plasmid 42230). As described by Cong et al. [11], oligonucleotides were designed with the help of the UCSC genome browser (http://genome.ucsc.edu) and purchased from Metabion. They were designed with a 4 bp overhang compatible with *Bbs*I digested overhangs. One μg backbone vector was digested with the FastDigest *Bbs*I (Fermentas) restriction enzyme for 30 min at 37 °C. The correct fragment was gel purified using the peqGOLD Gel Extraction Kit (Peqlab). Before proceeding with the ligation, the oligonucleotides were phosphorylated and annealed by mixing them with T4 PNK (NEB) (Supplementary materials). Ligation was performed at room temperature for 10 min. The complete ligation reaction was transformed into Stbl3 chemically-competent bacteria (Invitrogen). Plasmids were checked by PCR using oligonucleotides described in the Supplementary materials. The PCR-positive plasmid, termed px330-mCB2, was verified by sequencing.

2.3. Cell culture

Bruce4 ES cells [19,20] at passage 17 were grown on gelatinized cell culture plates at 37 °C and 5% CO_2 in ES cell culture medium (DMEM high glucose supplemented with 12,5% FBS, 1% sodium pyruvate 100×, 1% penicillin streptomycin mix 100×, 1% nonessential amino acids 100×, 0,1 mM β-mercaptoethanol, and 1000 U/ml leukemia inhibitory factor or LIF). The medium was changed daily. Cells were passaged at a confluence of 70–80% on new gelatinized cell culture dishes at ratio 1:5 and 1:10.

NIH3T3 cells [21] were grown in plastic T75 flasks with NIH3T3 culture medium (DMEM high-glucose supplemented with 10% FBS, 1% sodium pyruvate 100×, and 1% penicillin streptomycin mix 100×) at 37 °C and 5% CO_2. Cells were passaged at 70–80% confluency at a ratio of 1:3.

2.4. Electroporation of ES cells

Two hours before electroporation, the Bruce4 cell culture medium was replaced by fresh medium. Cells were harvested by trypsinization, centrifuged (950 *g*, 2 min, 4 °C) and washed twice with ice-cold HBSS (Hank's Balanced Salt Solution without calcium and magnesium). Throughout the electroporation procedure, ice-cold solutions were used and cells were kept on ice during waiting periods. Cells were centrifuged and re-suspended with HBSS at a concentration of 1.25×10^7 cells/ml. The electroporation was performed with 0.8 ml (1×10^7 cells) in pre-cooled 4 mm wide electroporation cuvettes, which already contained 20 μl of the linearized hCB2-neo (1 μg/μl) or the linearized hCB2-neo plus the px330-mCB2 (20 μg each). Electroporation was performed at 250 V, 500 μF with a Gene pulser Xcell Micropulser (Bio-Rad Laboratories). After a 10 min incubation period on ice, the mixture was dispensed on a 15 cm gelatinized cell culture dish with ES cell medium at room temperature.

Clones were selected by adding geneticin (G418) at a concentration of 170 μg/ml to the ES cell culture medium. The selection started 24 h after the electroporation and lasted for 10 days. The selection medium was changed daily. We picked 94 clones for each condition. Clones were trypsinized and replated on gelatinized 24-well plates for cell expansion and DNA preparation.

2.5. Lipofection of NIH3T3 cells

NIH3T3 cells were transfected with X-tremeGENE 9 DNA transfection reagent (Roche). Cells were plated in a 96-well plate at a density of 1.5×10^4 cells/well in 100 μl medium 18–24 h before transfection. Complexes were prepared at a 3:1 ratio of reagent to DNA, and 200 ng DNA were distributed in a drop-wise manner into the wells. Cells were incubated for 48–72 h and DNA was collected for the Surveyor assay (Transgenomics, Inc).

2.6. Genomic DNA isolation

Cultured cells were incubated for 2 h in 200 μl lysis buffer (Tris/HCl pH 8: 100 mM; EDTA: 5 mM; NaCl: 200 mM; SDS: 0.2% and proteinase K: 1 mg/ml) at 55 °C. The DNA was precipitated by adding the same volume of isopropanol to the lysate. After centrifugation (13000 *rpm*, 20 min) and careful removal of the supernatant, the DNA pellet was washed with 70% ethanol, air-dried and dissolved in TE buffer.

2.7. Surveyor assay

The Surveyor assay (Transgenomics, Inc) was performed according to the manufacturer's instructions. Briefly, the CRISPR/Cas9 target regions were amplified from the genomic DNA by PCR using primers surrounding the target region (Supplementary materials). SURVEYOR Enhancer S (1 μl) and SURVEYOR Nuclease S (2 μl) were added to the PCR reaction mixture. Tubes were gently mixed and incubated at 42 °C for 60 min. The reaction was stopped by adding stop solution (5.3 μl), gently mixed and subjected to electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide.

2.8. PCR analysis

2.8.1. Neo integration

DNA from positive clones was analyzed by PCR. The forward primer annealed at the end of the neo gene, whereas the reverse primer bound just after the integration site of the targeting construct. Oligonucleotide sequences are provided in the Supplementary materials. The expected PCR product had a size of 1953 bp.

PCR products were loaded on 1% agarose gel and visualized by ethidium bromide staining.

2.8.2. Bi-allelic recombination

The CB2 ORF was amplified from neo positive clones using primers annealing equally well to the mouse and the humanized CB2 gene locus. The products were then purified by ethanol precipitation, digested with SpeI (NEB), and analyzed on a 1% agarose gel.

3. Results

3.1. Targeting of CB2 coding exon with CRISPR/Cas9

Or aim here was to design the sgRNA such that Cas9 produces a double-strand break in the mouse target locus, but not in the targeting construct with the human sequences. We therefore first aligned the sequences of the Cnr2-ORF of the coding exon 2 from both species and found 82.4% homology. An important requirement for CRISPR/Cas to produce double-strand breaks is a short NGG protospacer-adjacent motif (PAM) immediately following the guide sequence. We identified this motive adjacent to a sequence that was highly divergent between mouse and human, with 6 mismatches in 20 bp (Fig. 1A). Also, the mismatches were at both ends of the sequence, which were reported to be more important for target specificity of sgRNAs than the central nucleotides [11]. This sequence therefore appeared to be well suited as a target for a sgRNA and the corresponding oligonucleotides were cloned into the px330 plasmid to obtain px330-mCB2 (Fig. 1B). This is the only cloning step required to direct the system to Cnr2 gene locus.

3.2. Surveyor assay

To validate that CRISPR/Cas did induce a double-strand brake at the desired position, a Surveyor assay was performed in NIH3T3 cells transfected with the px330-mCB2. This assay utilizes the fact that short insertions and deletions are generated during the repair of double strand breaks, resulting in sequence heterogeneity of a mixed cell population. This sequence heterogeneity can be detected after PCR amplification, denaturation and re-annealing using a surveyor nuclease, which cleaves misaligned single-stranded DNA. Primers flanking the px330-mCB2 target site were selected to generate an amplicon of 688 bp, or two fragments of

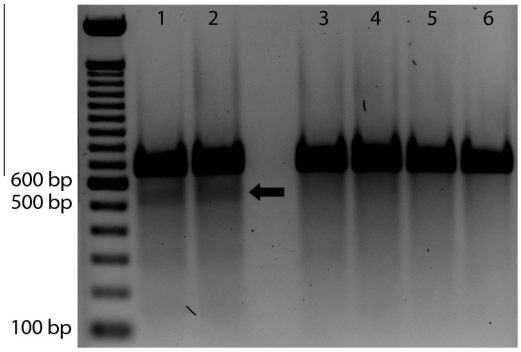

Fig. 2. Surveyor assay with NIH3T3 cells. Small insertions/deletions induced CRISPR/Cas mediated cleavage of the Cnr2 locus is revealed by Surveyor nuclease cleavage of PCR amplicons. Separation of the resulting fragment in a 2% agarose gel shows a main fragment of 688 bp from the wild type locus, which is present in all DNA samples, and a 525 bp fragment (arrow) from the mutant locus. This band is only present in lane 1 and 2, which contains DNA from cells transfected with px330-mCB2 cells. Lane 1: 2.5 µl genomic DNA; lane 2: 5 µl genomic DNA; lanes 3 and 4: 2.5 and 5 µl genomic DNA from cells transfected with the px330 empty plasmid (without guide sequence); lanes 5 and 6: 2.5 and 5 µl genomic DNA from wild type NIH3T3 cells.

525 and 163 bp when mismatches were present. As shown in Fig. 2 and 525 bp and 688 fragments were detected after transfection of px330-mCB2, thus indicating an efficient generation of double strand breaks at the target sequence. Please note that the signal from the 163 bp fragment was too faint for visualization.

3.3. Analysis of homologously recombined ES cells clones

We have made a total of 26 electroporations with the hCB2-neo gene replacement constructs into Bruce4 ES cells and isolated 1884 neomycin resistant clones. Of those, 5 were identified as homologous recombinants, indicating a targeting frequency of 1:377, or 0.27%. To determine if double strand breaks in the Cnr2 gene generated after px330-mCB2 transfection would enhance the frequency of homologous recombination, we performed two more electroporations, one with the linearized hCB2-neo construct alone and another one that also contained px330-mCB2. From each condition we harvested and analysed 94 individual clones. As shown in Fig. 3, PCR analysis identified no positive clones after electroporation of hCB2-neo alone, whereas 63 clones contained a targeted

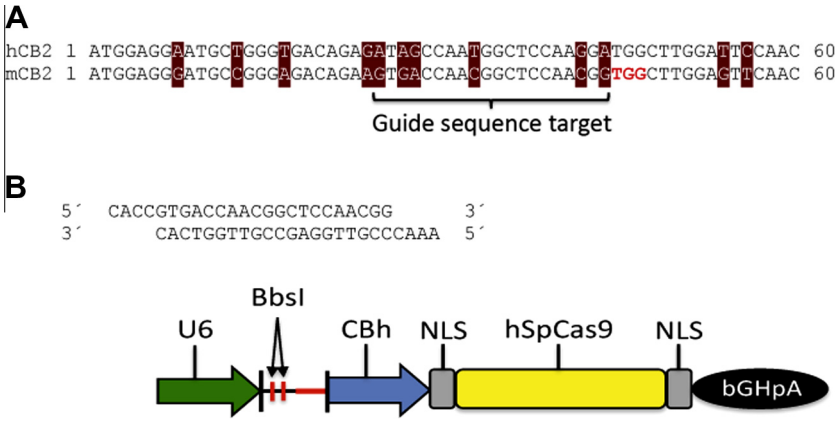

Fig. 1. CRISPR/Cas9 mouse CB2 ORF targeting. (A) Alignment of the first 60 nucleotides of the human and mouse Cnr2 open reading frames revealed several mismatches, which are highlighted by a red background color. Red letters represent the proto-spacer adjacent motifs (PAM). (B) Cloning strategy for CRISPR/Cas9 pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid. The oligonucleotides with the guide sequence containing 4 bp *Bbs*I-compatible overhangs were cloned into the BbsI site of the plasmid px330. U6: polymerase III promoter; *Bbs*I: restriction sites used for cloning; CBh: hybrid form of the chicken β-actin (CBA) promoter; NLS: nuclear localization sequence; hSpCas9: humanized *S. pyogenes* Cas9; bGHpA: bovine growth hormone polyadenylation signal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

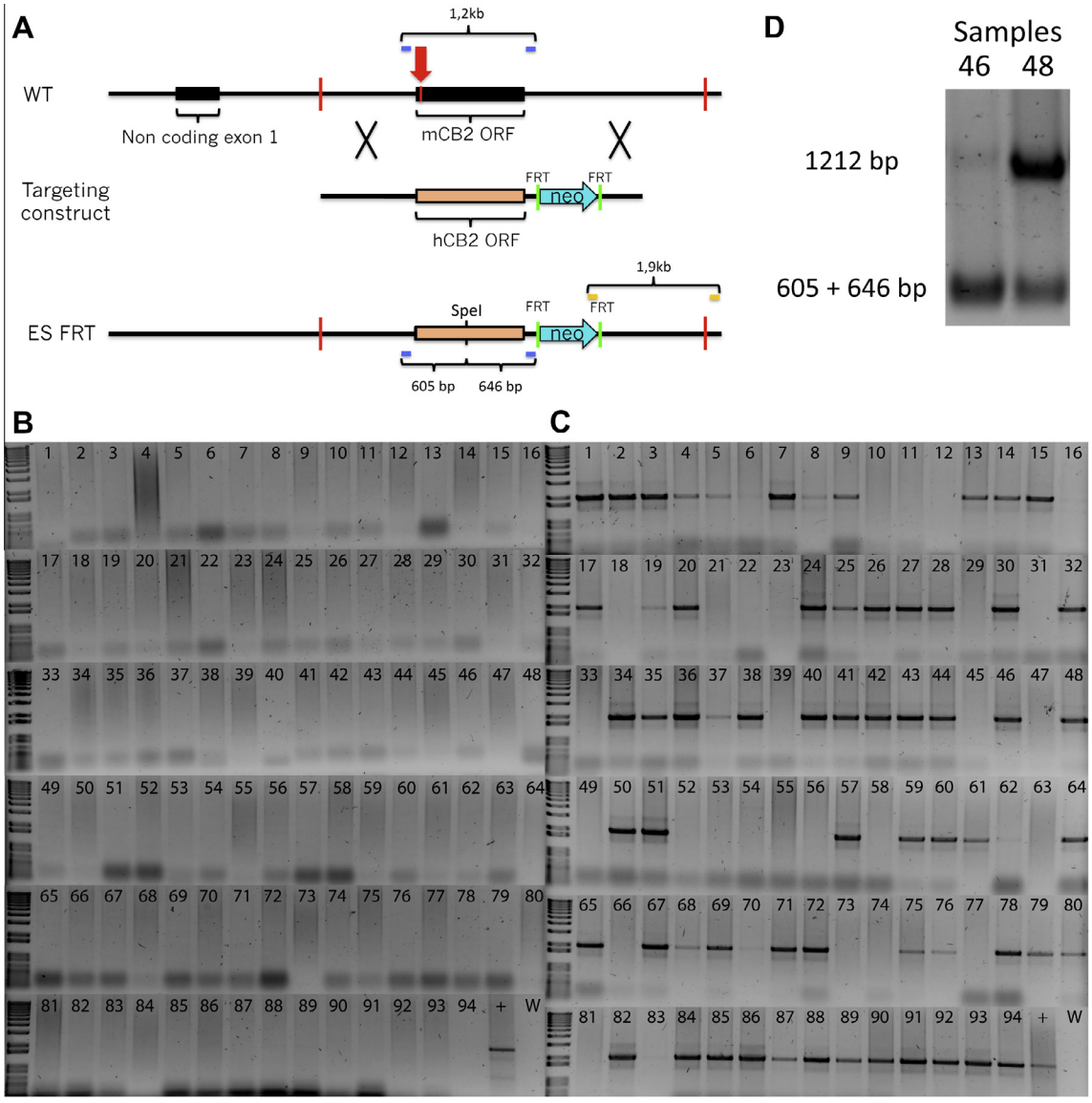

Fig. 3. Detection of homologous recombination. (A) PCR strategy to check the homologous FRT-neo-FRT integration in ES cells and bi-allelic recombination. WT represents the mouse wild type allele with a non-coding exon 1 and the open reading frame of exon 2. The small red bar and the red arrow at the beginning of the mouse CB2 open reading frame represent the guide sequence target. The targeting construct, hCB2-neo, is depicted with the human CB2 ORF in salmon color and the neomycin/kanamycin cassette in blue flanked by FRT sites represented by green bars. Crosses indicate the regions of homologous recombination. ES FRT indicated the "humanized" allele after homologous recombination with the targeting construct. Primers used for the analysis of the bi-allelic recombination are indicated as blue lines, while the yellow lines indicate primers used for the analysis of the neo integration. (B) Electroporation with the linearized hCB2-neo did not produce a PCR product of 1953 bp, indicative of homologous recombination, in any of the 94 selected ES cell clones. (C) After electroporation of the linearized hCB2-neo with the circular px330-mCB2 vector, numerous EC cell clones show the expected 1953 bp fragment. + denotes a recombined ES cell clone identified previously as a positive control and W indicates a negative water control. (D) PCR amplification of the CB2 ORF followed by SpeI digestion results in a 1212 bp fragment indicative of the mouse ORF, or two similar size fagments (605 and 646 bp) indicative of the humanized ORF. Please note that sample 46 contained only the humanized ORF, suggesting a bi-allelic homologous recombination. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cnr2 locus after co-electroporation with px330-mCB2. This corresponds to a targeting frequency of 1:1.5, or 67%. We next amplified the CB2 ORF from these clones and digested the products with SpeI, because this restriction site is only present in the human sequence. Four of these 63 clones had the human, but not the mouse sequence, thus indicating that both alleles were targeted.

4. Discussion

In this manuscript we demonstrate that CRISPR/Cas-mediated double strand breaks at the mouse Cnr2 locus dramatically increased the frequency of homologous recombination in neomycin resistant ES clones from 0.27% to 67%. Furthermore, 4% of all clones showed a bi-allelic recombination. Previous studies showed the utility of CRISPR/Cas or Talen technologies for enhancing the frequency of homology-directed repair with short single-stranded DNA sequences in mice [7]. In addition, CRISPR/Cas-mediated double strand breaks also enhanced the frequency of homologous recombination in plants and *C. elegans* [12–14] with double-stranded DNA. Here we now demonstrate that this technology can also boost gene replacements with large DNA constructs in the mouse genome. This new and simple technology thus holds a great promise to improve genetic engineering of the mouse genome.

We have previously used targeting constructs for the Cnr2 gene to generate a constitutive knockout [22] and, recently, to generate

conditional CB2 knockout mice. These targeting constructs contained negative selection cassettes and generated targeted ES clones with frequencies of approximately 3% (unpublished results). Assuming that the negative selection procedure provided a 10-fold enrichment of homologous recombinants [23], it thus seems that the targeting frequencies at the Cnr2 locus were similar with all different targeting constructs. This frequency was increased more than 200-fold by the CRISPR/Cas system. Importantly, 4 out of the 63 targeted clones no longer contained the mouse ORF, indicating that both alleles were targeted. It is thus possible to generated completely humanized cell lines with a single electroporation and normal selection conditions. We have not tried to combine the CRISPR/Cas-mediated stimulation of homologous recombination with the positive–negative selection strategy, simply because the observed frequency of positive clones was already so high that further improvements were pointless and probably offset by the additional investment in the negative selection procedure. However, such a combination may be useful in rare situations where homologous recombinants are very difficult to obtain.

One of the most appealing aspects of this technology is its simplicity. All that is required is the cloning of a short oligonucleotide and co-electroporation of the resulting plasmid. Although we have first assessed the ability of px330-mCB2 to induce double strand breaks in the Cnr2 locus, we feel that it may not be necessary to perform this control experiment. In fact, it was relatively difficult and time consuming to establish an assay that enabled us to detect small insertions/deletions generated by the repair of double strand breaks in a heterogeneous transiently transfected cell population. It was much easier to detect homologous recombinants. It remains to be determined if the CRISPR/Cas technology is more efficient than zinc-finger nucleases or TALENs [11] to stimulate homologous recombination. There is certainly evidence to suggest that this might be the case. Anyhow, considering the easiness to clone guide sequences in comparison to the rather cumbersome task of engineering TALENs or zinc-finger nucleases, it will be much easier to experiment with different sgRNAs, if necessary. It should be taken into consideration that it might be required to design the guide sequence such that it does not direct double strand breaks within the targeting construct. In practice, this should also not impose any problems, because it should always be possible to utilize sequences where the targeting construct diverges from the target locus, e.g. lox sites.

Acknowledgments

The authors would like to thank Dr. Eva Drews for critically reading this manuscript, Dr. Le Cong for providing his pX330-U6-Chimeric_BB-CBh-hSpCas9 to the scientific community. Research support was received from the Deutsche Forschungsgemeinschaft (FOR926), the Bundesministerium für Bildung und Forschung (NGFNplus) and the state of NRW (Biotech Pharma).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.138.

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