Comparative analysis of NHEJ and HDR repair pathways for genomic editing using CRISPR/Cas9 technology in ES cells

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discovery from charles river

1 Introduction

The error-prone non-homology end joining (NHEJ) repair pathway is often the pathway of choice when utilizing CRISPR/Cas9 to generate a genetic knockout. Despite the high efficiency of creating indel mutations, the pathway has its drawbacks. Deletions are often in-frame, which might result in semi-functional proteins. Indels can easily span from 1-200 bp, without any control on their exact length or location (upstream/downstream) which can make indel identification and validation an expensive and time consuming task.

The homology driven repair (HDR) pathway allows for precise editing of the genome by including a repair template in the process. Using a single-stranded template ensures that this template is not randomly inserted elsewhere in the genome or is targeted by the Cas9 enzyme. The introduced sequence also gives the opportunity for fast and efficient PCR validation of edited clones prior to sequencing. Here we performed a comparative analysis of the two repair mechanisms to investigate whether the HDR pathway is a good alternative to the NHEJ pathway for high frequency generation of genetic knockouts in mouse C57BL/6NCrl embryonic stem cells (ESC), but with the concomitant advantage of precise genome editing.

2 Double-strand break repair pathways

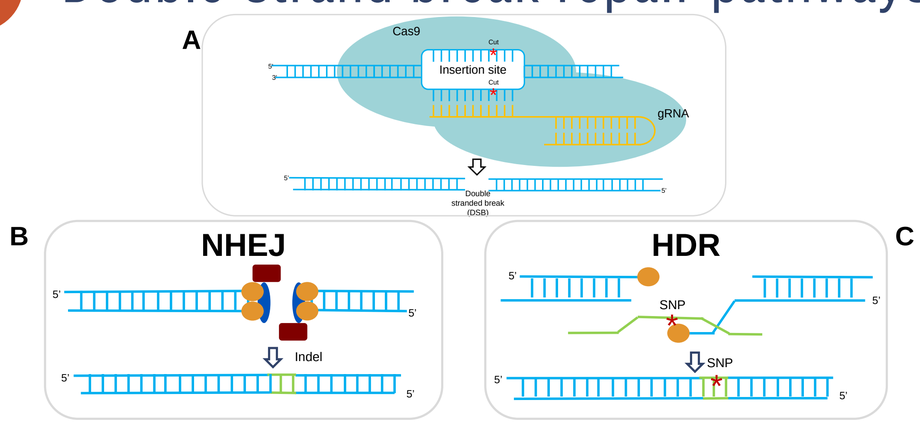


Figure 2: (A) Schematic drawing of CRISPR/Cas9-induced DSB and its sequential repair pathways (B) NHEJ is an error-prone repair pathway, repairing double-stranded breaks (DSB) through a 'paired end complex' which facilitates ligation of DNA breaks (C) The HDR pathway requires a homologous DNA strand which is used as a template for high fidelity DSB repair.

3 Method

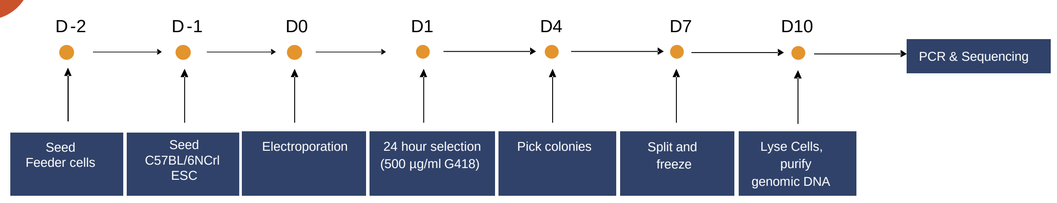


Figure 4: General experimental setup of CRISPR/Cas9 editing in mouse C57Bl/6NCrl ESC. Charles River constructed a two vector CRISPR/Cas9 system separating the sgRNA from the expression of the Cas9 protein for genomic editing. The system is designed for high throughput cloning of sgRNA making it suitable for producing an arrayed sgRNA library. Mouse C57Bl/6NCrl ESC were thawed and seeded on a feeder layer 24 hrs prior to electroporation on D-1. Trypsinized ESC were electroporated using the '*Mouse ES Cell Nucleofector® Kit*' from Lonza with up to 30 µg DNA per 1E6 ESC on D0. A 24 hrs selection period was initiated on D1 using 500 µg/ml G418. Three hundred single colonies were transferred to a 96 well on D4 for clonal expansion. At D7 colonies were divided for the purpose of clone preservation by freezing and for further expansion until D10 for genomic analysis.

4 Transfection efficiency in C57BL/6NCrl ESC

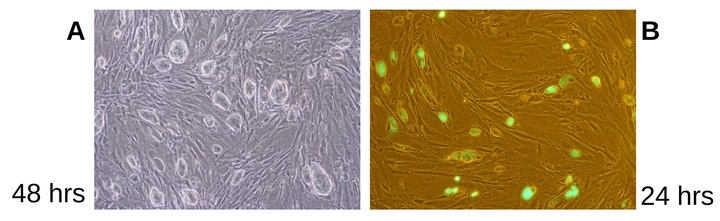


Figure 3: Transfection efficiency of C57BL/6NCrl ESC determined with a GFP plasmid. (A) Non-electroporated mouse ESC on feeder cells 48 hrs after seeding. (B) Mouse ESC electroporated with 10 µg MaxGFP® on top of feeder cells 24 hrs after seeding. Estimated transfection efficiency of 80% based on GFP expression. Magnification: 10x

5 Non homologous end joining

A

Genotype	No. of clones	%
Total Indels	154	73
Homozygous Indels	25	14

B

Figure 5: Generation of a genetic knockout in C57BL/6NCrl ESC via the NHEJ pathway. (A) A gene editing efficiency of 73% was achieved utilizing Charles Rivers two vector system. The frequency of homozygosity for confirmed indels was found to be 14%. (B) Sequencing identified 3 out 4 indels as small in-frame deletions.

6 Repair template length for HDR

A

HDR SNP Template	%
100 nt	57
50 nt	25

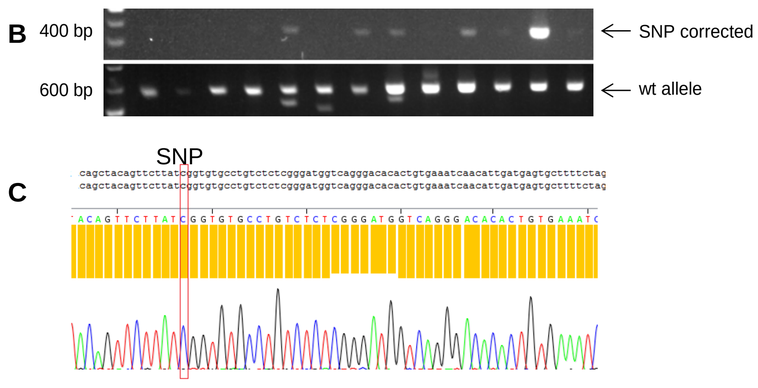
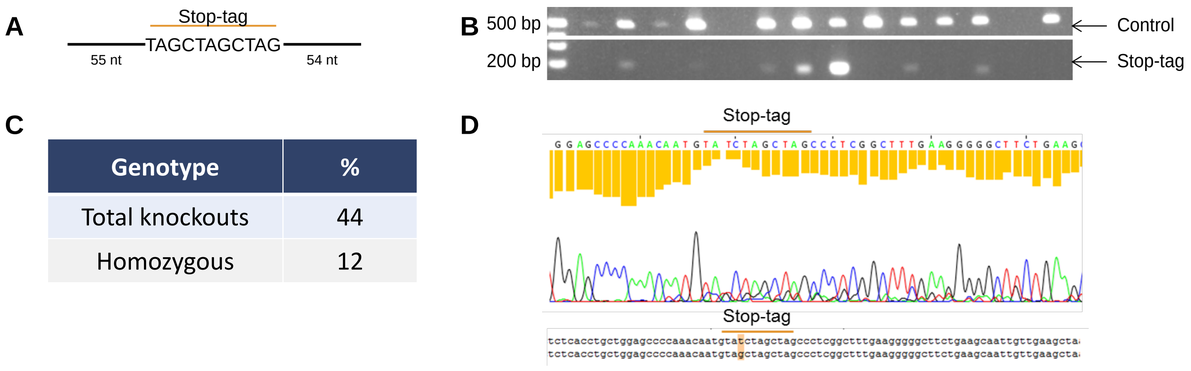


Figure 6: Effect of repair template length on gene editing frequency by HDR. (A) A single stranded repair template as small as 50 nucleotides (nt) introduced a point mutation via the HDR pathway with a gene editing frequency of 25%. Gene editing frequency was increased to 57% by lengthening the repair template to 100 nt. (B) PCR validation using a mutation-specific primer identified positive clones prior to sequencing (C) 2 out of 8 clones sequenced showed a homozygous genotype for the 100 nt template.

7 Homology driven repair



Genotype	%
Total knockouts	44
Homozygous	12

Figure 7. Generation of a genetic knockout in C57BL/6NCrl ESC via the HDR pathway. (A) A 120 nt single-stranded repair template with a Stop-tag flanked by homologous arms was designed to introduce a premature stop codon as well as a frameshift mutation upon gene integration. (B) PCR validation identified clones positive for the Stop-tag prior to sequencing. (C) A gene editing efficiency of 44% was achieved. (D) 1 out of 8 sequenced clones showed a homozygous genotype giving a homozygous frequency of approximately 12%.

8 Conclusion

In this study we demonstrated that a controlled genetic knockout can be efficiently generated by the HDR pathway. A relatively small single-stranded DNA template designed to introduce a premature stop codon in all three reading frames was sufficient for inducing homology directed repair in mouse C57BL/6NCrl ESC.

The overall HDR editing frequency was slightly lower compared to the frequency of indel mutations generated by the NHEJ pathway, however this is compensated by the elimination of small in-frame mutations and the opportunity for fast and efficient PCR validation of edited clones prior to sequencing. Furthermore, the frequency of homozygous mutations appeared to be comparable for both pathways.

The method described in this study is an efficient way to generate controlled gene knockouts by HDR in mouse ESC cells. All generated knockouts will be used for blastocysts injections to generate knockout mouse models..

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