C57BL/6NCrl mouse models generated by CRISPR/Cas9-mediated genome engineering

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discovery from charles river

1 Introduction

With the development of CRISPR/Cas9 gene-editing tools, generation of novel knock-out and knock-in mouse models can be done relatively fast and controllably compared to conventional methods. As a part of our ongoing R&D efforts involving CRISPR/Cas9 technology under a license from the Broad Institute, we set out to create two new C57BL/6NCrl knock-out mouse lines via genetic editing of embryonic stem cells. Repair of double-strand breaks (DSBs) by either non-homologous end-joining (NHEJ) or homology-directed repair (HDR) was targeted (**2**).

First, several mouse embryonic stem cell (ESC) lines were generated from C57BL/6NCrl mice. *In vitro* transfection of one of these ESC lines was optimized for CRISPR/Cas9-mediated gene-editing (**3-4**). Charles Rivers' two vector CRISPR/Cas9 system allows for high-throughput cloning of sgRNA in one vector and Cas9 expression from the other, also making it suitable for producing an arrayed sgRNA library.

For Proof-of-Concept purposes, mouse Leptin and Leptin Receptor genes were selected for gene-editing in this study, given their easy accessibility to measure the phenotype uniquely configured on a C57BL/6NCrl background. Results are presented and discussed below (**5-8**).

2 Double-strand break repair pathways

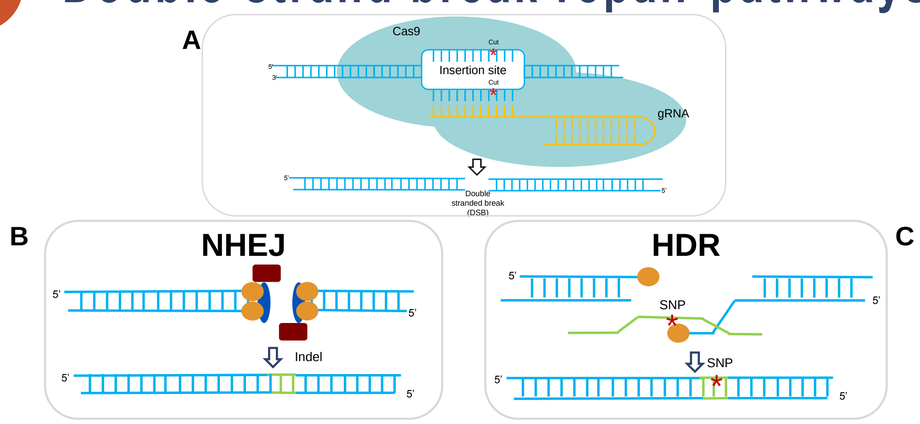


Figure 1. (A) Schematic drawing of CRISPR/Cas9-induced DSB and its sequential repair pathways. (B) In NHEJ, double-stranded breaks (DSB) are repaired through a 'paired end complex' facilitating ligation of DNA breaks. (C) The HDR pathway requires a homologous DNA strand which is used as a template for high fidelity DSB repair.

3 Method

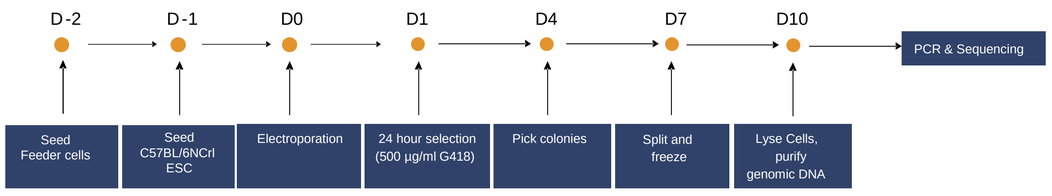


Figure 2. General experimental setup of CRISPR/Cas9-mediated gene editing in mouse C57BL/6NCrl ESCs. Mouse C57BL/6NCrl ESC were thawed and seeded on a feeder-layer 24 hrs prior to electroporation on D-1. Trypsinized ESCs were electroporated using up to 30 µg DNA per 1E6 ESCs on D0. A 24 hrs selection 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

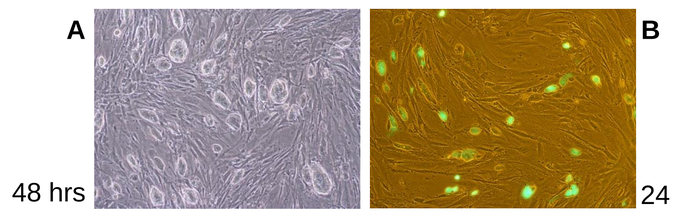


Figure 3. Transfection efficiency of C57BL/6NCrl ESC determined with a GFP plasmid. (A) Non-electroporated mouse ESC on feeder cells 48 hrs after seeding. (B) Mouse ESC electroporated with 10 µg GFP vector on top of feeder cells 24 hrs after seeding. Estimated transfection efficiency of 80% based on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

5 Leptin: Non-homologous end-joining

A

Genotype	No. of clones	%
Total Indels	179	73
Homozygous Indels	25	14
Homozygous frameshift mutation	6	24

B

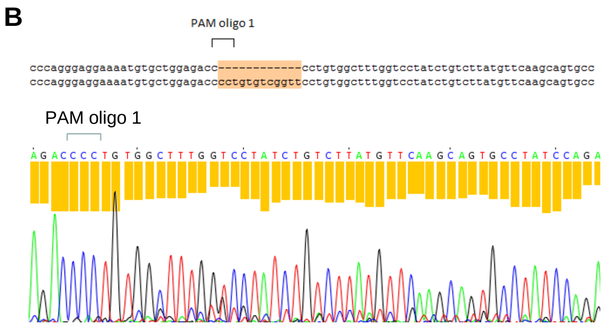
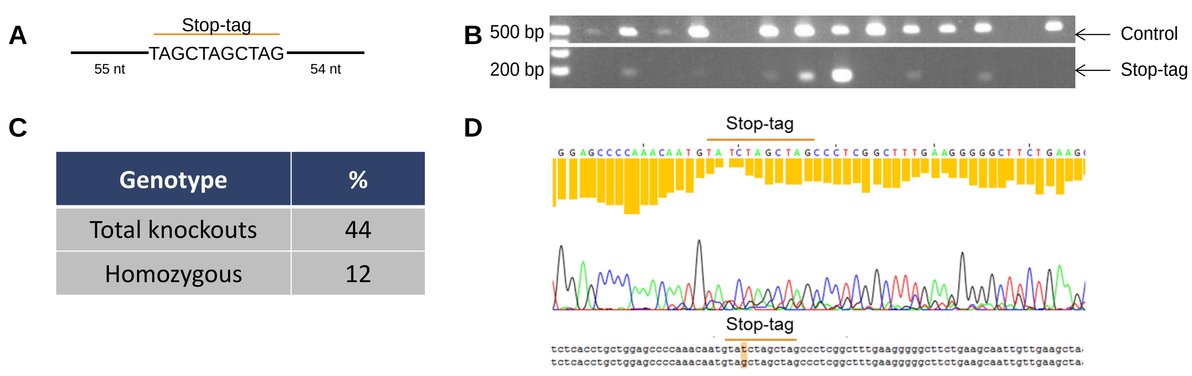


Figure 4. Genetic Leptin knockout in C57BL/6NCrl ESCs via the NHEJ pathway. (A) A gene editing efficiency of 73% was achieved utilizing the optimized Charles River two-vector system. The frequency of homozygosity for confirmed indels was found to be 14%. (B) Sequencing identified 3 out of 4 indels as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

6 Leptin Receptor: Homology-driven repair



C

Genotype	%
Total knockouts	44
Homozygous	12

Figure 5. Genetic Leptin Receptor knockout in C57BL/6NCrl ESCs via the HDR pathway. (A) A 120nt single-stranded repair template with a Stop-tag flanked by homology 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 were homozygous for the introduced mutation.

7 Blastocyst injection and germ-line transmission

Mutation	No. of blastocysts injected	% pups	No. of pups with chimerism				Total no. of F0 pups	% F0 pups with >75% chimerism	% Germline F1 pups
			0-25%	26-50%	51-75%	76-100%			
Leptin KO	350	16%	4	1	11	41	57	72%	30%
LeptinR KO	240	18%	2	0	15	28	44	64%	*ongoing*

Figure 6. Mutated clonal ESCs were injected into NMRI blastocysts. Over 75% chimerism was observed in 72% of Leptin-targeted offspring and in 64% of the pups with the Leptin Receptor mutation (F0). These animals were backcrossed with C57BL/6NCrl, upon which successful germ-line transmission was observed in the Leptin KO F1 generation, validating this novel line.

8 Conclusion

In this study we demonstrated genome engineering of novel C57BL/6NCrl embryonic stem cells using DSB repair by non-homologous end-joining (NHEJ) or homology-directed repair (HDR). Generation of knock-out mice was achieved by both methods.

The phenotype of these newly generated Leptin and Leptin Receptor knock-out lines is currently under study. Moreover, this Proof-of-Concept study paves the road for additional development of novel C57BL/6NCrl mouse models by *in vitro* CRISPR/Cas9-mediated gene editing in ES cells.

Please note that the Leptin and Leptin Receptor models presented here are only being developed for research purposes.
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