C57BL/6NCrl mouse models generated by CRISPR/Cas9-mediated gene-editing

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1 Introduction

With the development of CRISPR/Cas9 gene-editing tools, generation of novel knock-out and knock-in mouse models can be done more quickly and with greater precision compared to conventional methods. We set out to create new C57BL/6NCrl mouse lines via CRISPR/Cas9 technology to compare the efficiency of different CRISPR and genetic transfer methods. We used non-homologous end joining (NHEJ) or homology directed repair (HDR) CRISPR/Cas9 techniques combined with ES cell engineering, direct embryo cytoplasmic injection and electroporation genetic transfer. All putative founder mice were analyzed for screening CRISPR/Cas9 generated mutations by our sensitive platform that detects distinct heteroduplexes generated by each unique variant. These models were created as a part of our ongoing R&D efforts involving CRISPR/Cas9 technology licensed from the Broad Institute.

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. 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 on a C57BL/6NCrl background. Results are presented and discussed below.

2 Double-strand break repair pathways

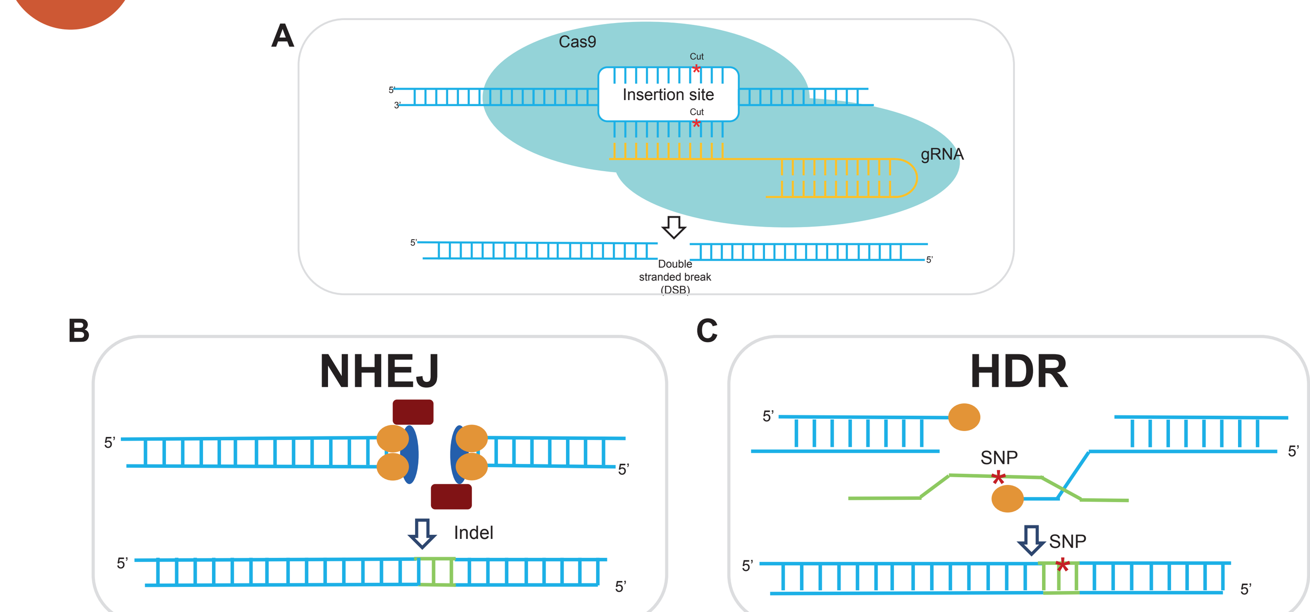

Figure 1. (A) Schematic drawing of CRISPR/Cas9-induced double strand breaks (DSB) and sequential repair pathways: (B) In NHEJ, double-stranded breaks (DSB) are repaired through a 'paired end complex facilitating ligation of DNA breaks. (C) The HDR pathway requires a homologous DNA strand which is used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

3 Leptin: Non-Homologous End-Joining

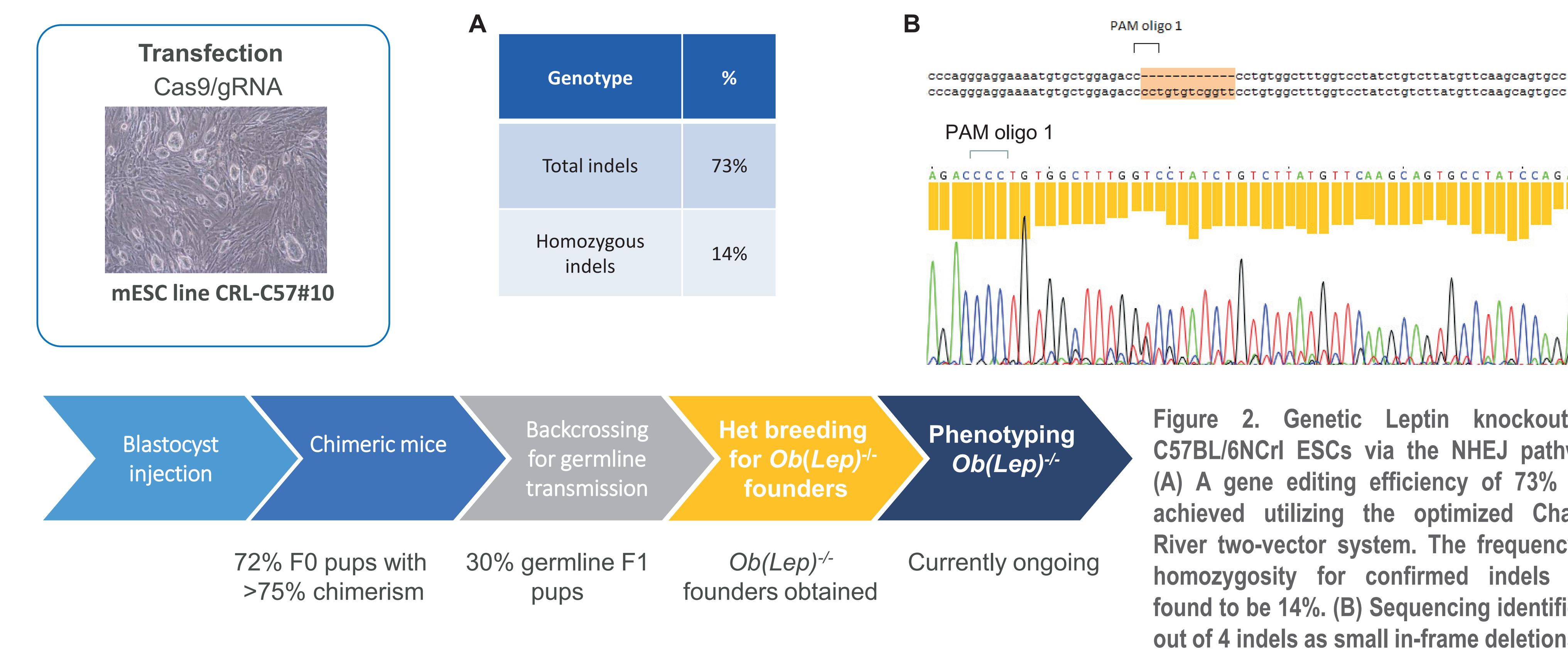

Figure 2. Genetic Leptin knockout in C57BL/6NCrl ESCs via the NHEJ pathway. (A) A gene editing efficiency of 73% was achieved utilizing the optimized Charles River two-vector system. The frequency of homozygosity for confirmed indels was found to be 14%. (B) Sequencing identified 3 out of 4 indels as small in-frame deletions.

4 Leptin Receptor: Homology-Directed Repair

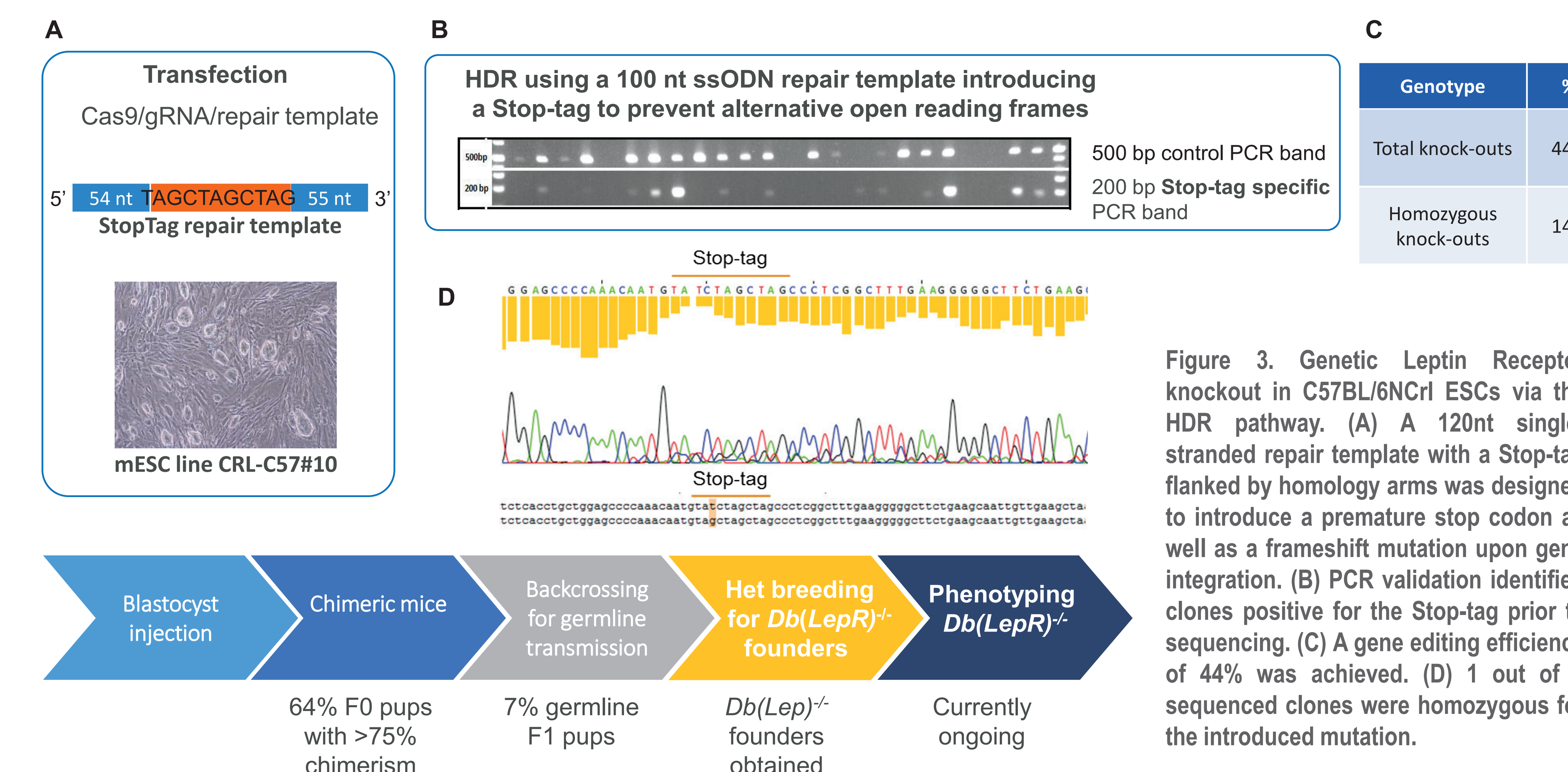

Figure 3. Genetic Leptin Receptor knockout in C57BL/6NCrl ESCs via the HDR pathway. (A) A 120nt single-stranded repair template with a Stop-tag flanked by homology arms was designed to introduce a premature stop codon as well as a frameshift mutation upon gene integration. (B) PCR validation identified 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.

5 Mosaicism and complex genotyping

CRISPR technology is very efficient at generating mutations at the target site. Mosaicism is common, where a founding animal has unique variants on each allele of the target gene. All putative founder mice were analyzed by an efficient method for screening CRISPR/Cas9 generated mutations. Our sensitive platform detects distinct heteroduplexes generated by each unique variant. Each variant generates a distinct heteroduplex banding patterns. The example in Figure 4 shows 7 unique variants, which were all confirmed by sequencing.

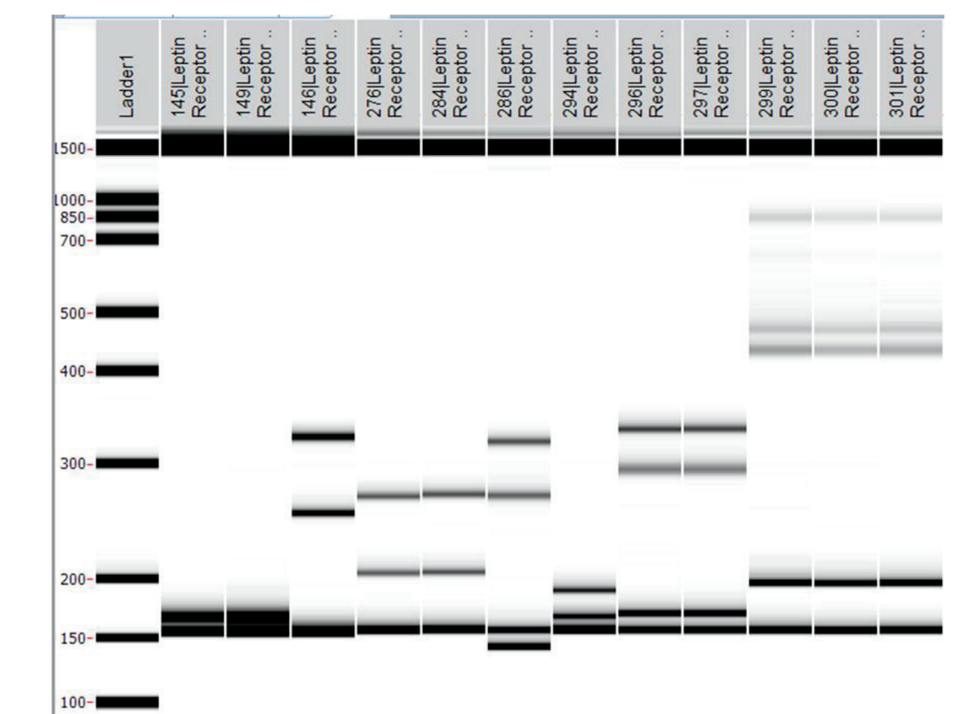

Figure 4. Sensitive assay for genetic analysis enables screens via heteroduplex formation. All putative founders and their F1 progeny were screened with this assay. Each unique variant of the target gene creates a distinct heteroduplex pattern in the presence of the wild type gene.

6 Conclusions

Method	# Embryos	# Embryos Transferred	# Pups	# with Deletions	% Delivery
Cytoplasmic Injection Leptin (NHEJ)	150	120	33	28	85%
Cytoplasmic Injection Leptin Receptor (HDR)	190	160	50	36	73%
Electroporation Leptin (NHEJ)	100	98	41	36	88%

Figure 5. Alternate Techniques. Utilizing the same gRNA and donor oligo that were successful in generating Leptin and Leptin Receptor knock-outs via ES cell targeting, we performed direct embryo injections and electroporation. Both alternate techniques were successful in generating knockout animals.

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. Further, we demonstrated that constructs successful in engineering ES cells could also be used in direct embryo injections and electroporation.

Generation of C57BL/6NCrl knock-out mice was achieved by all methods, ES cell editing, direct embryo injection and electroporation. The phenotype of these new knock-out lines is currently under study. Moreover, this proof-of-principle study paves the road for development of additional novel C57BL6/NCrl mouse models by CRISPR/Cas9-mediated gene editing.