SAGE LABS
Sigma Advanced Genetic Engineering

Zinc Finger Nuclease-mediated Genome Modification in Rodents

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Abstract

The creation of the first knockout rats via microinjection of zinc finger nucleases into single cell embryos is set to revolutionize the rat research world. As the first tool to manipulate the rat genome in a targeted fashion, the ZFN technology is a fast and reliable way to introduce desired mutations to the gene of interest.

We are in the process of generating suites of rat models in areas such as neurodegenerative diseases (DISC1, ApoE, BDNF, Park2, Park7, SNCA, LRRK2, PINK1), oncology (p53, PTEN), immunology (Rag1, Rag2, DNAPK, FOXN1), and toxicology (Mdr1a, Mrp1, Mrp2, BCRP). In general, 10-30% live births from microinjected embryos were founders with various lengths of deletions, and 100% modified alleles tested transmitted to germline.

In the mean time, ZFN technology also benefits the mouse genetics with its ability to allow modifications without the use of ES cells. We created the first ZFN-mediated knockout mice in both FVB/N and C57BL/6 background, where we observed deletions from 3 to over 1000 bp. All mutant alleles we bred went germline.

We also successfully achieved targeted integration using ZFNs, which opens doors to site-specific introduction of point mutations and creation of conditional knockouts.

Introduction

Zinc Finger Nucleases (ZFNs) are fusions of zinc finger proteins (ZFPs) and a nuclease domain, such as the DNA cleavage domain of a type II endonuclease, *FokI*. The ZFPs provide binding specificity, and the nuclease domain dimerizes and cleaves the DNA to generate double-strand breaks (DSBs), which are detrimental to the cell. The cell uses two main pathways to repair DSBs: high-fidelity homologous recombination and error-prone non-homologous end joining (NHEJ) (Figure 1). ZFNs have been applied in various species for knockouts, including zebrafish, rats, flies, and worms. ZFNs have also been shown to increase HR rate in cultured cells and flies over several order of magnitude. Here we report the generation of various mouse and rat models as well as explore the feasibility of targeted integration in embryos.

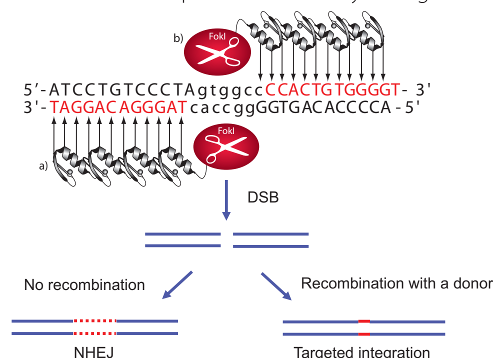


Figure 1. ZFN targeting mechanism. ZFNs bind to the target site, and *FokI* endonuclease domain dimerizes and makes a double strand break (DSB) between the binding sites. DSBs are repaired by either error-prone non-homologous end joining (NHEJ) pathway or high-fidelity homologous recombination. NHEJ introduces deletions or insertions, which change the spacing between the binding sites so that ZFNs might still bind but dimerization or cleavage cannot occur. In the presence of a donor DNA carrying homology flanking the target site, homologous recombination can use the donor as template to repair a DSB, achieving targeted integration.

Methods

Preparation of ZFN mRNA for injection: ZFN-encoding constructs, obtained from the Sigma CompoZr product line, were linearized and *in vitro* transcribed into 5'-capped and 3'-polyadenylated messenger RNA, which was validated for activity by transfection into cultured cells and detection of NHEJ.

Injection: mRNA was injected at 2 ng/ul (mice) or 5 ng/ul (rats) with or without 1 ng/ul donor DNA.

Single-strand DNA endonuclease assay: 300-600 bp regions flanking the target sites were PCR amplified, and the PCR reactions were put through the following program: 95°C, 10 min, 95°C to 85°C, at −2°C/s, 85°C to 25°C at −0.1°C/s, 4°C forever and digested with Nuclease S (Transgenomic, Omaha, NE) and resolved on 10% polyacrylamide TBE gel.

Southern: Fetal tissue or ear notches were used to prepare genomic DNA. Digested genomic DNA was resolved on 0.8% agarose gel, transferred to nylon membrane and hybridized to DIG-labeled probes (Roche, Indianapolis, IN).

Results

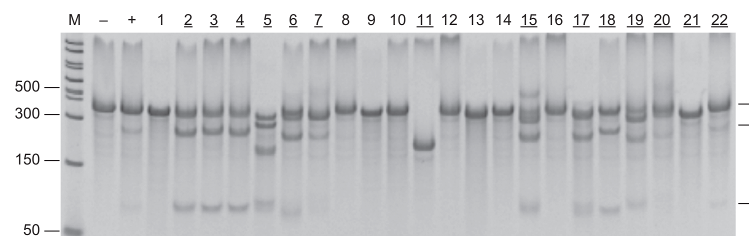


Figure 2. Identification of genetically engineered *Mdr1a* founders (FVB/N) using single-strand DNA endonuclease assay, which detects mismatches at the target site resulted from NHEJ-mediated insertions or deletions. Uncut and cleaved bands are marked with respective sizes in base pairs. Cleaved bands indicate a mutation is present at the target site. M, PCR marker. 1-44, 44 pups born from injected eggs. The numbers of founders are underlined.

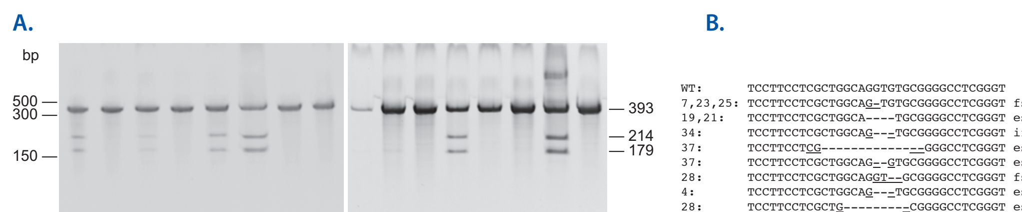


Figure 3. Identification of *Jag1* founders (C57BL/6). **A.** *Jag1* founders identified using the mutation detection assay. M, PCR marker. 1-38, pups born from two injection sessions. The numbers of founders are underlined. The sizes in base pairs of uncut and cut bands are labeled on the right. **B.** Genotype of the *Jag1* founders. Target site sequences of wild-type and founders are aligned. ZFN binding sites are in bold. A dash represents a deleted nucleotide. 1-4 bp of microhomology that was likely used by NHEJ is underlined. Frameshift (fs), exon skipping (es), and in-frame amino acid loss resulted from respective deletions were labeled to the right of each sequence.

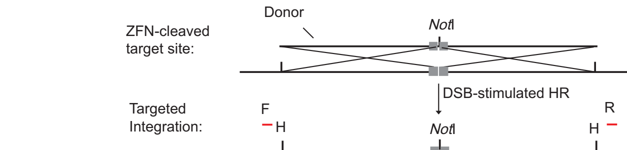
Founder	Deletion Size	Sequence
WT:		tctgctcttgttctccagtcttcctccagatgatattctggtaaggagccggcaagagggg
6	12	tctgctcttgttctccagtcttcct------------ctggtaaggagccggcaagagggg
18	8	tctgctcttgttctccagtcttcctcc--------ttctggtaaggagccggcaagagggg
33	3	tctgctcttgttctccagtcttcctccagat---attctggtaaggagccggcaagagggg
34	5	tctgctcttgttctccagtcttcctccagat-----tctggtaaggagccggcaagagggg
36	9	tctgctcttgttctccagtcttcctcc---------tctggtaaggagccggcaagagggg
	15	tctgctcttgttctccagtcttc---------------tggtaaggagccggcaagagggg
37	3	tctgctcttgttctccagtcttcctccagat---attctggtaaggagccggcaagagggg
38	14	tctgctcttgttctccagtcttcctccaga--------------ggagccggcaagagggg
40	11	tctgctcttgttctccagtcttcctccag-----------gtaaggagccggcaagagggg
53	5	tctgctcttgttctccagtcttcctccagat-----tctggtaaggagccggcaagagggg
74	8	tctgctcttgttctccagtcttcctcct-------tcctggtaaggagccggcaagagggg

Figure 4. Genotype of p53 knockout founder rats. ZFN binding sites are in red with spacer in bold. Each green dash represents a bp deletion.

Target	Efficiency (%)	Largest Deletion (bp)	Status
Mdr1a	14	274	F2
p53	31	24	F2
Mrp1	12	44	F1
Mrp2	1	726	F1
ApoE	20	44	F1
Rag1	20	811	F1
DISC1	10	20	F1
BCRP	56	1066	F1
PXR	4	1	F0
BDNF	24	14	F1
LRRK2	56	18	F1
Park2	18	167	F1
Park7	35	10	F0
Pink1	43	55	F1
SNCA	63	64	F0

Table 1. Models under construction. Targets in black are injected into Sprague Dawley background, and those in blue are in Long Evans Hooded rats, a suite of Parkinson's models funded by Michael J. Fox Foundation.

A.



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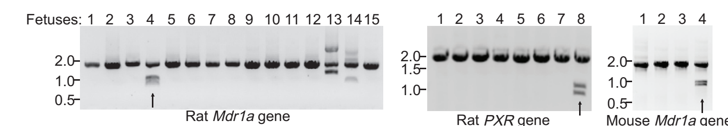

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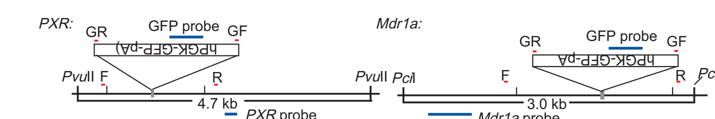

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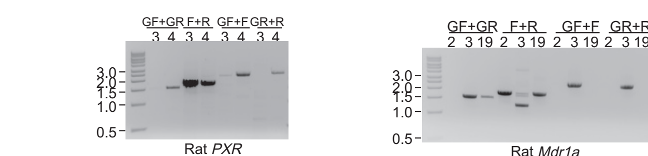

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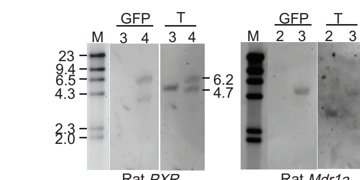

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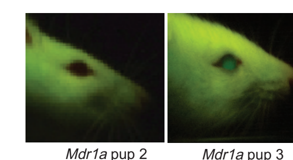

Figure 5. ZFN-mediated targeted integration in FVB/N and Sprague Dawley embryos. **A.** Schematic of donor and target site. Donors contain a *NotI* site inserted between the ZFN binding sequences (grey squares) with two flanking 800 bp homology. F and R, forward and reverse primers (red bar) outside of the homology. **B.** One pup (arrow) with two *NotI* insertion was identified in each target by using PCR followed by *NotI* digestion. **C.** Schematic of target site and GFP integration at *Mdr1a* and *PXR* loci. GF & GR, forward and reverse primers in GFP cassette. *PvuII* & *PciI* are restriction enzymes used in Southern analysis (1E), neither cuts the 1.5 kb insert. Probes used in Southern (blue bars) are marked at respective positions. **D.** PCR analysis of GFP integration in selected *Mdr1a* and *PXR* rat pups. Pup IDs are labeled under the primers used. **E.** Southern analysis of selected pups for GFP integration. GFP, GFP probe; T, target probe. **F.** GFP expression observed under UV light in *Mdr1a* pup #3.

Summary

Using ZFNs, gene disruption can be achieved readily in different backgrounds in both mice and rats, and homozygous F2s can be obtained in a matter of months.

Targeted integration is efficient in the rodent embryos in the absence of selection. Even though much needs to be optimized with ZFN-mediated homologous recombination in embryos, our initial success laid the foundation for various precise modifications of the genome and conditional knockout/knockin. We also hope that it will encourage researchers to adopt ZFN technology in other species.

Acknowledgement

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