Off- and on-target effects of genome editing in mouse embryos

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Abstract. Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas-based genome editing technology has enabled manipulation of the embryonic genome. Unbiased whole genome sequencing comparing parents to progeny has revealed that the rate of Cas9-induced mutagenesis in mouse embryos is indistinguishable from the background rate of de novo mutation. However, establishing the best practice to confirm on-target alleles of interest remains a challenge. We believe that improvement in editing strategies and screening methods for founder mice will contribute to the generation of quality-controlled animals, thereby ensuring reproducibility of results in animal studies and advancing the 3Rs (replacement, reduction, and refinement).

Key words: Cas9, CRISPR, Genome editing, Genotyping, Off-target effect

Off-target effects are minimal and manageable in mouse embryos

Early reports found off-target mutations at loci containing mismatches of less than 4 nucleotides in mice treated with a high concentration of Cas9 or an intentionally low-specificity guide RNA (gRNA) [12, 13]. In response, various web-based and stand-alone tools have been developed to prevent off-target effects [14–20]—especially important for targeting gene families or genes with pseudogenes. It is beneficial to use two or more tools to confirm gRNA specificity. For example, we found that a gRNA for the Xlr3a gene on mouse chromosome X—which also carries its family genes Xlr3b and Xlr3c—had a high specificity score according to one gRNA design tool, but had another sequence on-target to Xlr3b according to a second tool (Table 1). Cas9 introduced mutations to both on-target sequences that also transmitted to the germline. In these cases, alignment tools help to search for regions with similarity [21]. To prevent unconstrained Cas9 endonuclease activity, one should 1) select gRNAs that are as specific as possible (e.g., no off-target sites containing three or fewer mismatches) and 2) use Cas9 mRNA or protein instead of plasmids or strains that constitutively express Cas9 to limit exposure time to ribonucleoproteins [22]. To verify that off-target mutations can be transmitted to the germline from founder mice, we injected or electroporated C57BL/6N mouse zygotes with wild-type Streptococcus pyogenes Cas9 or D10A nickase as mRNA or protein, then performed Sanger sequencing of the PCR products. Only 1 locus in the N1 generation out of 906 loci, 112 gRNAs, and 59 genes was possibly mutated with Cas9 (Table 2), indicating off-target mutagenesis by Cas9 occurs at a minimal frequency in mouse embryos that falls below the detection threshold of biased methods when using carefully selected sequence-specific gRNAs.

Genome-wide unbiased procedures—such as GUIDE-seq, Digene-seq, and CIRCLE-seq—have also been developed to detect off-target effects [23–25]. Modified GUIDE-
seq and WGS have been shown to provide concordant results in genetically engineered rats and mice [5]. Pedigree-matched, unbiased WGS results from multiple labs have revealed that the rate of Cas9-induced mutagenesis in mouse embryos is indistinguishable from the background rate of de novo mutation [3–5, 26, 27]—over 100 single nucleotide variants and 3 to 4 indels every generation in separate colonies or over time [28]. The effect of colony variation is presumed to be larger than that of Cas9 off-target mutagenesis, as rare and unlinked off-target mutations can be easily segregated away in rodents through breeding.

In addition to D10A nickase and paired gRNAs [13, 27, 29], a number of strategies have been reported to enhance specificity, including using engineered high-fidelity Cas9 proteins (e.g., SpCas9-HF1, eSpCas9 (1.1), HypaCas9, Sniper-Cas9, and HiFi Cas9) and/or truncated gRNAs with 17–18 nucleotides [1, 30–34]. However, it should be noted that these options may result in low on-target efficiency [5], which is problematic in high-throughput production of gene-modified animals such as by the International Mouse Phenotyping Consortium (http://www.mousephenotype.org).

How can we screen out unexpected “on-target effects”? Compared with strategies to avoid off-target effects in embryonic genome editing, best practices to confirm results of on-target mutagenesis remain relatively unexplored. Nickase-mediated deletions in mouse zygotes extended up to 1 kb (Fig. 1), in agreement with reports that analyzed deletion or knock-in alleles in mice [35–37]. Droplet digital PCR or qPCR can be used as an alternative to WGS or standard PCR for copy counting of donor template DNA in founder mouse offspring, as template oligonucleotides can be randomly inserted into the genome [36, 38–40]. A report using several cell lines, including mouse ES cells, showed that Cas9 activity at on-target sites resulted in large deletions up to several kilobases long or complex lesions with segments from another chromosome in over 10% of the recovered alleles [11]. Long-range PCR with the PacBio system and/or long-read nanopore sequencing may reveal further consequences in the on-target region [11, 41]. However, we should consider the limitations of this study, which used only a limited number of targets and cells—either constitutively expressing Cas9, transfected with Cas9-expressing plasmid, or immortalized. Results can greatly differ between species, cell types, and methods used to introduce gRNAs and Cas9.

Future perspectives Continuous improvement to genome editing strategies and founder animal screening methods undoubtedly contribute to avoiding
extensive on-target DNA repair-associated damages and generating mutant animals with high-quality on-target alleles. Cautious assessment of on-target alleles will be imperative for studies utilizing animal models with in vivo delivery of CRISPR components, as selection or segregation of resultant alleles cannot be performed. We believe quality-controlled animals will certify reproducibility of animal studies and advance the 3Rs of ethical research practice (replacement, reduction, and refinement).

A cross-species perspective is truly important not only for those who engage in genome editing but also for those who study reproductive and developmental biology. A group triggered a scientific controversy by reporting that the dominant mechanism of the double-strand DNA break repair pathway at on-target sites in human embryos can be interhomolog recombination [42–45], the result of which has been found in mouse zygotes and ES cells [11, 46] (https://doi.org/10.1101/263699, https://doi.org/10.1101/362558). Further research into CRISPR-Cas-mediated genome editing would certainly contribute to the field.

**Materials and methods**

**Mouse strains**

C57BL/6Njcl and heterozygous CrIj:CD1-Foxn1<sup>tm</sup> mice were purchased from CLEA Japan (Tokyo, Japan) and Charles River Laboratories Japan (Yokohama, Japan), respectively, and maintained at RIKEN BioResource Research Center (BRC). All studies were reviewed and approved by the Institutional Animal Care and Use Committee of the RIKEN Tsukuba Branch.

**Injection and electroporation mix preparation**

Guide RNAs (gRNAs) were selected using either the CRISPR Design (http://crispr.mit.edu/), Benchling (https://benchling.com/), or CRISPOR (http://crispor.tefor.net/) websites [14, 20]. DNA templates for single-guide RNAs (sgRNAs) were produced using either DR274 (Addgene #42250, a gift from Keith Joung) [47] or overlapping oligonucleotides in a high fidelity PCR [48]. Cas9 and D10A nickase mRNA were generated using linearized T7-NLS hCas9-pA (RIKEN BRC #RDB13130, a gift from Tomoji Mashimo) [49] and pST1374-N-NLS-flag-linker-Cas9-D10A (Addgene #51130, a gift from Xingxu Huang) [50], respectively. Cas9 protein was purchased from Thermo Fisher (Waltham, MA, USA) or Integrated DNA Technologies (Skokie, IL, USA). Final injection mixes consisted of Cas9 mRNA (from 10 to 100 ng/μl) and sgRNA (from 5 to 50 ng/μl each) to a volume of 15 μl in DNase- and RNase-free water. Final electroporation mixes consisted of Cas9 mRNA (from 300 to 400 ng/μl) or Cas9 protein (from 100 to 200 ng/μl) and sgRNA (from 100 to 300 ng/μl each) to a volume of 50 μl in Opti-MEM I (Thermo Fisher).

**Microinjection with CRISPR/Cas9 reagents**

C57BL/6Njcl female mice, 25 to 31 days old, were injected with 7.5 IU/mouse of pregnant mare serum gonadotropin, followed 48 h later with 7.5 IU/mouse of human chorionic gonadotropin. The females were then mated to C57BL/6Njcl males, and fertilized oocytes were collected at 0.5 dpc. The sgRNA/Cas9 mixture was microinjected into the cytoplasm of pronuclear stage zygotes using piezoelectricity (Prime Tech; Ibaraki, Japan) [51]. The next day, approximately 20–28 zygotes were transferred into each pseudopregnant CrIj:CD1-Foxn1<sup>tm</sup> female.

**Electroporation with CRISPR/Cas9 reagents**

We performed in vitro fertilization according to a standard protocol [52]. In brief, collected oocytes and sperm suspensions were preincubated in human tubal fluid (HTF) medium at 37°C in humidified air containing 5% CO₂. At the time of insemination, preincubated sperm was transferred into droplets containing oocytes at a concentration of 100–300 spermatozoa/μl. After 3 to 4 h, fertilized oocytes were transferred into CZB medium containing 5.6 mM glucose, 0.1 mg/ml polyvinyl alcohol, and 3.0 mg/mL bovine serum albumin. The sgRNA/Cas9 mixture was electroporated into zygotes using a NEPA21 electroporator (Nepa Gene; Chiba, Japan) [53, 54].

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**Fig. 1.** Sequencing of 16 deletion bands following PCR from 14 founder mice for Tfr2 gene knockout. "0" marks the location of each cut (blue triangles) by Cas9 D10A nickase and a pair of gRNAs with 16-bp offset in exon 5. Blue and orange bars represent deletions and insertions, respectively.
Genotyping and analysis of off-target Cas9 activity

Genomic DNA was isolated from tail clips of mice using 10% Chelex 100 chelating resin (Bio-Rad; Hercules, CA, USA). On- and off-target regions were amplified by PCR (AmpliTaq Gold 360 Master Mix, Thermo Fisher) using primers approximately ± 300 bases offset. PCR products were treated with ExoSAP-IT (Thermo Fisher) and directly processed by Sanger sequencing (3500xL Genetic Analyzer, Thermo Fisher) using a PCR primer. Potential off-target sites (8-14 sites per gRNA) were chosen using the CRISPR Design, Benchling, Wellcome Trust Sanger Institute Genome Editing (http://www.sanger.ac.uk/hgt/wge/), and COSMID (https://crispr.bme.gatech.edu/) websites [17, 19].

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