Opinions and Hypotheses

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

Shinya AYABE[1), Kenichi NAKASHIMA[2) and Atsushi YOSHIKI[1)

[1)*Experimental Animal Division, RIKEN BioResource Research Center, Ibaraki 305-0074, Japan*
[2)*Gene Engineering Division, RIKEN BioResource Research Center, Ibaraki 305-0074, Japan*

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

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas-based genome editing technology has been widely used to manipulate the embryonic genomes of laboratory animals—including mice and rats, as well as livestock, fish, and humans. As with zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN), which have been used previously as genome editing tools, enormous efforts have been made to reduce off-target effects of CRISPR-Cas systems. Concerns for these potential off-target effects that induce DNA cleavage and produce mutations at sites in the genome other than at the desired target site [1, 2], have been discussed in the field of mouse model creation over the last few years. Although a study published in *Nature Methods* identified widespread off-target mutagenesis in Cas9-treated mouse embryos by whole-genome sequencing (WGS), replication studies did not support the original conclusion [3–5]. Additionally, the scientific community pointed out that the study failed to consider parent-offspring relationship [6–10]; therefore, the paper was retracted (https://doi.

org/10.1038/nmeth0518-394a). Recently, one group used mouse embryonic stem (ES) cells to show that on-target large deletions and rearrangements are other unpredictable risks [11], encouraging other researchers to also consider CRISPR-Cas-mediated on-target extensive DNA repair-associated damages —"on-target effects."

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, Digenome-seq, and CIRCLE-seq— have also been developed to detect off-target effects [23–25]. Modified GUIDE-

Table 1. Comparison of gRNA specificity for *Xlr3a* knockout mice using two web-based tools

	Benchling https://benchling.com/					CRISPOR http://crispor.tefor.net/				
	No. of mismatches					No. of mismatches				
	0	1	2	3	4	0	1	2	3	4
gRNA on-target site + PAM	No. of NGG genomic sites					No. of NGG genomic sites				
ATTCAGGATGTAGCCGGGCG TGG	Default setting					2	4	7	27	236
	1	0	0	0	> 50 [a]	*Xlr3a*				
	Xlr3a					*Xlr3b*				
	Advanced setting [b]									
	2	4	7	> 19 [a]	> 18 [a]					
	Xlr3a									
	Xlr3b									

[a] Benchling pares down the off-target list to 50 top possible off-target sites. [b] Benchling and crispr.mit.edu default to skipping repeats/low complexity regions when doing off-target search. Note that crispr.mit.edu will shut down in November 2018.

Table 2. Number of off-target mutations detected in N1 generation of genetically modified mice

Cas9 type	Method	No. of genes	No. of gRNAs	N1 animals screened	Off-target sites screened	Total sites screened	Off-target mutations
Cas9 mRNA	Cytoplasmic injection	25	47	89	384	1372	**1** [a]
	Electroporation	9	18	47	144	752	0
Cas9 protein	Electroporation	6	9	22	74	276	0
Cas9 nickase mRNA	Cytoplasmic injection	17	34	57	272	912	0
	Electroporation	2	4	9	32	144	0

[a] Indel mutation was detected in an off-target site with three mismatches (TAGTACAGATGTAATAGATT AGG, underline indicates mismatches) in 1 out of 6 N1 mice that were born from the *Ube2j2*-knockout founder mouse. The founder also possessed the mutation in mosaic. On-target sequence was GAGTACAGGTGTAATAGATG GGG.

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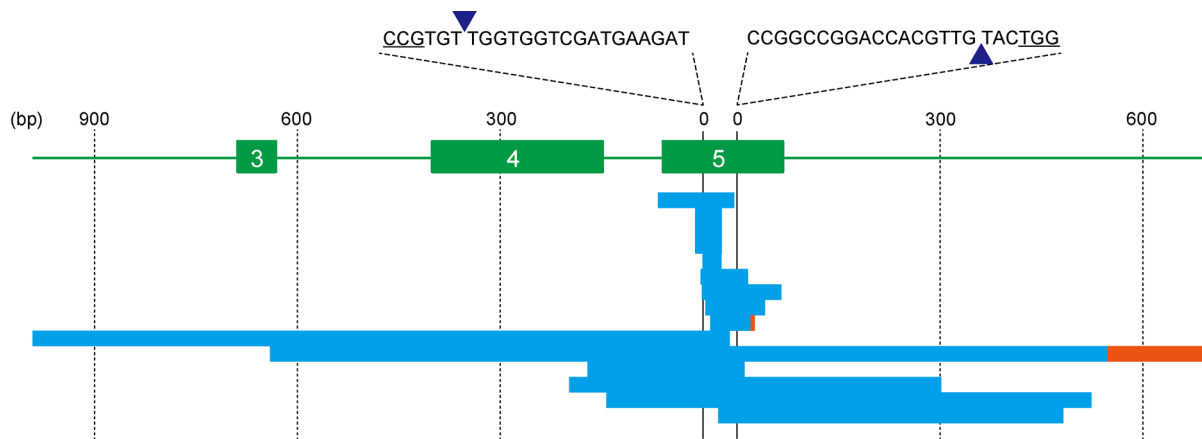
Fig. 1. Sequencing of 16 deletion bands following PCR from 14 founder mice for *Tfr2* gene knockout. "0" marks the location of each cut (blue triangles) by Cas9 D10A nickase and a pair of gRNAs with 16-bp offset in exon 5. Blue and orange bars represent deletions and insertions, respectively.

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 Crlj:CD1-*Foxn1nu* 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://crispr.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 Crlj:CD1-*Foxn1nu* 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% CO_2. 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].

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/htgt/wge/), and COSMID (https://crispr.bme.gatech.edu/) websites [17, 19].

References

1. Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol* 2014; 32: 279–284. [Medline] [CrossRef]

2. Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna JA, Liu DR. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat Biotechnol* 2013; 31: 839–843. [Medline] [CrossRef]

3. Willi M, Smith HE, Wang C, Liu C, Hennighausen L. Mutation frequency is not increased in CRISPR-Cas9-edited mice. *Nat Methods* 2018; 15: 756–758. [Medline] [CrossRef]

4. Iyer V, Boroviak K, Thomas M, Doe B, Riva L, Ryder E, Adams DJ. No unexpected CRISPR-Cas9 off-target activity revealed by trio sequencing of gene-edited mice. *PLoS Genet* 2018; 14: e1007503. [Medline] [CrossRef]

5. Anderson KR, Haeussler M, Watanabe C, Janakiraman V, Lund J, Modrusan Z, Stinson J, Bei Q, Buechler A, Yu C, Thamminana SR, Tam L, Sowick MA, Alcantar T, O'Neil N, Li J, Ta L, Lima L, Roose-Girma M, Rairdan X, Durinck S, Warming S. CRISPR off-target analysis in genetically engineered rats and mice. *Nat Methods* 2018; 15: 512–514. [Medline] [CrossRef]

6. Lareau CA, Clement K, Hsu JY, Pattanayak V, Joung JK, Aryee MJ, Pinello L. Response to "Unexpected mutations after CRISPR-Cas9 editing in vivo". *Nat Methods* 2018; 15: 238–239. [Medline] [CrossRef]

7. Nutter LMJ, Heaney JD, Lloyd KCK, Murray SA, Seavitt JR, Skarnes WC, Teboul L, Brown SDM, Moore M. Response to "Unexpected mutations after CRISPR-Cas9 editing in vivo". *Nat Methods* 2018; 15: 235–236. [Medline] [CrossRef]

8. Kim ST, Park J, Kim D, Kim K, Bae S, Schlesner M, Kim JS. Response to "Unexpected mutations after CRISPR-Cas9 editing in vivo". *Nat Methods* 2018; 15: 239–240. [Medline] [CrossRef]

9. Wilson CJ, Fennell T, Bothmer A, Maeder ML, Reyon D, Cotta-Ramusino C, Fernandez CA, Marco E, Barrera LA, Jayaram H, Albright CF, Cox GF, Church GM, Myer VE. Response to "Unexpected mutations after CRISPR-Cas9 editing in vivo". *Nat Methods* 2018; 15: 236–237. [Medline] [CrossRef]

10. Lescarbeau RM, Murray B, Barnes TM, Bermingham N. Response to "Unexpected mutations after CRISPR-Cas9 editing in vivo". *Nat Methods* 2018; 15: 237. [Medline] [CrossRef]

11. Kosicki M, Tomberg K, Bradley A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat Biotechnol* 2018; 36: 765–771. [Medline] [CrossRef]

12. Fujii W, Kawasaki K, Sugiura K, Naito K. Efficient generation of large-scale genome-modified mice using gRNA and CAS9 endonuclease. *Nucleic Acids Res* 2013; 41: e187. [Medline] [CrossRef]

13. Iyer V, Shen B, Zhang W, Hodgkins A, Keane T, Huang X, Skarnes WC. Off-target mutations are rare in Cas9-modified mice. *Nat Methods* 2015; 12: 479. [Medline] [CrossRef]

14. Concordet JP, Haeussler M. CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Res* 2018; 46(W1): W242–W245. [Medline] [CrossRef]

15. Bae S, Park J, Kim JS. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics* 2014; 30: 1473–1475. [Medline] [CrossRef]

16. Labun K, Montague TG, Gagnon JA, Thyme SB, Valen E. CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Res* 2016; 44(W1): W272-6. [Medline] [CrossRef]

17. Cradick TJ, Qiu P, Lee CM, Fine EJ, Bao G. COSMID: A web-based tool for identifying and validating CRISPR/Cas off-target sites. *Mol Ther Nucleic Acids* 2014; 3: e214. [Medline] [CrossRef]

18. Naito Y, Hino K, Bono H, Ui-Tei K. CRISPRdirect: software for designing CRISPR/Cas guide RNA with reduced off-target sites. *Bioinformatics* 2015; 31: 1120–1123. [Medline] [CrossRef]

19. Hodgkins A, Farne A, Perera S, Grego T, Parry-Smith DJ, Skarnes WC, Iyer V. WGE: a CRISPR database for genome engineering. *Bioinformatics* 2015; 31: 3078–3080. [Medline] [CrossRef]

20. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* 2013; 31: 827–832. [Medline] [CrossRef]

21. Kent WJ. BLAT—the BLAST-like alignment tool. *Genome Res* 2002; 12: 656–664. [Medline] [CrossRef]

22. Kim S, Kim D, Cho SW, Kim J, Kim JS. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res* 2014; 24: 1012–1019. [Medline] [CrossRef]

23. Tsai SQ, Nguyen NT, Malagon-Lopez J, Topkar VV, Aryee MJ, Joung JK. CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. *Nat Methods* 2017; 14: 607–614. [Medline] [CrossRef]

24. Kim D, Bae S, Park J, Kim E, Kim S, Yu HR, Hwang J, Kim JI, Kim JS. Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods* 2015; 12: 237–243: 1: 243. [Medline] [CrossRef]

25. Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, Aryee MJ, Joung JK. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol* 2015; 33: 187–197. [Medline] [CrossRef]

26. Nakajima K, Kazuno AA, Kelsoe J, Nakanishi M, Takumi T, Kato T. Exome sequencing in the knockin mice generated using the CRISPR/Cas system. *Sci Rep* 2016; 6: 34703. [Medline] [CrossRef]

27. Mianné J, Chessum L, Kumar S, Aguilar C, Codner G, Hutchison M, Parker A, Mallon AM, Wells S, Simon MM, Teboul L, Brown SD, Bowl MR. Correction of the auditory phenotype in C57BL/6N mice via CRISPR/Cas9-mediated homology directed repair. *Genome Med* 2016; 8: 16. [Medline] [CrossRef]

28. Uchimura A, Higuchi M, Minakuchi Y, Ohno M, Toyoda A, Fujiyama A, Miura I, Wakana S, Nishino J, Yagi T. Germline mutation rates and the long-term phenotypic effects of mutation accumulation in wild-type laboratory mice and mutator mice. *Genome Res* 2015; 25: 1125–1134. [Medline] [CrossRef]

29. Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 2013; 154: 1380–1389. [Medline] [CrossRef]

30. Vakulskas CA, Dever DP, Rettig GR, Turk R, Jacobi AM, Collingwood MA, Bode NM, McNeill MS, Yan S, Camarena J, Lee CM, Park SH, Wiebking V, Bak RO, Gomez-Ospina N, Pavel-Dinu M, Sun W, Bao G, Porteus MH, Behlke MA. A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. *Nat Med* 2018; 24: 1216–1224. [Medline] [CrossRef]

31. Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* 2016; 529: 490–495. [Medline] [CrossRef]

32. Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F. Rationally engineered Cas9 nucleases with improved specificity. *Science* 2016; 351: 84–88. [Medline] [CrossRef]

33. Chen JS, Dagdas YS, Kleinstiver BP, Welch MM, Sousa AA, Harrington LB, Sternberg SH, Joung JK, Yildiz A, Doudna JA. Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. *Nature* 2017; 550: 407–410. [Medline] [CrossRef]

34. Lee JK, Jeong E, Lee J, Jung M, Shin E, Kim YH, Lee K, Jung I, Kim D, Kim S, Kim JS. Directed evolution of CRISPR-Cas9 to increase its specificity. *Nat Commun* 2018; 9: 3048. [Medline] [CrossRef]

35. Shin HY, Wang C, Lee HK, Yoo KH, Zeng X, Kuhns T, Yang CM, Mohr T, Liu C, Hennighausen L. CRISPR/Cas9 targeting events cause complex deletions and insertions at 17 sites in the mouse genome. *Nat Commun* 2017; 8: 15464. [Medline] [CrossRef]

36. Codner GF, Mianné J, Caulder A, Loeffler J, Fell R, King R, Allan AJ, Mackenzie M, Pike FJ, McCabe CV, Christou S, Joynson S, Hutchison M, Stewart ME, Kumar S, Simon MM, Agius L, Anstee QM, Volynski KE, Kullmann DM, Wells S, Teboul L. Application of long single-stranded DNA donors in genome editing: generation and validation of mouse mutants.

BMC Biol 2018; **16**: 70. [Medline] [CrossRef]

37. **Parikh BA, Beckman DL, Patel SJ, White JM, Yokoyama WM.** Detailed phenotypic and molecular analyses of genetically modified mice generated by CRISPR-Cas9-mediated editing. *PLoS One* 2015; **10**: e0116484. [Medline] [CrossRef]

38. **Mianné J, Codner GF, Caulder A, Fell R, Hutchison M, King R, Stewart ME, Wells S, Teboul L.** Analysing the outcome of CRISPR-aided genome editing in embryos: Screening, genotyping and quality control. *Methods* 2017; **121−122**: 68–76. [Medline] [CrossRef]

39. **Gertsenstein M, Nutter LMJ.** Engineering point mutant and epitope-tagged alleles in mice using Cas9 RNA-guided nuclease. *Curr Protoc Mouse Biol* 2018; **8**: 28–53. [Medline] [CrossRef]

40. **Lanza DG, Gaspero A, Lorenzo I, Liao L, Zheng P, Wang Y, Deng Y, Cheng C, Zhang C, Seavitt JR, DeMayo FJ, Xu J, Dickinson ME, Beaudet AL, Heaney JD.** Comparative analysis of single-stranded DNA donors to generate conditional null mouse alleles. *BMC Biol* 2018; **16**: 69. [Medline] [CrossRef]

41. **Jain M, Koren S, Miga KH, Quick J, Rand AC, Sasani TA, Tyson JR, Beggs AD, Dilthey AT, Fiddes IT, Malla S, Marriott H, Nieto T, O'Grady J, Olsen HE, Pedersen BS, Rhie A, Richardson H, Quinlan AR, Snutch TP, Tee L, Paten B, Phillippy AM, Simpson JT, Loman NJ, Loose M.** Nanopore sequencing and assembly of a human genome with ultra-long reads. *Nat Biotechnol* 2018; **36**: 338–345. [Medline] [CrossRef]

42. **Ma H, Marti-Gutierrez N, Park SW, Wu J, Lee Y, Suzuki K, Koski A, Ji D, Hayama T, Ahmed R, Darby H, Van Dyken C, Li Y, Kang E, Park AR, Kim D, Kim ST, Gong J, Gu Y, Xu X, Battaglia D, Krieg SA, Lee DM, Wu DH, Wolf DP, Heitner SB, Belmonte JCI, Amato P, Kim JS, Kaul S, Mitalipov S.** Correction of a pathogenic gene mutation in human embryos. *Nature* 2017; **548**: 413–419. [Medline] [CrossRef]

43. **Egli D, Zuccaro MV, Kosicki M, Church GM, Bradley A, Jasin M.** Inter-homologue repair in fertilized human eggs? *Nature* 2018; **560**: E5–E7. [Medline] [CrossRef]

44. **Adikusuma F, Piltz S, Corbett MA, Turvey M, McColl SR, Helbig KJ, Beard MR, Hughes J, Pomerantz RT, Thomas PQ.** Large deletions induced by Cas9 cleavage. *Nature* 2018; **560**: E8–E9. [Medline] [CrossRef]

45. **Ma H, Marti-Gutierrez N, Park SW, Wu J, Hayama T, Darby H, Van Dyken C, Li Y, Koski A, Liang D, Suzuki K, Gu Y, Gong J, Xu X, Ahmed R, Lee Y, Kang E, Ji D, Park AR, Kim D, Kim ST, Heitner SB, Battaglia D, Krieg SA, Lee DM, Wu DH, Wolf DP, Amato P, Kaul S, Belmonte JCI, Kim JS, Mitalipov S.** Ma et al. reply. *Nature* 2018; **560**: E10–E23. [Medline] [CrossRef]

46. **Wu Y, Liang D, Wang Y, Bai M, Tang W, Bao S, Yan Z, Li D, Li J.** Correction of a genetic disease in mouse via use of CRISPR/Cas9. *Cell Stem Cell* 2013; **13**: 659–662. [Medline] [CrossRef]

47. **Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK.** Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 2013; **31**: 227–229. [Medline] [CrossRef]

48. **Bassett AR, Tibbit C, Ponting CP, Liu JL.** Highly efficient targeted mutagenesis of Drosophila with the CRISPR/Cas9 system. *Cell Reports* 2013; **4**: 220–228. [Medline] [CrossRef]

49. **Yoshimi K, Kunihiro Y, Kaneko T, Nagahora H, Voigt B, Mashimo T.** ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes. *Nat Commun* 2016; **7**: 10431. [Medline] [CrossRef]

50. **Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, Wang L, Hodgkins A, Iyer V, Huang X, Skarnes WC.** Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nat Methods* 2014; **11**: 399–402. [Medline] [CrossRef]

51. **Yang H, Wang H, Jaenisch R.** Generating genetically modified mice using CRISPR/Cas-mediated genome engineering. *Nat Protoc* 2014; **9**: 1956–1968. [Medline] [CrossRef]

52. **Hasegawa A, Mochida K, Matoba S, Yonezawa K, Ohta A, Watanabe G, Taya K, Ogura A.** Efficient production of offspring from Japanese wild-derived strains of mice (Mus musculus molossinus) by improved assisted reproductive technologies. *Biol Reprod* 2012; **86**: 167: 1–7. [Medline] [CrossRef]

53. **Sato Y, Tsukaguchi H, Morita H, Higasa K, Tran MTN, Hamada M, Usui T, Morito N, Horita S, Hayashi T, Takagi J, Yamaguchi I, Nguyen HT, Harada M, Inui K, Maruta Y, Inoue Y, Koiwa F, Sato H, Matsuda F, Ayabe S, Mizuno S, Sugiyama F, Takahashi S, Yoshimura A.** A mutation in transcription factor MAFB causes Focal Segmental Glomerulosclerosis with Duane Retraction Syndrome. *Kidney Int* 2018; **94**: 396–407. [Medline] [CrossRef]

54. **Kaneko T, Sakuma T, Yamamoto T, Mashimo T.** Simple knockout by electroporation of engineered endonucleases into intact rat embryos. *Sci Rep* 2014; **4**: 6382. [Medline] [CrossRef]