Genome-Wide Off-Target Analysis in CRISPR-Cas9 Modified Mice and Their Offspring

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ABSTRACT The emergence of the CRISPR-Cas9 system has triggered a technical revolution in mammalian genome editing. Compared to traditional gene-targeting strategies, CRISPR-Cas9 technology offers a more efficient and cost-effective approach for generating genetically modified animal models. However, off-target cleavage in CRISPR-mediated genome editing is a major concern in the analysis of phenotypes as well as the selection of therapeutic targets. Here, we analyzed whole-genome sequencing (WGS) data from two knock-out (KO) mouse strains generated by using the CRISPR-Cas9 system targeting the Mmd1 and Paqr8 loci. A total of nine individuals were sequenced including two parents, four F1 offspring and three uninjected control mice. Using GATK and bcftools software, we identified two off-target events in the founder mice. The two CRISPR-Cas9-induced off-target events were predictable using Cas-OFFinder and were not passed on to the offspring that we investigated. In addition, our results indicated that the number of CRISPR-Cas9-induced mutations was not statistically distinguishable from the background de novo mutations (DNMs).

CRISPR-Cas systems have become the most powerful tools for genome editing and have been used for the modification of genomes in a wide variety of organisms (Hsu et al. 2014; Doudna and Charpentier 2014; Hwang et al. 2013). Type II CRISPR-Cas9 is the most commonly used genome editing system, involving Cas9 nuclease from Streptococcus pyogenes and a 17-20 nt single guide RNA (sgRNA) complementary to a target site (protospacer) adjacent to a 5'-NGG protospacer adjacent motif (PAM) (Jinek et al. 2012; Mulepati et al. 2014; Shah et al. 2013). However, the possibility of inducing off-target mutations has limited the use of CRISPR-Cas9 technology in research and clinical applications (Cho et al. 2014; Fu et al. 2013; Lessard et al. 2017; Scott and Zhang 2017). Many efforts have been made to minimize the off-target effects of CRISPR-Cas systems by designing high-fidelity nucleases such as eSpCas9 (Slaymaker et al. 2016), SpCas9-HF1 (Kleinstiver et al. 2016), HypaCas9 (Chen et al. 2017), HeFspCas9 (Kulcsár et al. 2017), HiFiCas9 (Vakulskas et al. 2018), Sniper Cas9 (Lee et al. 2018) and EvoCas9 (Casini et al. 2018). It has also been reported that the use of Cas9 nickase (Trevino and Zhang 2014; Shen et al. 2014; Ran et al. 2013) or Cas9 from Streptococcus canis (ScCas9) (Chatterjee et al. 2018) can decrease off-target effects to a certain extent. Furthermore, properly timed addition of Cas9 inhibitors could reduce off-target editing (Harrington et al. 2017; Shin et al. 2017b). A recent report suggested that precise editing mainly depends on the fourth nucleotide upstream of the PAM (Chakrabarti et al. 2019), which could be used in sgRNA design to decrease off-target effects. Nevertheless, the mechanism responsible for CRISPR-Cas-mediated off-target effects remains unclear, and the detection of unintended off-target mutations is of concern.

Unbiased genome-wide methods for detecting DNA double-strand breaks (DSBs) and identifying off-target cleavage events have been established. These methods include BLESS (Crosetto et al. 2013), Digenome-seq (Kim et al. 2015, 2016), GUIDE-seq (Tsai et al. 2015), HTGTS (Frock et al. 2015), CIRCLE-seq (Tsai et al. 2017) and VIVO (Akcakaya et al. 2018). A new method for detecting in vivo off-target effects referred to as DISCOVER-seq...
(Wienert et al. 2019) has been reported recently. Furthermore, the analysis of editing fidelity could be more precise if pedigree-matched controls are included (Iyer et al. 2018). These methods show high sensitivity in detecting CRISPR-Cas9-induced off-target effects; however, they have limitations such as abundant false positives, cellular model restriction and complex experimental designs.

Here, we assessed off-target events in CRISPR-Cas9-modified knock-out (KO) mice and their offspring by whole-genome sequencing (WGS) analysis and using GATK and bcftools. As part of a broader program of studying the regulation of sex development, we are interested in exploring the developmental roles of several candidate genes obtained from an expression screen, including Mmd and Paqr8 (Zhao et al. 2018), via CRISPR-induced loss-of-function experiments, which provide an opportunity for the in-depth study of many technical issues, such as the type and frequency of off-target events in CRISPR experiments. We sequenced nine individual mice, including two CRISPR-Cas9 edited Mmd and Paqr8 KO mice (founders) and their offspring (F1), together with three un.injected controls. We confirmed one CRISPR-Cas9-induced off-target event in each CRISPR-Cas9-edited KO mouse, which all occurred at sites predicted during sgRNA design. We also confirmed that the off-target events identified in parent mice were not passed on to the next generation. More importantly, we found that the frequency of CRISPR-mediated mutagenesis was not statistically distinguishable from the background rate of de novo mutations (DNMs).

**MATERIALS AND METHODS**

**Production of genetically modified mice**

SgRNAs were selected using the online CRISPR design tool CRISPOR (Hauessler et al. 2016) (http://crispor.tefor.net/). Genetically modified mice were produced using a previously described method (Yang et al. 2014). In brief, C57BL/6 female mice and CD1 mouse strains were used as embryo donors and foster mothers, respectively. Cas9 protein, tracrRNA and crRNAs were purchased from Integrated DNA Technologies, Singapore. To prepare the Cas9 ribonucleoprotein (RNP), tracrRNA and crRNAs were annealed to form an RNA duplex, which was subsequently mixed with Cas9 protein, followed by incubation at 37°C for 15 min. For microinjection, the Cas9 RNP containing Cas9 protein (30 ng/µL) and RNA duplexes (10 ng/µL each) was injected into the pronuclei of zygotes. The injected zygotes were cultured overnight in EmbryoMax Advanced KSOM medium (Merck Millipore, MR-101-D) at 37°C under 5% CO2 to the two-cell stage. Then, 16-20 two-cell-stage embryos were transferred to the oviducts of pseudopregnant CD1 females at 0.5 dpc. All animal procedures were conducted at the University of Queensland and were approved by the Animal Care Committee at the University of Queensland Animal Ethics Committee.

**WGS, variant calling and filtration**

Whole-genome sequencing libraries of nine samples were prepared using standard protocols for the Illumina X10 platform, generating 2,492 gigabytes of raw data. The Illumina raw reads were trimmed using Cutadapt (Martin 2011) to remove adaptors and bases of low quality. Then, the cleaned reads were mapped to the reference genome from the Ensembl database (http://ftp.ensembl.org/pub/release-89/) using the BWA mem program (Li and Durbin 2009). Sequence variants were called for all parents and offspring using GATK and bcftools. Variant calling and filtration using GATK software were performed with the UnifiedGenotyper and VariantFiltration commands, respectively. The parameters for single-nucleotide variants (SNVs) were set as follows: QD < 2.0, FS > 40.0, MQ > 40.0, MQRankSum < -12.5, ReadPosRankSum < -8.0, and those for indels were QD < 2.0, FS > 200.0, ReadPosRankSum < -20.0. The bcftools version and command options were as follows: bcftools-1.9 mpileup, bcftools mpileup –Ou, bcftools call –cv, bcftools norm –f, bcftools filter –Oz -s LOWQUAL -e “QUAL < 10 or DP < 10”. Bcftools mpileup configured for sensitivity required a minimum allele fraction of 0.05. Bcftools called between 220,709 and 252,140 variants per sample with a genotyping quality ≥ 10 and a median of 245,475 variants. All variant loci were required to have a total depth of at least 10 reads for further analysis. DNM calling for all variants was performed independently for each parent/offspring. The following criteria were applied to remove false positives and obtain final variants: (1) A minimum variant allele fraction of 5% was set to allow mosaic alleles. (2) Any variant coincident with an allele reported in the C57BL/6NJ strain in the Mouse Genomes Project was removed. (3) All individual repeat tracks within the University of California Santa Cruz (UCSC) RepeatMasker track

![Figure 1](image)

**Figure 1** Generation of Mmd and Paqr8 KO alleles using CRISPR/Cas9. (A) Targeting strategy of the Mmd gene. PCR genotyping primers indicated in the scheme produced bands with correct size (525 bp) in targeted mice, but not in WT samples. (B) Targeting strategy of the Paqr8 gene. PCR genotyping primers indicated in the scheme produced a band of 841 bp in KO allele, and 1818 bp in WT allele. Black arrows indicate sgRNAs; Green and Red arrows indicate PCR primers; M, DNA marker; KO, knock-out samples; WT, wild type samples.
RESULTS

Targeting of Mmd and Paqr8 genes

To generate Mmd or Paqr8 KO alleles, we employed a pair of sgRNAs to delete one or more coding exons in each gene. The two sgRNAs (sgRNA-m-5’ and sgRNA-m-3’) used for disrupting the Mmd gene were located in introns 3 and 6, respectively, resulting in the deletion of a 3448 bp region including exons 4-6 (Figure 1A). In the targeting of Paqr8, both sgRNAs (sgRNA-p-5’ and sgRNA-p-3’) were located within exon 3, and a 977 bp region was deleted (Figure 1B).

The Cas9 protein and the sgRNAs targeting Mmd or Paqr8 were coinjected into 40 C57BL/6 mouse zygotes via pronuclear microinjection. The injected zygotes were allowed to develop to the 2-cell stage and were then transferred into 0.5-day postcoital pseudopregnant CD1 females. After natural delivery, a total of three founder mice with the desired mutant alleles were identified using PCR and Sanger sequencing (Table 1). The primers used for PCR genotyping are shown in Supplementary Table 1. We then tested germline transmission by

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Table 1 CRISPR/Cas9-mediated gene targeting in C57BL/6 mice

| Gene | Injected Zygotes | Transferred 2-cell embryos (Recipients) | Newborns | Mouse with correct mutant allele(s) |
|------|------------------|----------------------------------------|----------|-----------------------------------|
| Mmd  | 40               | 34 (2)                                 | 4        | 2                                 |
| Paqr8| 40               | 38 (2)                                 | 4        | 1                                 |

(approximately 1.2 Gb) were merged, and all variants inside or 1 bp adjacent to merged repeats were removed. (4) Any de novo variant shared by two or more samples was removed.

Data Availability

The WGS data used in this study can be accessed at http://gsa.big.ac.cn/, with the accession of CRA001780. All data necessary for confirming the conclusions of the article are present within the article, figures, tables and supplementary tables. Supplemental material available at FigShare: https://doi.org/10.25387/g3.8137763.

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Figure 2 Analysis of CRISPR-Cas9-induced off-target events using WGS. (A) Mice used in this study. (B) WGS coverage. Nine mice were subjected to WGS, yielding a median depth of 101.6X. (C) Work flow of variant calling and filtering. (D) Final SNV counts summary. (E) Final indel counts summary.
backcrossing male founder with wild-type (WT) C57BL/6 females and successfully generated heterozygous F1 individuals.

**WGS data analysis**

We sequenced the whole genomes of nine mice (Figure 2A), including two founder mice (M01 and P01), four heterozygous mice (M02, M03, P02 and P03) and three uninjected control mice (WT1, WT2 and WT3). Sequencing was performed on the Illumina X10 WGS platform, yielding a median sequencing depth of 101.6x per genome (Figure 2B). We first normalized the WGS data by deleting regions of low-quality reads using Cutadapt software, setting the threshold at 30 (Martin 2011). Unclear reads and fragments of less than 60 bp were also deleted from the data. After data processing, we acquired an average of 241.32 GB of clean data for each sample. The work flow of variant calling and filtering is indicated in Figure 2C. We performed variant calling using both GATK (UnifiedGenotyper and VariantFilteruration) software (McKenna et al. 2010) and bcftools (mpileup and bcftools call) (Li 2011), resulting in median values of 240,156 variants per sample (129,970 SNVs/110,186 indels) and 238,913 variants per sample (127,508 SNVs/111,405 indels), respectively (Table 2). We then filtered the high-quality calls using reference mouse genome GRCm38 (Ensembl), the UCSC database and the International Mouse Phenotyping Consortium (IMPC) database for all samples, removing repeats and coincident regions. After visual inspection of the filtered variants, false positives were removed, and candidate DNMs for each sample were acquired (Table 2). As a result, median values of 1020 candidate DNMs (708 SNVs/312 indels) and 470 candidate DNMs (312 SNVs/158 indels) were obtained using the GATK method and bcftools, respectively. Furthermore, we compared the candidate DNMs of M01 and P01 mice to those of three WT mice and removed coincident mutations. Similarly, in the case of F1 offspring mice (M02, M03, P02 and P03), we removed the coincident mutations found in WT mice. Final SNV and indel counts were then obtained (Figure 2D and 2E). As a result, the median number of final SNVs was 141, and the median number of final indels was 11 (Table 2).

### Statistical analysis of variants from KO and WT samples

We next conducted a Kruskal-Wallis Rank test using the candidate and final variant counts (Table 3). The results showed no significant differences between the Mmd, Paqr8 and WT groups ($P = 0.1499$ and $P = 0.6701$ for candidate SNV counts and indel counts, respectively; $P = 0.0181$ and $P = 0.2881$ for final SNV counts and indel counts, respectively). Additionally, a Wilcoxon rank test was performed to identify significant differences between the CRISPR-Cas9 edited groups (Table 3). The results showed no significant difference between the two groups.

### Table 2 Summary of high-quality variant counts, filtered DNMs and final variants

| Mouse sample | Treatment Group | Relationship | Quality passed variants (GATK) | Candidate de novo mutations (GATK) | Candidate de novo mutations (bcftools) | Final SNVs | Final Indels |
|--------------|-----------------|--------------|--------------------------------|------------------------------------|--------------------------------------|------------|-------------|
| M01          | Cas9 RNP        | Parent       | 129169 113307                  | 130516 117545                      | 647 301                              | 141 21     |             |
| M02          | Offspring       |              | 128666 116517                  | 126096 123203                      | 644 231                              | 37 1       |             |
| M03          | Offspring       |              | 130771 114722                  | 127508 120029                      | 644 229                              | 28 3       |             |
| P01          | Cas9 RNP        | Parent       | 130152 115350                  | 135322 116818                      | 708 312                              | 155 12     |             |
| P02          | Offspring       |              | 128514 110186                  | 126265 11366                       | 392 221                              | 46 5       |             |
| P03          | Offspring       |              | 129970 102837                  | 126670 94039                      | 399 162                              | 45 4       |             |
| WT1          |                 |              | 130319 109723                  | 134782 110693                      | 1447 439                            | 200 23     |             |
| WT2          |                 |              | 128871 109005                  | 124798 109253                      | 935 320                             | 168 11     |             |
| WT3          |                 |              | 130665 112541                  | 131262 111405                      | 1064 385                            | 188 18     |             |
| Median       |                 |              | 129970 110186                  | 127508 111405                      | 647 301                            | 141 11     |             |

### Table 3 Statistical analysis of final variant counts

| Groups compared (mouse number) | Value compared | Test                          | Result                          | Null Hypothesis                   | Reject Null Hypothesis |
|--------------------------------|----------------|-------------------------------|---------------------------------|-----------------------------------|------------------------|
| Mmd(3), Paqr8(3), WT (3)       | Candidate SNV  | Kruskal-Wallis Rank Sum       | (G) chi-squared = 5.4222, df = 2, p-value = 0.06646 | No significant difference in treatment group means | No                     |
|                                | Candidate indel counts | Kruskal-Wallis Rank Sum | (B) chi-squared = 5.6471, df = 2, p-value = 0.0594 | No significant difference in treatment group means | No                     |
|                                | Final SNV      | Kruskal-Wallis Rank Sum       | (G) chi-squared = 3.8222, df = 2, p-value = 0.1479 | No significant difference in treatment group means | No                     |
|                                | Final indels   | Kruskal-Wallis Rank Sum       | (B) chi-squared = 5.6, df = 2, p-value = 0.06081 | No significant difference in treatment group means | No                     |
| Mmd(3), Paqr8(3)               | Final SNV      | Wilcoxon Rank Sum             | W = 2, p-value = 0.4            | No significant difference in treatment group means | No                     |
|                                | Final indel    | Wilcoxon Rank Sum             | W = 3, p-value = 0.7            | No significant difference in treatment group means | No                     |
**Determination of Cas9-induced off-target effects**

To investigate whether our strategy can identify Cas9-induced off-target effects, we compared the final variants with the expected off-target effects for each sgRNA predicted by Cas-OFFinder software (Bae et al. 2014). A total of 114 off-target sites were predicted for the targeting of the *Mmd* gene (31 and 83 for sgRNA-m-5' and sgRNA-m-3', respectively), while 163 loci were predicted for the *Paqr8* gene (55 and 111 for sgRNA-p-5' and sgRNA-p-3', respectively) (Supplementary Table 1). We identified one indel in each founder mouse (M01 and P01), which was shown by both our final variants and the predicted lists of Cas-OFFinder, indicating real Cas9-induced off-target events. The indels were located on chr14 and chr2 and were generated by sgRNA-m-3' and sgRNA-p-5', respectively (Table 4). These mutations might be filtered out among the final variants of the offspring mice using our filtering strategy. Thus, we performed PCR verification and Sanger sequencing to confirm the off-target mutations in the offspring mice. We did not find those mutations in M02, M03, P02 and P03, indicating that the off-target mutant alleles were not passed on to the F1 mice that we inspected.

**DISCUSSION**

We generated *Mmd* and *Paqr8* KO mouse strains using the CRISPR-Cas9 system. To increase the targeting efficiency, we applied a two-sgRNA system with the aim of deleting multiple exons of the *Mmd* gene and part of exon 1 of the *Paqr8* gene. We performed pronuclear microinjection of the Cas9 protein in our experiments, which may contribute to minimizing off-target effects because the protein can be degraded a few hours after microinjection. We obtained four newborns from 40 injected zygotes for each strain, with up to 50% of the newborn mice harboring biallelic mutations in the targeted genes, suggesting that the two-sgRNA system is highly efficient in generating KO mice. However, the use of two sgRNAs may increase Cas9-induced off-target events. Although no unexpected phenotypes were observed in the founder mice, we conducted WGS analysis to identify off-target sites.

The mechanism responsible for Cas9-induced off-target effects could be highly related to the tolerance of sgRNA mismatches, especially the seed sequence of the sgRNAs, which is the 6-11 nt region immediately upstream of the PAM sequence (Zhang et al. 2015; Shin et al. 2017a). Moreover, GC contents and the positions of cytosine and adenine are suggested to contribute to Cas9-induced off-target effects (Zhang et al. 2015). Thus, most CRISPR design tools score sgRNAs based on the number of nucleotide mismatches, the position and the distance to the PAM, providing potential off-target sites predicted by algorithms. A recent study presented a binding energy model for Cas9 RNP in which energy parameters were introduced into sgRNA design to increase specificity (Alkan et al. 2018). Nevertheless, there could be other factors involved in off-target cleavage that have not yet been incorporated into current algorithms. In our study, although the two confirmed off-target sites were both predicted by Cas-OFFinder, there is still a lack of evidence showing the accuracy of current algorithms.

Previous studies have addressed concerns by using WGS for detecting DNMs, which may represent mutations introduced by the CRISPR-Cas9 system (Iyer et al. 2015; Mianné et al. 2016). To overcome the defects of studies using only strain-matched controls, pedigree-matched controls were introduced for the analysis of off-target events (Iyer et al. 2018). Furthermore, a new method referred to as GOTI (Genome-wide Off-target analysis by Two-cell embryo Injection) was developed to detect off-target mutations (Zuo et al. 2019). However, these methods require the sequencing of all parents to establish pedigree trees or to perform microinjection at the two-cell stage. In this study, we sequenced the whole genomes of one parent and two offspring from each strain, together with three uninjected mice. We found that the data obtained from those mice were sufficient to analyze off-target events when two widely used analysis software platforms, GATK and bcftools, are used. Variant calling was independently performed using each software platform, and high-quality variants were then filtered using the UCSC and IMPC databases, resulting in candidate DNMs. Final variants were then obtained by comparing the parents and/or controls using the criteria mentioned in the section Materials and Methods. Two off-target sites were successfully identified during the analysis, indicating that a few background controls together with a partial pedigree tree may fulfill the detection of off-target sites induced by CRISPR-Cas9. We have shared our WGS data at Genome Sequence Archive (see Data Availability), which can be utilized by other researchers.

Intriguingly, statistical analysis showed that there was no significant difference between Cas9-induced mutagenesis and background DNMs, which is consistent with previous studies (Iyer et al. 2018; Akcakaya et al. 2018). In addition, off-target and on-target mutations could segregate through meiosis and homologous recombination, especially when they are located on different chromosomes. The two off-target effects identified in the current study were located on chr14 and chr2, whereas *Mmd* and *Paqr8* are located on chr11 and chr1, respectively. Although the off-target mutations are heritable, we did not find them in the F1 mice that we examined. Nevertheless, it is highly likely that a two-sgRNA system may increase Cas9-induced off-target effects; therefore, it is not recommended to use more than two sgRNAs unless large deletions are being generated.

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