CRISPR for genetically modified mice? Not so fast...

The use of genetically modified mice as model organisms for the study of human disease remains as relevant as ever[1] and CRISPR-based genome editing is rapidly changing the field. However, whereas the potential of CRISPR technology is very exciting, there are some limitations to its usefulness and drawbacks inherent to the technology can easily be overlooked.

CRISPR based genome editing typically relies on the induction of a double strand break within the target genomic sequence by a nuclease, such as Cas9, as directed by a guide RNA[2,3]. The nuclease is delivered to a zygote or early stage embryo and may either be delivered as protein in complex with sgRNA, as mRNA that is subsequently translated, or DNA. The nuclease may remain present in cells for some time, particularly when expressed from plasmid DNA, and is not 100% efficient. Consequently, the occurrence of nuclease-mediated double strand breaks is stochastic and can occur across several early cell divisions[4]. Repair of these double strand DNA breaks occurs via a number of competing pathways that have evolved to maintain integrity of the genome, such as non-homologous end-joining (NHEJ), microhomology-mediated end joining (MMEJ), and homology directed repair (HDR)[5]. With the exception of HDR, repair pathways are mutagenic and repair of CRISPR-mediated double stand breaks by NHEJ typically results in small insertions and deletions (INDELS) of less than 20 bp. CRISPR-mediated double strand breaks can induce large deletions (kilobases), complex genomic rearrangements, and chromosomal truncations, that are often overlooked by the genotyping methods employed[6,7].

Consequently, CRISPR founder animals are almost always mosaic in that they harbour multiple, and potentially complex, alleles within a single individual[8,9]. It is therefore necessary to have a genotyping strategy that allows for the detection of the desired allele amongst the mixture of different alleles[10]. Founder animals can be screened for the presence of the desired allele and its presence verified in the F1 offspring, assuming the allele contributes to the germline. Depending on the nature of the study, this can add considerable additional time and cost and increase animal numbers. For example, the authors of one study generating large knockouts concluded that while CRISPR was faster at generating the desired genomic deletions, understanding the nature of the alleles it generated may ultimately involve more time and effort than using classical Cre-loxP systems[11].

In many instances CRISPR is used to generate non-conditional knockout lines where the generation of multiple alleles may not be of primary concern provided that they are predicted to function as null alleles. However, CRISPR is also commonly used for the generation of knock-in (KI) lines via the repair of double strand breaks utilising HDR with a repair template. As HDR typically occurs at a lower rate than competing repair pathways[12], the efficiency of generating KI lines by CRISPR can vary. For example, the generation of conditional knockout (conKO) lines by CRISPR requires the simultaneous insertion of two loxP sites. An initial approach utilised two guide RNAs and two single-stranded oligonucleotides each encoding a loxP site[13]. However, a consortium of core facilities has reported the generation of only 15 conKO mice from a total of 17,887 engineered zygotes, indicating that the technique is very inefficient and difficult to reproduce[14]. Subsequently, an improved technique utilising a single long single-stranded oligonucleotide to introduce the two loxP sites in a single HDR event has been described[15,16]. However, the technique still requires electroporation or pro-nuclear injection of a large numbers of zygotes in order to generate correctly targeted conKO (floxed) mice, and the use of lssODNs is limited by size and sequence constraints. Analysis of founder mice generated by this technique shows that they are typically mosaic with a range of alleles, again highlighting the need for extensive genotyping so that mice harbouring additional or incorrect alleles are not used in experimentation[17].

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A recent study systematically compared the efficiency of CRISPR to classic gene targeting by homologous recombination for the generation of large KIs (>1.5kb)[18]. The authors analysed 221 experiments that targeted 128 loci in murine embryonic stem (ES) cells. While CRISPR often produced more targeted ES cells than classic gene targeting when screen by overlapping PCR, further investigation by Southern hybridisation revealed that many of these clones had large genomic deletions and rearrangements. When this result was taken into consideration, classic gene targeting was found to produce clones with the correct integration of the KI sequence at a higher frequency than CRISPR. The authors stated that the observation of genomic deletions and rearrangements occurring with CRISPR, "ultimately rendered CRISPR/Cas9 less efficient than classical homologous recombination for the production of fully validated clones".

In addition to these limitations to CRISPR-mediated genome engineering, it is also possible that the nuclease may induce a double strand break at a region within the genome other than the target site. There has been concern that such off-target effects could confound experimental results in instances where off-target mutations result in a phenotype. Recent modifications to the CRISPR technique, such as improved gRNA design and the development of improved nucleases with higher specificity have alleviated some of this concern and recent studies have shown that off target mutations occur less frequently in engineered animals as opposed to cell lines[19]. However, off-target mutations do still occur: Whole-genome sequencing of ten genome engineered mouse embryos found 43 off-target mutations of which only 30 were predicted by an *in silico* prediction tool[20]. Where they do occur, off target mutations will segregate from the targeted allele, if not genetically linked, with subsequent breeding. The possible confounding effect of off target mutations remains a consideration for lines generated in a pre-clinical setting and remains a significant concern for the application of genome engineering as a human therapeutic[21].

Successful generation of an animal model by CRISPR depends on many variables[22]. These factors include the locus being targeted, the developmental stage of the embryo being targeted, the experimental design and sequence of the guide RNA, the nuclease being used, the template is used (if any) for HDR, and the method of delivery of CRISPR reagents. Mosaicism of the founder animals should be expected and a means of genotyping either the founders and/or their offspring is required to select animals that harbor the desired alleles. In instances where the desired allele is present at low frequency, a large number of founder or F1 mice may need to be generated. The possibility of off-target effects should also be considered and investigated where appropriate. When these factors are considered, the generation of genetically modified animal lines by CRISPR may not always be as technically simple or cost effective as commonly expected.

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