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Analysing the outcome of CRISPR-aided genome editing in embryos: Screening, genotyping and quality control

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ABSTRACT

The application of CRISPR/Cas9 technology has revolutionised genetics by greatly enhancing the efficacy of genome editing in the early embryo. Furthermore, the system has enabled the generation of allele types previously incompatible with *in vivo* mutagenesis. Despite its versatility and ease of implementation, CRISPR/Cas9 editing outcome is unpredictable and can generate mosaic founders. Therefore, careful genotyping and characterisation of new mutants is proving essential. The literature presents a wide range of protocols for molecular characterisation, each representing different levels of investment. We present strategies and protocols for designing, producing and screening CRISPR/Cas9 edited founders and genotyping their offspring according to desired allele type (indel, point mutation and deletion).

Contents

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeat; Cas9, CRISPR associated 9; ddPCR, droplet digital polymerase chain reaction; DNA, deoxynucleotide acid; DSB, double stranded break; HDR, homology directed repair; indel, insertion/deletion; ND, not determined; NHEJ, non-homologous end joining; nt, nucleotide; PAM, protospacer adjacent motif; PCR, polymerase chain reaction; QC, quality control; sgRNA, single guide RNA; ssODN, single-stranded oligo-deoxynucleotide; WT, wild-type.

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1. Introduction

Originally identified as a bacterial adaptive immune system, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR Associated 9 (Cas9) system has recently been adapted and re-deployed to efficiently modify eukaryotic genomes [1]. Since the system's first description as a genome editing tool, a wide range of allele types have been obtained. Modifications reported range from simple indels [2], precise introduction of point mutations [3], insertion of loxP sites or tag sequences [4], or larger cassettes such as cDNA for expression [5]. For each of these experiments, the mechanism of editing involves scanning of the DNA by the CRISPR/Cas9 complex prior to cutting and repair [6]. The complex molecular events involved in the process are starting to be unravelled and this knowledge exploited for a more efficient application of the system [7].

Although not highlighted in the initial reports of genome editing by CRISPR/Cas9 [3], we and others have described that the CRISPR-aided mutagenesis is an unpredictable process. Founder animals are often mosaic and sequence changes additional to those intended can be found associated at the site of repair (illegitimate repair [8]). The literature offers a wide range of protocols for analysing the outcome of CRISPR aided-mutagenesis, but many do not allow for a comprehensive characterisation of the complexity of mutagenesis events generated with the system, nor for the identification of unforeseen additional sequence changes. Table 1 summarises different techniques that can be employed to characterise mouse mutants generated by the microinjection of CRISPR/Cas9 reagents and shows examples of references that apply them.

Here we describe the use of the CRISPR/Cas9 technology for the modification of the mouse genome to produce indels, point mutations and deletions and analysing the outcome of these experiments. We have drawn on our experience of the generation and quality control of large numbers of mouse mutants, many of them produced within the International Mouse Phenotyping Consortium (IMPC) [13], and delineated methods for efficient screening, genotyping and ascertaining of the quality of new alleles produced. We detail strategies to analyse the genetic complexity of the animals produced by CRISPR/Cas9 editing according to the type of allele to be generated. We propose that F_0 animals (born from microinjection) can be screened for the presence of an allele of interest, but the genotype of new mutant lines is proven in the subsequent generation only. The approach we propose can be employed for the mutagenesis of other species.

2. Materials and methods

2.1. Mutation design and generation of reagents for microinjection

The sequences of oligonucleotides, protospacers and donor DNAs used within the examples presented in this study are shown in Supplemental Table 1.

2.1.1. Design tool and choice of guide

Single guide RNA (sgRNA) sequence selection is carried out using any of the following online tools:

- http://tefor.net/crispor/crispor.cgi [14]
- http://crispr.mit.edu/ [15]
- http://www.sanger.ac.uk/htgt/wge/ [16]

SgRNA sequences are selected with as few predicted off-target events as possible, particularly on the same chromosome as the intended modification. When selecting sgRNAs to introduce indels or point mutations, the region for guide selection is more limited as the cut site is to be within a given sequence or as close to the point at which the single base change is to be introduced. For exon deletion projects, where the intention is to generate a null allele, the exon chosen should be represented in all transcripts and its removal should result in a frame shift. The number of guide sequences selected is dependent upon the intention of each project i.e. to introduce point mutations and indels into genomic sequence, a single sgRNA is required, whereas in our high-throughput pipeline, exon deletion projects generally employ four sgRNAs with two each side of the targeted deletion.

2.1.2. Donor oligonucleotides

Sequences for donor templates are designed with homology arms at least 60 nt in size flanking the intended point mutation. These are generally centred on the cutting site but might be offset towards a point mutation to favour a recombination that includes the desired change. Wherever possible, silent point mutations are introduced to the ssODN sequence to disrupt the PAM sequence and prevent re-cutting of the modified allele by Cas9. If altering the PAM sequence in the donor is not possible, further changes in the seed sequence to which the sgRNA will bind will be introduced to the donor. Modifications to introduce restriction digest sites to aid with subsequent animal genotyping can also be incorporated into the ssODN. Single-stranded oligo-deoxynucleotide donor sequences were ordered as Ultramer™ DNA oligonucleotides