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Editing the Mouse Genome Using the CRISPR-Cas9 System

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Abstract

The ability to modify the murine genome was perhaps one of the most important developments in modern biology. However, traditional methods of genomic engineering are costly and relatively clumsy in their approach. The utilization of programmable nucleases, which include zinc finger nucleases (ZFNs) and *transcription activator-like effector nucleases* (TALENs) was a significant step in improving the precision of 'genome editing' technology as it allowed precise control of gene targeting. However, the design and use of ZFNs or TALENs remains cumbersome and prohibitively expensive. The CRISPR/Cas9 system is the next installment in the line of programmable nucleases, and provides highly efficient and precise genome editing capabilities, whilst using reagents that are simple to design and cheap to generate. Furthermore, with the CRISPR/Cas9 system it is possible to move from a hypothesis to an *in vivo* mouse model in less than a month. The simplicity, cost effectiveness and speed of the CRISPR/Cas9 system allows researchers to tackle questions that would otherwise not be technically or financial viable. In this article we will discuss practical considerations for the use of Cas9 in genome engineering in mice and will provide detailed protocols for its implementation.

Cas9 background and principle components

Genetically modified mice are a cornerstone of biomedical research as they provide essential tools to understand gene function and to model complex human diseases. Until recently, genetically engineered mice were generated through genetic modification of mouse embryonic stem (ES) cells by homologous recombination (HR). Targeted ES cells are expanded and injected into wild-type mouse blastocysts with the expectation that they will

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contribute to the germ line of chimeric mice. Chimeric mice are then bred to wild-type mice to generate progeny containing the targeted locus (Thomas and Capecchi 1987). This process is extremely costly, time-consuming and in some cases uncertain. Although, this procedure usually takes 9–12 months, the generation of mice carrying multiple targeted loci or challenging targeting locations can substantially add more time, effort and economic cost.

In the last decade, different methods have been developed to generate mutant mice in a rapid and efficient manner. The most successful approaches utilize programmable nucleases, such as zinc finger nucleases (ZFNs) and *transcription activator-like effector nucleases* (TALENs) (Boch et al. 2009), which are injected directly into mouse one-cell embryos; a procedure that greatly accelerates the process of generating genetically modified mice by avoiding the use of ES cells [for a recent review see Kim *et al* (Kim and Kim 2014)]. Once injected, these nucleases have the capability to generate double strand breaks (DSB) at predefined sites in the genome, which are then repaired by error-prone non-homologous end joining (NHEJ), resulting in either Insertion or deletion (indel) mutations; indels located within protein-coding exons can cause frameshifts resulting in a knock-out (KO) allele (Kim and Kim 2014). Alternatively, if a single-stranded DNA (ssDNA) or a circular donor plasmid with homology regions flanking the DSB is introduced into the one-cell embryo in combination with these nucleases, a defined DNA sequence can be inserted into the genome by high-fidelity homologous directed repair (HDR), allowing the generation of knock-in (KI) mice carrying point mutations, tags, conditional alleles or fluorescent proteins (Kim and Kim 2014).

The most recently developed genome-editing tool is the CRISPR-associated protein 9 (Cas9) nuclease. The CRISPR/Cas system functions as an RNA-based adaptive immune system in bacteria and archaea (Barrangou et al. 2007). In *Streptococcus pyogenes*, a type II CRISPR/Cas system composed of Cas9, CRISPR RNAs (crRNAs) and a trans-activating crRNA (tracrRNA) target and degrade nucleic acids from foreign plasmids or bacteriophages (Deltcheva et al. 2011; Jinek et al. 2012). In this system, the Cas9 nuclease is guided to invading foreign nucleic acids by crRNAs that are partially complementary to the target sequence, and the tracrRNA plays a pivotal structural role for the proper activity of the Cas9 nuclease. The repurposing of Cas9 to generate site-specific DSB in mammalian genomes was a turning point in genome editing (Cho et al. 2013; Cong et al. 2013; Mali et al. 2013b; Wang et al. 2013; Yang et al. 2013). Part of this repurposing was the fusion of the crRNA and tracrRNA to form a single-guide RNA (sgRNA) (Jinek et al. 2012). To direct Cas9 to a specific genomic region, the sgRNA is designed so that the 20 nucleotides at its 5' are homologous to the genomic target sequence. In addition, the genomic sequence must be immediately followed by a protospacer adjacent motif (PAM) sequence, a 3-bp (NGG) motif present in the target sequence but not the sgRNA (Figure 1).

Like previous programmable nucleases, CRISPR/Cas9 provides highly efficient and precise genome editing capabilities in mice (Wang et al. 2013; Yang et al. 2013). However, the significant advantage that Cas9 offers is that it uses reagents that are simple, cheap and quick to design and generate. Furthermore, it is also significantly more efficient than ZFN and TALENs (Yasue et al. 2014). In addition, the CRISPR/Cas9 system allows many targeting applications, such as the targeting of multiple loci simultaneously, the generation

of conditional alleles, and the production of mice carrying endogenous reporters (Wang et al. 2013; Yang et al. 2013). Moreover, these modifications can be made in pure inbred strains of mice (i.e C57BL/6) as well as directly in established mutant strains, dramatically reducing time and cost required to generate/modify complex animal models.

We adopted this revolutionary technology in late 2012, since then we have generated more than one hundred novel genetically engineered mouse strains. In concordance with previous reports, we have observed high success rates in all the potential types of genome targeting events. In the accompanying protocol, we describe in detail the optimal conditions to generate mice carrying point mutations, chromosomal deletions, conditional alleles, fusion tags or endogenous reporters.

Cas9 genome editing applications

In this section we will discuss general considerations for the main Cas9 mediated genome editing applications, as outlined below (Figure 2).

Gene knockout through indel generation

One main use of genome modification has been in the generation gene knockout animals. Double stranded breaks generated by Cas9 are repaired by the error prone NHEJ pathway resulting in indel generation. When targeted within the coding region of a gene indels frequently results in a frame shift and loss of function. This is the most simple and efficient form of Cas9 mediated genome editing, requiring only injection of Cas9 and a single sgRNA.

Point mutations/Small insertions

A powerful use of the Cas9 system is the precise editing of the mouse genome to introduce specific nucleotide changes (Wang et al. 2013). This enables disease modeling by allowing exact nucleotide changes engineered to mimic human disease mutations. In addition, the creation/destruction of specific genomic sequences, such as transcription factor binding sites, allows interrogation of their function. Cas9 can also be used to introduce short artificial sequences, such as HA-tags or loxP sites, into the genome at precise locations (Yang et al. 2013). Precise genome editing requires three components; Cas9, an sgRNA and a single stranded DNA (ssDNA) oligo containing the desired nucleotide modifications. As described above Cas9 is directed by an sgRNA to generate a DSB at a specific location in the genome. In the presence of a ssDNA oligo, with homology flanking the DSB, the host DNA repair machinery is able to perform homology directed repair (HDR) using the donor oligo as a template; any mutation/artificial sequence included in the donor oligo will be copied into the genome at this exact location. Donor ssDNA oligos typically contain 50–60 bases either side of the region to be edited. The addition phosphorothioate linkages at the 5' and 3' terminal nucleotides can increase *in vivo* stability of the oligos potentially increasing the efficiency of the reaction. Using this approach we have successfully inserted an artificial 100nt sequence into the genome.

To prevent Cas9 from re-cutting the edited sequence and introducing an indel, sgRNAs should be designed to place the modified region as close to the PAM sequence as possible.

This increases the chance that the modification is correctly incorporated, as well as reducing the probability of Cas9 re-cutting (by altering the sgRNA binding site). When making modifications at multiple sites, such as flanking an exon with loxP sequences, a separate sgRNA and donor oligo is required for each modification site. It is important to remember that these are independent targeting events and may not occur on the same copy of the chromosome and therefore may segregate on breeding.

Large deletions

For some applications the ablation of large chromosomal regions is desired, however, such deletions are difficult to achieve with classical targeting methodologies, often requiring multiple rounds of recombination in ES cells (Hacisuleyman et al. 2014). However, large deletions are relatively simple to achieve using the CRISPR/Cas9 system. By using two sgRNAs, to generate DSBs flanking a region of interest, it is possible to efficiently delete the intervening sequence through the NHEJ repair process (Yang et al. 2013; Krishnaswamy et al. 2015). Although efficiency is likely influenced by the linear distance between the sgRNAs (and potentially 3-dimensional structure of the genome) we have deleted genomic regions of up to 200kb using this approach.

Large Insertions

To introduce large DNA sequences at precise locations (e.g. fluorescent protein), a plasmid that encodes the DNA sequence to be inserted flanked by >2kb of homology is general used (Yang et al. 2013). However, we have been able to introduce large fragments of DNA with smaller homology regions (~500 bp). The DNA sequence of interest should be inserted as close as possible to the generated DSB, and If possible the sgRNA target sequence should be modified in the targeting vector to prevent cutting of the donor DNA or re-cutting of the genome after HDR. We have had success using both cytoplasmic and pronuclear injections for large insertions. However, to reduce random insertion, it is best to use circular plasmids rather than linearized DNA.

Design of sgRNAs to maximize cutting and minimize off-targets

The design of sgRNAs is relatively simple, requiring only that the 20nt homology be immediately followed in the genome by an NGG PAM sequence. However, certain nucleotides are favored/disfavored at different positions along the sgRNA and this should be considered during sgRNA design (Doench et al. 2014). Most importantly, careful selection of sgRNAs is paramount to minimize potential off-target cutting; candidate sgRNAs should be blasted against the genome and an ideal sgRNA would only be present as a single site within the genome and should have at least 5 mismatches to any similar sites in the genome. A number of web sites offer free tools to assist with sgRNA design. The use of truncated sgRNA has been shown to reduce off-target cutting, however, we have little experience with this approach (Fu et al. 2014). Alternatively, off-target effects can be reduced by using the Nickase Cas9 mutant, which can only generate double stranded breaks when two gRNA targets are close to each other (Mali et al. 2013a). Nickase can be used in place of regular Cas9 in any of the applications described above, however this requires replacing every sgRNA with two sgRNAs (Lee and Lloyd 2014; Rong et al. 2014; Shen et al. 2014). We

have had similar success rates using the nickase Cas9 mutant when compared to the wild-type Cas9 in the generation of mice with indel mutations or small deletions.

Considerations for screening

For screening indels or small insertions, a simple PCR across the targeted region followed by a Surveyor assay can be used for initial screening. To confirm sequence of mutated alleles, cloning of the genotyping PCR product followed by sequencing is often required. For genotyping large deletions, primers spanning the excised region provide a simple assay for deletion. However, NHEJ across large deletions can result in loss of additional nucleotides proximal to each DSB; therefore PCR primers should be placed at least 100bp outside of the expected cut sites. Although founder animals may sometimes carry homozygous modifications, they are frequently mosaic, with different cells in the same animal carrying different modifications; we have detected founders carrying 5–6 unique alleles. It is therefore important to cross founder animals to wild type mice and then screen the various alleles after segregation. This is especially important when screening to select animals in which loxP sites have integrated on the same copy of the chromosome.

Conclusions and future perspectives

The CRISPR/Cas9 system has revolutionized genome engineering, overcoming many of the problems associated with previous programmable nucleases. However, Cas9 mediated targeting is still limited to sites containing a PAM seq (NGG). An expanding toolbox of CRISPR/Cas from different bacterial species, with alternative PAM sequence requirements, will provide greater targeting flexibility (Hou et al. 2013). Finally, the current the bottleneck in Cas9 targeting is the highly technical and time-consuming process of microinjection. In the future, high throughput methods of delivery will replace microinjection opening up this technology to even more labs.

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Figure 1. Basic components of the Cas9 system

The Cas9 nuclease generates double strand breaks by using its two catalytic domains (HNH and RuvCI) to cleave each strand of a DNA target site next to a PAM sequence (red) and matching the 20-nucleotide sequence of the single guide RNA (sgRNA). The sgRNA includes a fused RNA sequence derived from CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA) that binds and stabilizes the Cas9 nuclease.

Figure 2. Procedures to generate genetically modified mice using the CRISPR/Cas9 genome editing system

Isolated zygotes are co-injected with Cas9 mRNA and sgRNAs to generate mice carrying indel mutations or targeted chromosomal deletions. Alternatively, the Cas9 mRNA and sgRNAs are co-injected in combination with donor ssDNAs or circular plasmids to generate mice harboring point mutations, tags, loxP sites or large DNA fragments such as a fluorescent protein.