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Gene editing in mouse zygotes using the CRISPR/Cas9 system

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ABSTRACT

The generation of targeted mouse mutants is a key technology for biomedical research. Using the CRISPR/Cas9 system for induction of targeted double-strand breaks, gene editing can be performed in a single step directly in mouse zygotes. This article covers the design of knockout and knockin alleles, preparation of reagents, microinjection or electroporation of zygotes and the genotyping of pups derived from gene editing projects. In addition we include a section for the control of experimental settings by targeting the Rosa26 locus and PCR based genotyping of blastocysts.

1. Introduction

Engineering of the mouse germline is a key technology in biomedical research for studying the function of genes in health and disease. In the first two decades of this technology, gene targeting was based on the use of embryonic stem (ES) cell lines, relying on the spontaneous recombination of vector encoded sequences with the ES cell genome, followed by the injection of engineered ES cells into blastocysts to obtain germline chimaeric mice. This approach was displaced in recent years by the ascent of sequence-specific, programmable nucleases which are introduced directly into mouse zygotes. In such one-cell embryos nucleases are used for creating targeted, highly recombinogenic double-strand breaks (DSBs) in genes of interest. These targeted DSBs are processed by DNA repair enzymes, often resulting in a variety of sequence modifications. The two step procedure of nuclease induced DSB induction followed by mutagenic DNA repair is commonly referred to as 'gene editing'. Since gene editing is directly applied in mouse zygotes it has become the preferred standard procedure for generating knockout and knockin mice which saves time and efforts as compared to classical gene targeting in ES cells.

1.1. Gene editing in mouse zygotes

Nuclease mediated gene editing in zygotes was demonstrated using zinc-finger and TAL nucleases between 2009 and 2013 [1]. However, these types of nucleases require the recoding of protein sequences for each new target site and the construction of two molecules which act as dimers. Therefore, it was a breakthrough that in 2013 the CRISPR/Cas9 nuclease system of *Streptococcus pyogenes*, a defense mechanism against pathogenic DNA, was adapted for generating double strand breaks in the genome of mammalian cells, enabling targeted genome editing at high efficiency [2]. Using this system, two basic components are introduced into cells to achieve gene editing: the Cas9 protein, harboring two nuclease domains, and a pair of short RNAs, a crRNA and tracrRNA which hybridize to each other and associate with Cas9. If desired, a DNA molecule serving as repair template can be added as a third component. For many applications the crRNA and tracrRNA are conveniently fused into a single guide (sg) RNA molecule. The first twenty nucleotides of sgRNA direct Cas9 to a specific complementary DNA target sequence via RNA-DNA hybridization. If the target sequence is located upstream of an invariant PAM sequence ('protospacer adjacent motif'; 5′-NGG-3′ in case of Cas9 from *S. pyogenes*), the Cas9 nuclease function is activated and creates a

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DSB located 3 bp upstream of the PAM site. Using this system Cas9 induced DSBs can be directed to any DNA sequence of the formula $N_{1\text{-}20}$-NGG by simply altering the first 20 nucleotides of the sgRNA which correspond to the target DNA sequence (Fig. 1). The advantage of CRISPR/Cas9 is that new target sites can be easily addressed by modification of the short target RNA sequence whereas the Cas9 protein component is invariant. Since 2013 numerous studies confirmed the utility and efficacy of CRISPR/Cas9 gene editing in mouse zygotes enabling the direct production of knockout and knockin mutants in a single step [3] (Fig. 2). Initially Cas9 mRNA and sgRNAs were generated by *in vitro* transcription and delivered into zygotes by pronuclear or cytoplasmic microinjection as used for the production of transgenic mice or nuclear transfer. Both delivery routes can lead to comparable results [4] and whether pronuclear or cytoplasmic injection is chosen mostly depends on individual skills and the available equipment. The procedure can be further streamlined by the use of recombinant Cas9 protein and chemically synthesized RNAs which are commercially available. Furthermore, the electroporation of zygotes in batches has been recently developed as an alternative to the tedious microinjection procedures [5].

1.2. Gene editing by repair of targeted double-strand breaks

Gene editing occurs by targeted induction of DSBs which is followed by DNA repair. Two main DNA repair pathways exist in mammalian cells. In most cases DSBs are repaired by the non-homologous end joining (NHEJ) pathway that religates open DNA ends by DNA ligase IV without the use a repair template [6]. DSBs which are processed by NHEJ repair frequently exhibit the random deletion and/or insertion of nucleotides (indels). If these indels are located within coding regions and generate a shift in the reading frame (i.e. the size of the indel is not a multiple of three) a knockout mutation occurs. NHEJ repair is active in all phases of the cell cycle. Alternatively DSBs can be repaired by homology directed repair (HDR), which requires a DNA molecule as repair template and is restricted to the S and G2 phases of the cell cycle [7]. For the repair of spontaneously occurring DSBs, cycling cells use the intact region of the sister chromatid as HDR template. To achieve gene editing of targeted DSBs, HDR can be hijacked by the addition

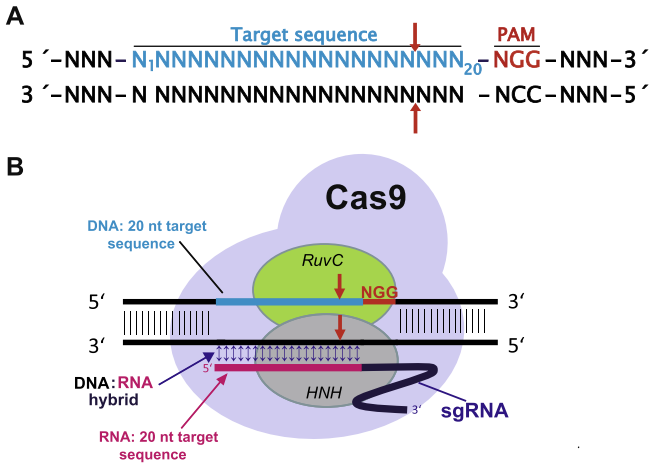

Fig. 1. The CRISPR/Cas9 nuclease system A: Cas9 and sgRNA recognize 20 nt target sequences located upstream of the invariant 3 bp NGGPAM ('protospacer adjacent motif', red letters). The double-strand break occurs 3 bp upstream of the NGG PAM sequence (red arrows). B: Cas9 bound to sgRNA screens DNA for sequences complementary to the first 20 nt of the sgRNA. If a target sequence is located upstream of the PAM site, each of the RuvC and HNH nuclease domains of Cas9 cut one strand of the target DNA (red arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

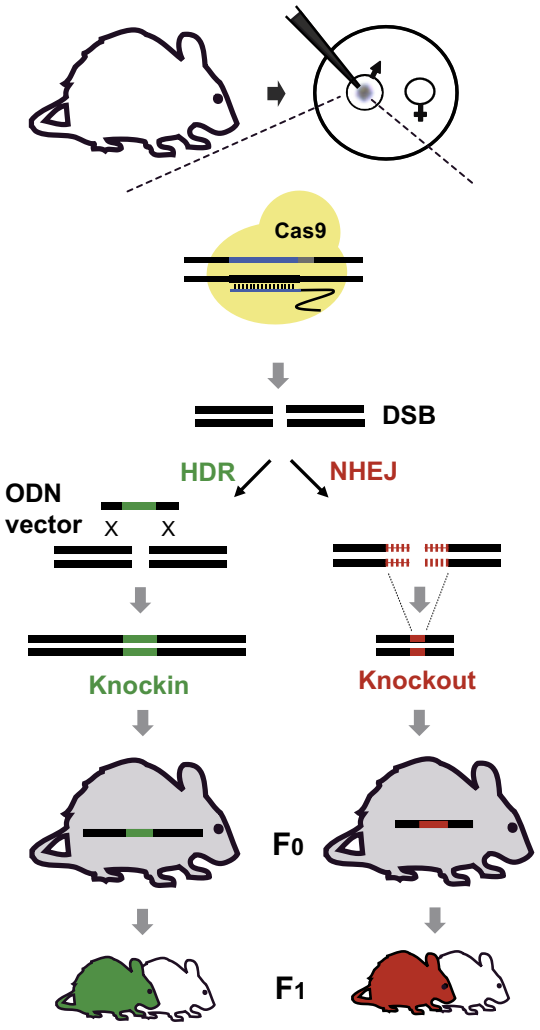

Fig. 2. Gene editing in mouse zygotes using CRISPR/Cas9. Mouse zygotes are microinjected into the (larger) male pronucleus with Cas9 protein or mRNA, sgRNA and a repair template plasmid vector or oligonucleotide (ODN). The double-strand break (DSB) in the target gene can be either repaired by NHEJ, leading to small sequence deletions and gene knockout alleles. Precise sequence modifications (Knockin alleles) are introduced by HDR with the homology regions of DNA template molecules (ODN or plasmid vector). Mice derived from microinjected zygotes (F_0 generation) represent a variety of mutant founder alleles. Mating of the F_0 founders transfers individual mutant alleles to their F_1 progeny.

of artificial DNA template molecules which include homology regions identical to the sequences located up- and downstream of the DSB, flanking a heterologous sequence modification or insertion. In the repair process sequence conversion extends from the template's homology regions into the heterologous sequence and transfers the genetic modification into the target gene (knockin), enabling the introduction of preplanned mutations such as codon replacement or the insertion of a reporter gene. Large sequence insertions require the construction of plasmid-based gene targeting vectors which include homology regions of several thousand basepairs, whereas small sequence modifications can be introduced using synthetic single-stranded DNA oligonucleotides with lengths of 100–150 nt. In cycling cells both mechanisms work side by side, but mostly DSBs are repaired by prevailing NHEJ pathway whereas HDR occurs less frequently. Therefore, the induction of DSBs in the pronuclei of zygotes leads to a variety of individually repaired alleles at the target locus and to a group of heterogeneous founder mutants (F_0 generation; Fig. 2) which must be screened for