Innovative cloning-free CRISPR/Cas9 system: Efficiently creating targeted model mice

Kohichi Tanaka

Professor of Molecular Neuroscience at TMDU

Dr. Tanaka received his MD and PhD from Niigata University. His postdoctoral research was performed with the Neural Network Team at RIKEN. He became a section chief of Neurodegenerative Diseases at the National Institute of Neuroscience in 1993 and assumed his present post at TMDU in 1998.

Genetic engineering in mice

The mouse has become the most commonly used animal in the biological and medical sciences because its genome can be specifically modified with one-nucleotide precision. Recent advances in genomic microarray and next generation sequencing technologies have identified many genetic variants associated with common and complex human diseases. To determine whether these variants are causal for specific human diseases, we need to investigate their biological functions. One possible approach to address this is the use of mouse models that incorporate the identified genetic variants. Although traditional gene targeting in embryonic stem (ES) cells is suitable for carrying any desired genetic modifications, it is laborious and time-consuming.

The development of engineered zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and, most recently, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated endonucleases (Cas) is revolutionizing genetic engineering in mice. These technologies depend on the cell processes triggered by the DNA double strand break (DSB) in specific DNA sequences (Fig.1). The non-homologous end-joining (NHEJ) pathway, which repairs DNA damage in the absence of template DNA, results in the introduction of random insertions or deletions that disrupt gene function. In contrast to NHEJ, homology-directed repair (HDR) uses a DNA donor template that is homologous to the DSB site to achieve precise recombination. These methods provide exciting and groundbreaking opportunities, enabling direct and rapid gene targeting in fertilized mouse eggs, with no need for ES cells. Using *in vivo* genome editing, genetically engineered mice can be created in months rather than years.

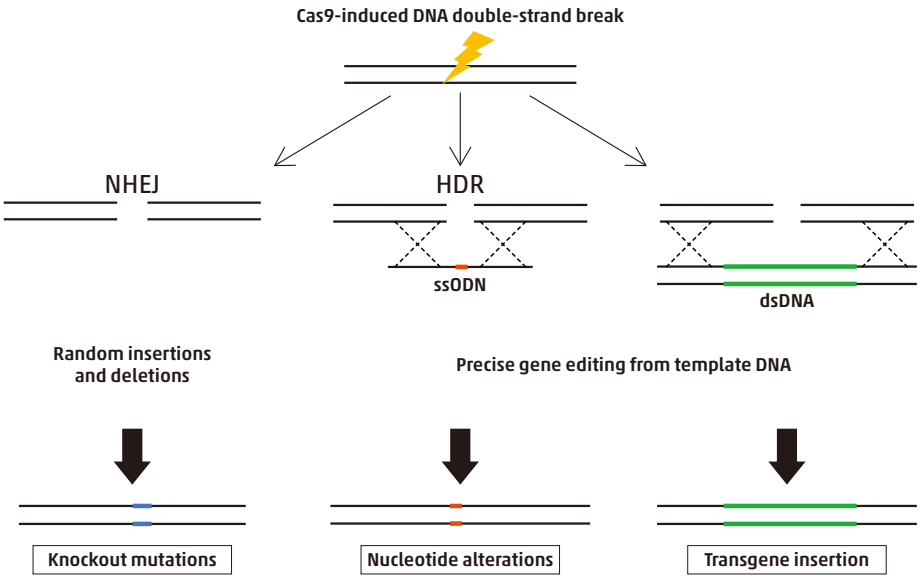


Fig. 1. Cas9-induced genome editing

Cas9 induces double-strand (ds) DNA breaks. Then, DNA is repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ results in the introduction of random insertions or deletions that may disrupt gene function. HDR with exogenous 'repair templates', such as single-stranded donor oligonucleotides (ssODN) or ds DNA, can lead to the introduction of precise nucleotide substitutions or transgene insertion. Dashed lines indicate homologous recombination.

Cloning-free CRISPR/Cas system

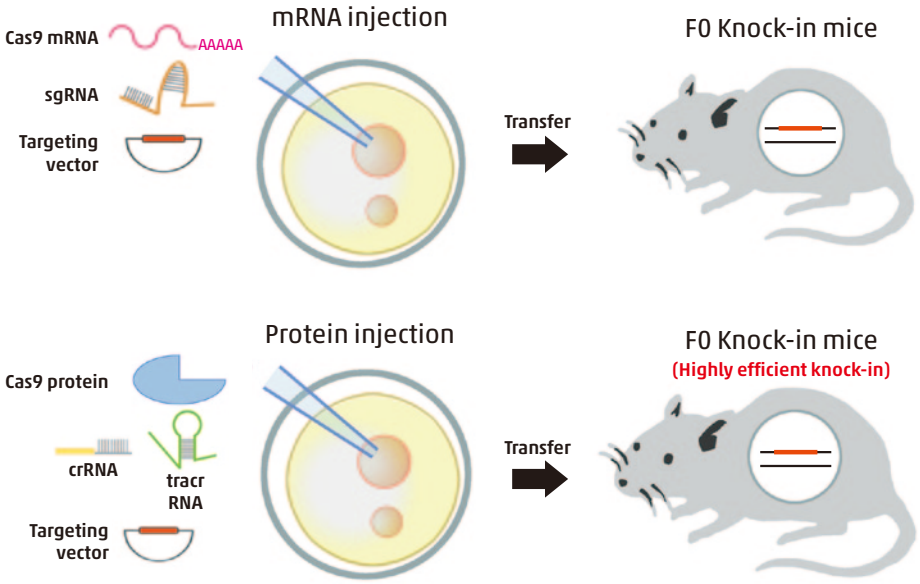
In contrast to ZFNs and TALENs, the most recent CRISPR/Cas system is remarkably

simple and efficient. Consequently, a flood of studies using CRISPR/Cas-mediated *in vivo* genome editing have reported the production of knockout mice and knock-in mice that carry single nucleotide substitutions using oligo DNA donors. Yet there has been only one report of the successful production of knock-in mice that carry reporter gene cassettes, which are essential tools for analyzing complex tissues, such as brain, *in vivo*. In that case, the reported efficacy of the targeted insertion of the reporter gene was only about 10%. Moreover, since founder F0 mice are often mosaic, transmission of the targeted allele to the next F1 generation is not guaranteed. The low success rates and the mosaicism of gene-cassette carrying knock-in mice limit the applicability of CRISPR/Cas-mediated *in vivo* genome editing.

The CRISPR/Cas system was initially reported as an adaptive immune system in bacteria, consisting of three components, including Cas9 nuclease and two small RNAs—the CRISPR RNA (crRNA), which guides the Cas9 complex to the target sequence, and trans-activating crRNA (tracrRNA), which binds to crRNA and forms a ribonucleoprotein complex with Cas9 nuclease. When it was harnessed as a genome editing tool, the dual-crRNA:tracrRNA was engineered as a chimeric single guide RNA (sgRNA). The CRISPR/Cas system consisting of two components - Cas9 nuclease and sgRNA - became the standard approach in the field of genome editing due to its enhanced convenience and robust targeting. However, it is still unknown whether the commonly used sgRNA works more efficiently than the dual-crRNA:tracrRNA, especially for the production of knock-in mice that carry reporter gene cassettes.

I have now overcome this issue by developing an innovative, highly efficient CRISPR/Cas system (Fig.2), which resulted in the targeted insertion of a long gene cassette, including enhanced green fluorescent protein (EGFP), into the mouse genome in fertilized eggs with efficiency of up to approximately 50%. I reproduced the natural state of the CRISPR/Cas system, which consists of three components: Cas9 protein, chemically synthesized crRNA, and tracrRNA, instead of the commonly used two-component system consisting of Cas9 mRNA and sgRNA. This has led to extremely high efficiency in several respects. First, the direct delivery of Cas9 protein, chemically synthesized crRNA and tracrRNA, and targeting vector into the pronuclei of zygotes allowed for the highly efficient generation of knock-in mice carrying gene cassettes in the endogenous gene. Second, the CRISPR/Cas vector construction and in vitro RNA transcription could be omitted by using commercially available Cas9 protein and chemically synthesized crRNA and tracrRNA, leading to a cloning-free CRISPR/Cas system. Third, the Cas9 protein-RNA complex was rapidly degraded in embryos, thus reducing the likelihood of off-target effects and mosaicism. Thus, the cloning-free CRISPR/Cas system further provides highly convenient and accurate gene modification, and its successful transmission to the next generation.

Fig. 2. Cloning-free CRISPR/Cas9 system



The application for the cloning-free CRISPR/Cas9 system

This improved CRISPR/Cas system will be useful for a variety of applications, including the creation of humanized mice for the modeling of genetic diseases, drug metabolisms, immunity, and infectious diseases. Further, accurate targeted insertion will improve the safety of gene therapy in human patients in the future. Taken together, our streamlined cloning-free CRISPR/Cas-mediated *in vivo* genome editing system provides highly efficient and extremely convenient one-step generation of knockout and knock-in animals, leading to the acceleration of *in vivo* functional genomic research.

Cloning-free CRISPR/Cas system facilitates functional cassette knock-in mice, *Genome Biol*, doi: 10.1186/s13059-015-0653-x.