Development of the Generation Methods of Genome-modified Animals Using Engineered Endonucleases

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Genetically modified animals are useful when performing functional analysis of genes or genome-sequences *in vivo*. Instead of the conventional gene-targeting method using embryonic stem cells, genome-modified animals can be generated efficiently by using engineered endonucleases. My colleagues and I have developed and improved the engineered endonucleases-mediated genome-modification system.

Custom-designed zinc finger nucleases (ZFN) is a useful engineered endonuclease functional in various cells including mammalian embryos, but the previously reported ZFN-preparation methods have demerits in terms of cost, time, labour, and repeatability. We therefore established a novel construction method for ZFN expression plasmid vectors, which enables the repeated construction of intended ZFNs inexpensively in a short period of time. With this method, named OLTA (OverLap extension PCR and TA-cloning), and zygote-mediated genome-modification, we could generate various kinds of knockout mice in a month.

Next, we optimized the CRISPR/Cas system to zygote-mediated genome-modification by improving Cas9 and gRNA constructs and experimental conditions, and established a highly efficient system useful for multi-loci modification. By using this optimized tool, we generated large-scale genome-modified mice or multi-knockout mice by one-step, and confirmed the transmission of these mutation alleles to the next generations. This tool could be used for the oocytes and zygotes in diverse mammals such as rat or porcine.

Compared to knockout, the designable locus of gRNA is limited when generating knock-in animals, particularly the single-nucleotide substitutions or short sequence-insertions. If novel CRISPR/Cas systems capable of recognizing DNA sequences which are ignored by conventional engineered endonucleases can function with similar high efficiency as in conventional CRISPR/Cas within zygote, the broad use of the zygote-mediated genome-modification can be expected. We attempted to apply the offset-nicking method and orthologous CRISPR/Cas to zygote-mediated genome-modifications, and successfully generated knockout and knock-in mice.

We also developed a CRISPR/Cas9 system that could express functional gRNAs and Cas9 with a single RNA polymerase II promoter and induce multi-loci disruptions in specific cells. We expect this system is applicable to transgenic-mediated conditional knockout by linking it to a spatiotemporal expression promoter.

These systems can be applied to the reverse genetics studies in various cells and animals, and we expect that these will contribute to the advancement of agricultural studies.

References

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