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Review

Mouse Model for Optogenetic Genome Engineering

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Optogenetics, a technology to manipulate biological phenomena thorough light, has attracted much attention in neuroscience. Recently, the Magnet System, a photo-inducible protein dimerization system which can control the intracellular behavior of various biomolecules with high accuracy using light was developed. Furthermore, photoactivation systems for controlling biological phenomena are being developed by combining this technique with genome-editing technology (CRISPR/Cas9 System) or DNA recombination technology (Cre-loxP system). Herein, we review the history of optogenetics and the latest Magnet System technology and introduce our recently developed photoactivatable Cre knock-in mice with temporal-, spatial-, and cell-specific accuracy.

Key words: optogenetics, Cre recombinase

Optogenetics

Recently, the new technology of optogenetics has been actively applied to research. The term optogenetics is a newly coined word derived from opto, a word for light, and genetics. Optogenetics enables cell-specific photoactivatable ion channel and ion pump expression to artificially control their functions (membrane potential) using photic stimulation. Optogenetics originated with the discovery of a membrane protein, which deforms in response to light irradiation, in the photosynthetic *Chlamydomonas* [1]. Currently, three types of photoactivatable proteins, Channelrhodopsin 2, Halorhodopsin [2] and Archaerhodopsin [3] are frequently used in optogenetics research. Light irradiation between 400 and 600 nm induces protein deformation, leading to cell membrane depolarization or hyperpolarization due to ion influx or efflux from the cell (Na^+ influx into the cell and H^+, K^+ efflux for Channelrhodopsin2; Cl^- influx i for Halorhodopsin; H^+ efflux for Archaerhodopsin). Deisseroth and Boyden *et al.* first reported that neural activities could be artificially controlled by expressing Channelrhodopsin in nerve cells [4,5]. When light is irradiated onto nerve cells where Channelrhodopsin is expressed, cations flow into the cell through Channelrhodopsin leading to cell membrane depolarization, resulting in the generation of an action potential across the nerve cell membrane. This technology developed in the 2000s has quickly spread throughout the neuroscience field and is now an essential tool for studying the function of specific neural circuits in the brain. More recently, this technology was first applied in monkeys [6,7], demonstrating the possibility of optogenetic control of biological phenomena in primates. Thus, in the future, it can even be applied to humans.

"Magnet System" Technology

Focusing on a cryptochrome (Vivid, VVD) present in *Neurospora crassa*, Sato *et al.* successfully developed

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a photoactivatable protein called "Magnet System" by introducing an amino acid mutation in the VVD [8]. VVD is one of the smallest photoreceptors currently used as an optogenetic tool and its cofactor, flavin adenine dinucleotide, is abundantly present in eukaryotic cells. The VVD can switch from a monomer to a homodimer in response to blue light [9,10]. The Magnet System is composed of newly-developed photoswitch proteins named pMag/nMag, characterized by binding to each other in response to blue light (470±20 nm), and once the irradiation is stopped and their binding affinity lost, they dissociate. For instance, by genetically linking the photoswitch proteins (pMag, nMag) to the N-terminal fragment (X) and the C-terminal fragment (Y) of a target protein, respectively, binding (functional) and dissociation (nonfunctional) of the target protein can be controlled at will depending on the presence or absence of light irradiation. This system is advantageous in that it enables: (1) to control and analyze the behaviors of living cells and animals; (2) to control and analyze the target cells in milliseconds; and (3) to control them reversibly and non-invasively. Sato *et al.* reported various types of light-based technologies developed using the Magnet System, including a photoactivatable genome-editing tool, namely, the Photoactivatable-Cas9 (PA-Cas9) [11]. To control Cas9 activity, and therefore genome editing, with light, the Cas9 protein was divided into an N-terminal and C-terminal fragment, to which the pMag and nMag photoswitch proteins were respectively linked. PA-Cas9 allows genome editing in any target point and timing; thereby, the off-target effect, cause of concern from the conventional CRISPR/Cas9 system technology, may be attenuated. Recently, Takao *et al.* successfully performed genome editing using PA-Cas9 in mice on the Lif gene, which controls fertility [12]. Prior studies have reported light control of biological phenomena at the cellular level; however, in this study, uterine function and *in vivo* control were achieved for the first time in mice. This finding demonstrated that a photo-responsive system is also useful for *in vivo* investigations. Sato *et al.* also developed the Split-CRISPR-Cas9-based photoactivatable transcription system (CPTS) 2.0 technology, which enables control gene expression using light [13], leading to successful induction of differentiation from induced pluripotent stem cells to neural cells by photic stimulation.

Cre-loxP System

The widely used Cre recombinase (Cre)-loxP system is a DNA recombination system found first in bacteriophage P1. The Cre-loxP system can site-specifically remove all bases of a target gene between two loxP sequences when the DNA recombination enzyme Cre acts on the loxP sequence, which includes 34 bases. Thus, it is an essential research tool for the analysis of *in vivo* gene functions in mice. Crossing a mouse with Cre recombinase expressed downstream of a tissue-specific promoter, and a mouse (flox mouse) in which the loxP sequence is knocked in at both ends of the gene exon to be deleted, allows Cre-dependent tissue-/cell- specific gene deletion (conditional knock out). Our group also used this system to produce a flox mouse, which is a Runt-related transcription factor 2 (Runx2, a transcription factor essential to skeleton formation, a constitutive knockout mouse dies shortly after birth) knockout mouse, for the first time and has advanced analyses using various types of cell-type-specific Cre mouse lines [15-19]. Further, Cre activity may be temporally controlled by administering a Cre-carrying virus (*e.g.*, an adeno-associated virus vector) to any target site instead; however, direct administration has invasiveness issues, which are to be avoided for immune and cancer studies. A widely used non-invasive system, the tamoxifen (TAM) dependent (CreERT2) system, was developed by fusing an estrogen receptor mutant with the Cre recombinase. However, TAM is an antiestrogen, and as such, has its own pharmacological effects. Until recently, no other non-invasive and temporospatial-specific *in vivo* DNA recombination system based on the Cre system has been reported.

Photoactivatable-Cre System

In 2016, Sato *et al.* developed a photoactivatable-Cre (PA-Cre) system to artificially control gene recombination using "blue light" by combining the Cre-loxP and the Magnet systems [14]. In the PA-Cre system, the fragmented photoswitch proteins (pMag/nMag) were linked to the N-terminal fragment (CreN) and C-terminal fragment (CreC) of the inactivated Cre recombinase, respectively, to dimerize the Cre recombinase. Only when blue light is irradiated, binding between pMag and nMag allows the inactivated Cre recombinase fragments to come together, thus inducing

the formation of an activated Cre recombinase [8]. The PA-Cre System has the advantage of greatly improved DNA recombination efficiency in response to light irradiation. In addition, binding a photo-responsive o-nitrobenzyl caged biomolecule to the catalytic site of the Cre recombinase can better control light induced Cre recombinase activity [20]. The initial irradiation-dependent systems had the disadvantage of a certain cytotoxicity risk due to DNA damage by irradiated ultraviolet (UV) light, but this disadvantage is not present in the PA-Cre System as it employs no UV light. The development of a genetically modified mouse incorporating the PA-Cre system would enable non-invasive, temporal- and tissue-/cell-specific genetic control by irradiating light from outside of the body enabling to further broaden the scope of *in vivo* gene analysis technology. Our team developed such a model, as we will describe next.

Development of tTA-dependent Photoactivatable Cre-loxP Rrecombinase Knock-in (TRE-PA-Cre) Mouse

We focused on the tetracycline-induced expression system (Tet-On/Off) to add cell-type specificity to the spatiotemporal-specific PA-Cre System. The Tet-On/Off system enables reversible regulation of the expression of a target gene by administering doxycycline (DOX), a tetracycline-derivative antibiotic. In the Tet-On system, a target gene is expressed in the presence of DOX, an in the Tet-Off system gene expression occurs in the absence of DOX. The Tet-On/Off system uses Tet Repressor (TetR) and Tet Operator (TetO) sequences, which act on the *E. coli's* tetracycline resistance operon; the TetR is characterized by binding to the TetO sequence in the absence of tetracycline but not in its presence.

At first, the authors constructed a vector (TRE-PA-Cre vector; Fig. 1) with a tetracycline response element (TRE, a sequence composed of multiple TetO sequences in tandem) upstream of the PA-Cre gene (a nMag-fused CreN-terminal gene (CreN-nMag) and a pMag-fused CreC-terminal gene (CreC-pMag) linked through an intracellular self-cleaving peptide sequence (P2A) and conducted an *in vitro* functional verification test. A TRE-PA-Cre vector, CAG-FLEX-tdTomato vector (Cre-dependent tdTomato expression vector), and a Tetracycline Transactivator (tTA) were introduced into HEK293T cells and tdTomato expression observed under a fluorescence microscope to examine the influence of DOX addition/blue LED irradiation. The results showed that tdTomato was expressed where tTA was introduced and blue light was irradiated without adding DOX, while tdTomato was not expressed where tTA was not introduced and blue light was irradiated without adding DOX, and where tTA was introduced and blue light was irradiated with DOX added. In summary, the TRE-PA-Cre system enables control of Cre activity in a tTA-, DOX- and light-irradiation-dependent manner.

Next, the knock-in site for the TRE-PA-Cre system mouse was selected. Insertion of the TetO sequence into a random chromosome is likely to fail, while the insertion (knock-in) into a site in the vicinity of a housekeeping gene (*e.g.*, Actb) can be successfully achieved [21, 22]. In fact, in a variety of cell types the efficiency of gene transduction by tTA was improved downstream of the polyA site of the Actb gene [23].

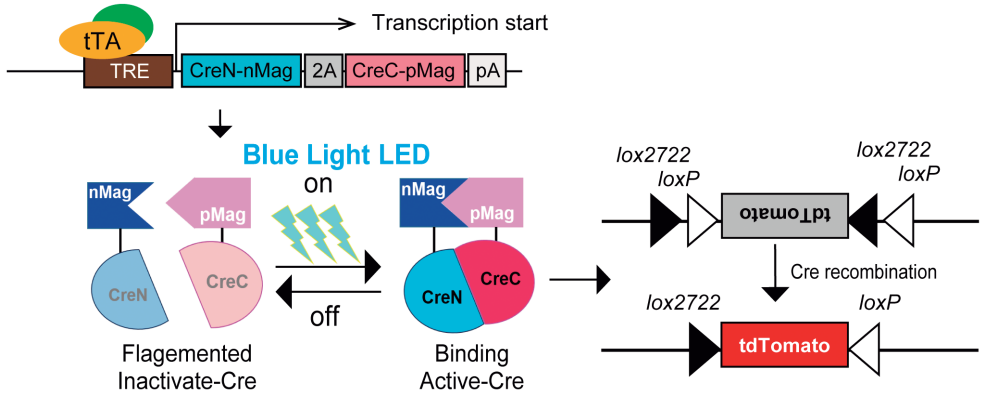

Fig. 1 Schematic presentation of the TRE-PA-Cre system. (Takao *et al.*, BBRC, 2020 [25], partially modified, reproduced with permission.)

This is probably because transcription is unlikely induced by TetO even in the presence of tTA once the TetO sequence is inserted into a silent site, where transcription from the chromosome does not occur. Based on the decisive reason, a TRE-PA-Cre targeting vector (Actb-TRE-PA-Cre) to knock in the Actb locus was prepared (Fig. 2).

To produce a TRE-PA-Cre mouse, chemically-synthesized Actb crRNA and tracrRNA, Cas9 proteins, and the Actb-TRE-PA-Cre targeting vector were injected into a fertilized egg by a rapid CRISPR/Cas9 knock-in technlogy [24]. The genome-modified fertilized egg was transplanted into the fallopian tube of a female mouse. PCR analysis of a newly born mouse demonstrated successful production of a mouse with accurate insertion of the TRE-PA-Cre sequence in the Actb 3′UTR locus (TRE-PA-Cre mouse).

Subsequently, it was evaluated whether the produced TRE-PA-Cre mouse would express PA-Cre in a tTA dependent manner and show *in vivo* activated Cre functionality in response to blue light. First, the TRE-PA-Cre mouse was crossed with a ROSA26-loxP-stop-loxP-tdTomato (Rosa26-tdTomato) mouse, capable of Cre-dependent tdTomato expression, to produce a TRE-PA-Cre; ROSA26-tdTomato mouse. Next, the tTA expression vector was introduced into the liver of the TRE-PA-Cre; ROSA26-tdTomato mouse by the hydrodynamic tail vein technique, which enables rapid vector transduction into the liver. Starting at 6 h after introduction of the tTA expression vector, the mouse was bred for 18 h under fluorescent or blue LED light. Thereafter, the liver was isolated and observed under a fluorescent microscope to look for tdTomato expression. Although tdTomato was not expressed in the liver of the mouse bred under fluorescent light, robust tdTomato expression was observed in the liver of the

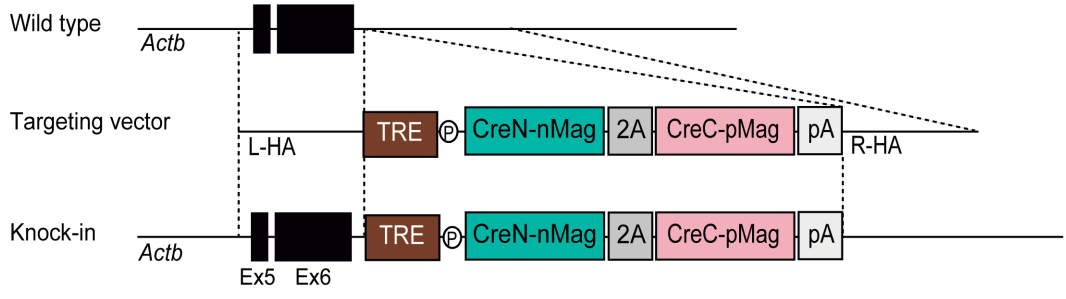

Fig. 2 Procedure for producing the TRE-PA-Cre knock-in-mouse. The targeting strategy for producing the TRE-PA-Cre mouse (Takao *et al.*, BBRC, 2020 [25], partially modified, reproduced with permission.)

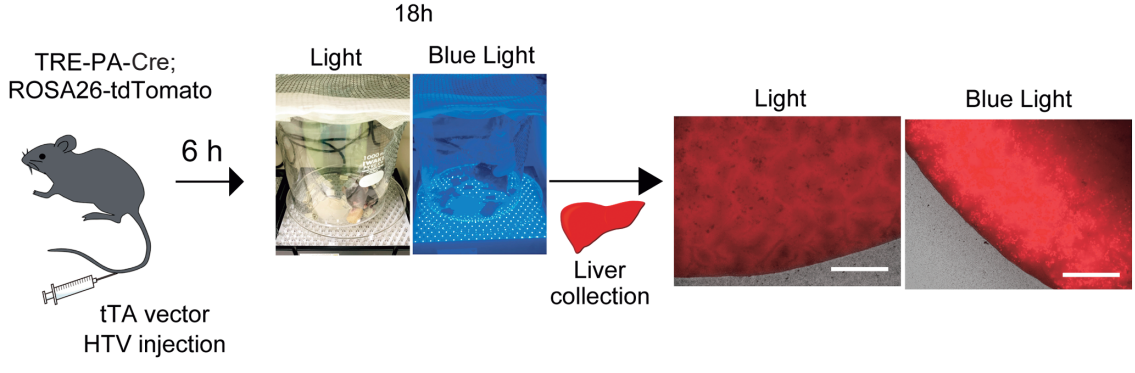

Fig. 3 Analysis using the TRE-PA-Cre knock-in-mouse. The TRE-PA-Cre mouse was crossed with the ROSA26-loxP-stop-loxP-tdTomato (Rosa26-tdTomato) mouse, which belongs to the Cre reporter line, to produce a TRE-PA-Cre: ROSA26-tdTomato mouse. The tTA expression vector was introduced into the liver of the produced mouse by the hydrodynamic tail vein injection method. 6 h after the injection, blue LED light (470 ± 20 nm, 134 W) was irradiated to the animals for 18 h. Then, the liver was collected to observe the expression of tdTomato under a fluorescent microscope. Bar: 1 mm. (Takao *et al.*, BBRC, 2020 [25], partially modified, reproduced with permission.)

mouse bred under blue light (Fig. 3). These findings demonstrated that our TRE-PA-Cre mouse can induce *in vivo* DNA recombination in a tTA-/light-irradiation-dependent manner [25]. The TRE-PA-Cre mouse can be obtained from the Riken BioResource Research Center (RIKEN BRC, https://mus.brc.riken.jp/ja/) (RBRC11090).

Conclusion

In this review, we discussed the Magnet system as novel technology for optogenetics, and introduced the photoactivatable Cre-loxP system with tetracycline system that we have developed (TRE-PA-Cre mouse). Crossing the TRE-PA-Cre mouse with a cell-specific tTA expression mouse enables cell-type- and time specific *in vivo* Cre-dependent DNA recombination. In addition, DOX administration to this mouse allows to switch off PA-Cre expression, leading to the suppression of light-irradiation-independent DNA recombination reaction (PA-Cre leakage). Thus, our mouse model would enables persistent labeling of the specifically marked cell, thus allowing lineage tracking, with potential applications to immunology as well as developmental biology and stem cell biology studies.

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