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TECHNICAL REPORT

Photoactivatable Cre knock-in mice for spatiotemporal control of genetic engineering in vivo

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Abstract

Although the Cre-loxP recombination system has been extensively used to analyze gene function in vivo, spatiotemporal control of Cre activity is a critical limitation for easy and precise recombination. Here, we established photoactivatable-Cre (PA-Cre) knock-in (KI) mice at a safe harbor locus for the spatial and temporal regulation of Cre recombinase activity. The mice showed whole-body Cre recombination activity following light exposure for only 1 h. Almost no leaks of Cre recombination activity were detected in the KI mice under natural light conditions. Spot irradiation could induce locus-specific recombination noninvasively, enabling us to compare phenotypes on the left and right sides in the same mouse. Furthermore, long-term irradiation using an implanted wireless LED substantially improved Cre recombination activity, especially in the brain. These results demonstrate that PA-Cre KI mice can facilitate the spatiotemporal control of genetic engineering and provide a useful resource to elucidate gene function in vivo with Cre-loxP.

Introduction

The Cre-loxP conditional gene regulation system is an important technology that can be used to understand the spatiotemporal functions of a targeted gene in vivo. In tissues or cells where Cre recombinase is expressed, a genomic region flanked by two loxP sequences can be removed by Cre-mediated recombination. The expression of Cre is controlled by tissue-specific and chemically inducible promoters, such as the tetracycline (Tet)-on/off gene expression system or tamoxifen-inducible Cre-ERT2 system, and many Cre-transgenic and knock-in (KI) mice have been established for conditional knockout (KO) models [1–3]. However, these approaches have several problems with spatiotemporal resolution. For example, tissue-specific promoters often induce Cre expression in other tissues or cells as well as targeted tissues, causing unexpected phenotypes [1]. In addition, chemically inducible Cre shows leaky chemical-independent Cre activity. As well as leaks, the use of chemicals introduces limitations related to cytotoxicity and target site accessibility [4, 5]. Under these circumstances, it is difficult to choose ideal Cre driver strains from databases such as the Jackson Laboratory's Cre Portal [2, 6]. Thus, a novel approach for the spatiotemporal regulation of Cre is still needed.

The recent innovation of photoactivatable tools has allowed rapid and reversible control of protein activity to be achieved with spatiotemporal precision [7–10]. Photoactivatable recombinase systems have been developed as highly efficient tools to optogenetically control genome engineering in mammalian cells [11–14]. These systems are based on the reassembly of split recombinase fragments by light-inducible dimerization tools such as the Magnet system, which consists of two photoswitches called positive Magnet and negative Magnet [15]. The photoactivatable-

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Cre (PA-Cre) system enables the highly efficient induction of DNA recombination under blue light illumination in vitro. Furthermore, induction of the system in the liver via hydrodynamics-based delivery enables in vivo recombination through external illumination. While there are several transgenic models harboring PA-Cre in species such as zebrafish [16], use of the method in mammals is primarily limited to hepatocytes and neurons via the use of adeno-associated virus vectors, which have low transduction efficiencies [12, 17]. Therefore, there is an immediate need for a more versatile system to enable efficient PA-Cre-mediated genetic engineering for in vivo applications.

To facilitate broader applications of the PA-Cre system in vivo, we established a KI mouse strain at the *Rosa26* safe harbor site using the "two-hit by [guide] (g)RNA and two oligos with a targeting plasmid" (2H2OP) method [18]. PA-Cre recombination activity in KI strains was characterized in detail by crossing Cre-reporter KI mice expressing green fluorescent protein (GFP) before and red fluorescent protein (DsRed) after Cre-mediated recombination [19]. We also applied blue spotlights and wireless LED devices to specifically illuminate several tissues in adult mice for the efficient spatiotemporal control of genetic engineering in vivo.

Materials and methods

Construction of plasmids, RNAs, and single-stranded oligodeoxynucleotides (ssODNs)

For the KI PA-Cre-expressing plasmid, previously reported PA-Cre sequences from the donor [12] were amplified and integrated into *Xho*I and *Bgl*II sites in the pCAGGS vector (RDB08938, Riken BRC DNA bank, Tsukuba, Japan) using an In-Fusion HD Cloning Kit (Takara Bio Inc., Shiga, Japan). Cas9 mRNA was transcribed in vitro using an mMESSAGE mMACHINE T7 Ultra Kit (ThermoFisher Scientific Inc., Waltham, MA, USA) from a linearized plasmid (ID#72602; Addgene, Watertown, MA, USA) and purified using a MEGAclear kit (ThermoFisher Scientific). Guide (g)RNAs targeting the mouse *Rosa26* locus, and the donor plasmid and ssODNs to integrate donor DNA were also prepared as previously reported [18]. Sequences of target sites and ssODNs are listed in Supplementary Table 1.

Animals

C57BL/6JJcl mice were obtained from CLEA Japan Inc. (Tokyo, Japan). The floxed reporter KI strain (C57BL/6N-Gt(ROSA)26Sor$^{tm1(CAG-EGFP/tDsRed)Utr/Rbrc}$) was provided by Riken BRC (ID# RBRC04874). Animals were kept under conditions of 50% humidity and a 12:12 h light:dark cycle. They were fed a standard pellet diet (MF, Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum. Animal care and experiments conformed to the Guidelines for Animal Experiments of Osaka University, and were approved by the Animal Research Committee of Osaka University.

Generation of PA-Cre KI mice

Briefly, mouse eggs were collected from female C57BL/6JJcl mice that were superovulated by injection with pregnant mare serum gonadotropin (Aska Pharmaceutical Co., Tokyo, Japan) and human chorionic gonadotropin (Aska Pharmaceutical Co.), and zygotes were prepared by in vitro fertilization. Then, 50 ng/μL Cas9 mRNA, 25 ng/μL gRNAs, 25 ng/μL ssODNs, and 2 ng/μL donor DNA were microinjected into the male pronuclei of embryos using a micromanipulator (Narishige, Tokyo, Japan). Two-cell embryos were transferred into pseudopregnant females and founder pups were obtained.

For genotyping analysis, DNA was extracted from tail biopsies using the KAPA Express Extract DNA Extraction Kit (Kapa Biosystems, London, UK), and PCR was performed with Tks Gflex DNA Polymerase(Takara Bio Inc.). The cycling protocol was 95 °C, 2 min; then 35 cycles of 98 °C, 10 s; 60 °C, 10 s; 68 °C, 30 s. PCR products were electrophoresed on a 2% agarose gel, then directly sequenced using the BigDye Terminator v3.1 cycle sequencing mix and an Applied Biosystems 3130 DNA Sequencer according to the manufacturer's protocol (ThermoFisher Scientific). Primer sequences are listed in Supplementary Table 1.

Southern blotting

Southern blotting was performed to identify the targeted KI at the *Rosa26* locus as previously reported [18]. Briefly, genomic DNA was purified from the liver of all founders using phenol/chloroform extraction. After digestion of the DNA with appropriate restriction enzymes (*Eco*RV and *Bgl*II to confirm the correct KI and *Dra*I to determine the single copy number KI), 10-μg samples of digested DNA were separated by electrophoresis on 0.8% agarose gels and blotted onto positively charged nylon membranes (Roche, Basel, Switzerland). UV-crosslinked membranes were hybridized with ^{32}P radioisotope-labeled DNA probes for Cre, and 5′ and 3′ loci of *Rosa26*, then washed and exposed to X-ray film. All processes were performed at the Institute of Immunology Co., Ltd. (Tokyo, Japan).

Reverse transcription (RT)-quantitative (q)PCR

To investigate the expression pattern of PA-Cre among tissues, total RNA was purified from several tissues of PA-

Cre KI and C57BL/6 mice by the Maxwell RSC simplyRNA Tissue Kit (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. cDNA was synthesized with the ReverTra Ace qPCR RT Master Mix (TOYOBO Co. Ltd, Osaka, Japan) and real-time RT-PCR was performed using the SsoAdvanced Universal SYBR Green Supermix and a CFX Connect device (BIO-RAD Inc., Hercules, CA, USA). The number of target molecules was normalized against those of *Gapdh* and *Actb* as internal controls. Sequences of the primers used are listed in Supplementary Table 1.

Photoactivation of Cre in KI mice by blue light sources

The PA-Cre KI strain was mated to the floxed reporter KI strain, and F_1 mice were used in all experiments. To activate Cre protein by blue light throughout the mouse body, pups at postnatal day 1 (P1) were placed on a transparent thermal plate with a glass surface (Kitazato Corp., Tokyo, Japan) and exposed to 200 W/m^2 blue light from an LED array (ISL-150×150-HBB; CCS Inc., Kyoto, Japan) for 30 min. The procedure was repeated after 2 h to avoid the potential risk of cellular damage especially in eyes by strong blue light stimulation [20]. All treated mice were kept under natural light conditions before autopsy and histological analysis. F_1 mice that were not subjected to blue light illumination were also kept under natural light conditions and dissected at several time points to detect leaky Cre activation.

To demonstrate noninvasive Cre activation in adult mice, we prepared an inhalational anesthesia system (Natsume Seisakusho Co., Ltd., Tokyo, Japan) and fiber blue light illuminator device (PJ2-3005-4CA-PE, HLV3-22BL-4-NR, and HFS-14-500; CCS Inc.). Hair around the targeted regions (top of the head, abdomen, and groin) was shaved under anesthesia, and the regions were illuminated on the skin by maximum blue light output for 1 h. Autopsies were performed 2 weeks after illumination.

To investigate blue light illumination of tissues in freely moving mice, we obtained a wireless LED lighting system (X-Base, XB001WPS30, and XB004LBS; Happinet Co., Tokyo, Japan). We coated the X-Base wireless blue LED with a clear coating of nail varnish to prevent it from getting wet and then transplanted it under the skin at the head, abdomen, and groin. Treated mice were kept in a cage on the X-Base Wireless Power Station for 2 weeks. If mice removed an LED by scratching, it was retransplanted at the same position.

Autopsy and histological analysis for the detection of Cre activation

Mice under 3 weeks of age were sacrificed by cervical dislocation under anesthesia with isoflurane. Mice older than 3 weeks were treated by perfusion fixation with 4% paraformaldehyde phosphate buffer solution (Nakarai Tesque Inc., Kyoto, Japan) under anesthesia with a mixture of medetomidine, midazolam, and butorphanol. Tissues were resected and observed macroscopically under fluorescent light. They were then washed with phosphate-buffered saline and fixed in 4% paraformaldehyde phosphate buffer solution for at least 24 h. A section of each tissue was also collected for genetic analysis.

All collected tissues were embedded in optimal cutting temperature compound medium (Sakura Finetek Japan Co., Ltd, Tokyo, Japan) and frozen 10-μm sections were prepared using a cryostat (CM3050S, Leica Microsystems, Wetzlar, Germany). GFP and DsRed fluorescence in sections was analyzed using the All-in-One fluorescence microscope (BZ-X810; Keyence Co., Osaka, Japan). We determined the area of GFP and DsRed expression in five images from each tissue via BZ-X analysis software (Keyence Co.), and calculated the recombination efficiency using the average ratio of DsRed-expressing area to total tissue area in each image. Statistical analyses were performed using Student's *t* tests following Bonferroni correction.

Genetic analysis for the detection of Cre activation

Genomic DNA from 10 mg of each sample was extracted using the NucleoSpin Tissue XS kit (Takara Bio Inc.) according to the manufacturer's protocol. Target loci were amplified using Tks Gflex DNA Polymerase (Takara Bio Inc.). The cycling protocol was 95 °C, 2 min, then 35 cycles of 98 °C, 10 s; 60 °C, 10 s; 68 °C, 30 s. The PCR amplicons were detected by the MultiNa microchip electrophoresis system and DNA-2500 Kit (Shimadzu, Kyoto, Japan) according to the manufacturer's protocol. Primer sequences are listed in Supplementary Table 1.

Results

Generation of PA-Cre KI mice

To generate KI mice expressing the PA-Cre protein in their entire body, a 7.0 kb PA-Cre expression plasmid under the control of the CAG promoter was introduced at the *Rosa26* locus using the previously reported 2H2OP method (Supplementary Fig. 1a). We co-injected a mix of 50 ng/μL of Cas9-poly(A) mRNA, 25 ng/μL of each of the two gRNAs, 25 ng/μL of each of the two ssODNs, and 2 ng/μL of the CAG-PA-Cre plasmid into 438 C57BL/6J embryos. Following transplantation of 316 two-cell embryos, 3 of 22 founders showed specific amplification of PA-Cre sequences (Supplementary Fig. 1b). Primer sets designed for either side of each *Rosa26* and CAG-PA-Cre junction (F1-R3 or

F3-R1 in Supplementary Fig. 1c) amplified the junction sequences in Founder No. 7. Sequencing analysis of the PCR product demonstrated precise ligation at the upstream sites and ligation with a 32-bp insertion at the downstream sites. Southern blotting confirmed the integration of one copy of the plasmid into the *Rosa26* region (Supplementary Fig. 2). Crossing the No. 7 founder and wild-type C57BL/6 mice resulted in faithful transmission of the PA-Cre allele to the next generation.

To confirm the expression pattern of PA-Cre integrated at the *Rosa26* locus, we performed RT-PCR on RNA extracted from several tissues. PA-Cre was shown to be stably expressed throughout the entire body, although a variation in expression intensity was detected among tissues (Supplementary Fig. 3). PA-Cre expression in the lung was about 15 times higher than in the brain, which showed the lowest expression. These variations were expected because of CAG promoter activity at the *Rosa26* locus. Based on these results, the PA-Cre KI strain established from the No. 7 founder was named C57BL/6J-*Gt(ROSA)26Sor*$^{tm1(CAG\text{-}PA\text{-}Cre)Iexas}$ and characterized in subsequent experiments.

Optogenetic recombination in PA-Cre KI mice

To verify whether Cre-mediated recombination could be induced by blue light illumination of a PA-Cre KI mouse, the PA-Cre KI strain was crossed with color-convertible Cre-reporter KI mice (C57BL/6N-*Gt(ROSA)26Sor*$^{tm1(CAG\text{-}EGFP,tdsRed)Utr}$) carrying the CAG promoter and *loxP*-flanked enhanced GFP and DsRed sequences at the *Rosa26* locus. In F_1 mice, non-recombined cells expressed GFP, whereas recombined cells (by Cre activation) expressed DsRed protein. Therefore, PA-Cre activated cells could be visually detected via blue light as DsRed-expressing cells in the entire body (Fig. 1a).

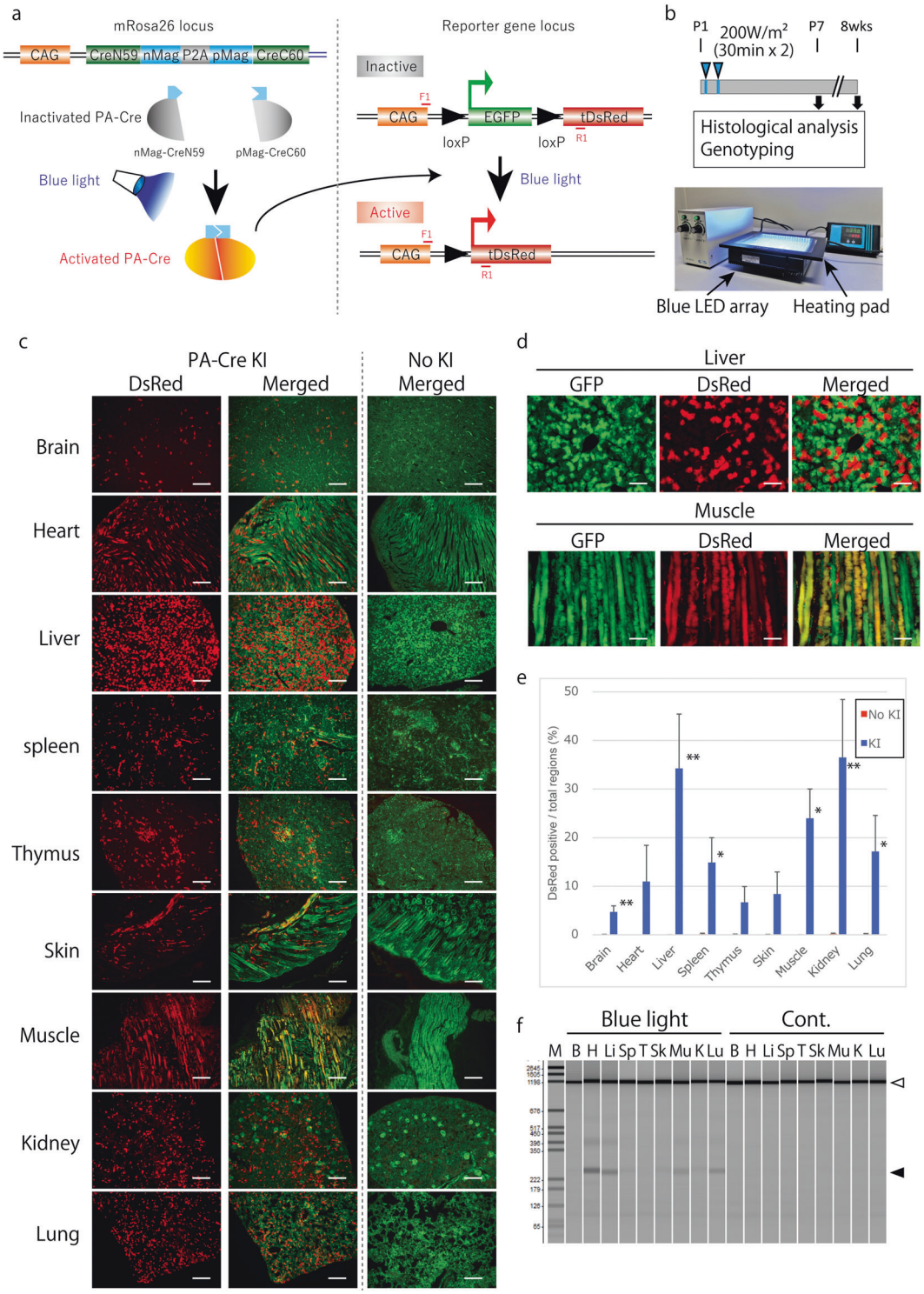
An autopsy was conducted on F1 pups 1 week after exposing them to blue light (Fig. 1b). The whole bodies of the mice were macroscopically red (Supplementary Fig. 4), and histological analysis of nine representative tissues demonstrated that DsRed expression was significantly increased, indicating that efficient recombination had occurred via activated PA-Cre (Fig. 1c). High-magnification images demonstrated that cells expressing either GFP or DsRed were detected randomly in tissues such as the liver, whereas both GFP- and DsRed-expressing cells were detected in skeletal muscles of the thigh. This is likely because skeletal muscle fibers are multinucleate cells so would include both alleles recombined by PA-Cre and non-recombined alleles (Fig. 1d).

Measurement of the red fluorescence conversion in each tissue revealed >20% recombination via PA-Cre in the liver (34.2 ± 11.2%), muscle (24.0 ± 6.0%), and kidney (36.5 ± 11.9%) (Fig. 1e), which are tissues located subcutaneously and thus easily exposed to blue light. In contrast, the brain (4.7 ± 1.2%), heart (11.0 ± 7.5%), spleen (14.9 ± 5.1%), skin (8.4 ± 4.5%), thymus (6.7 ± 3.2%), and lung (17.1 ± 7.4%) showed relatively low recombination activity. The variation in Cre activity among tissues may reflect differences in PA-Cre expression, as we show by RT-PCR, as well as the reduced likelihood of blue light reaching deeper tissues such as those located in the skull or under the ribs. Overall, recombination activity was significantly higher in all tissues of KI mice than in those of non-KI mice. Genetic analysis by PCR detected the expected short band representing Cre-mediated recombination in the genome of all tissues which confirmed our histological analysis (Fig. 1f). These results reveal that blue light illumination of the entire P1 body for 1 h induces PA-Cre-mediated recombination at an efficiency exceeding 30% in several tissues.

To investigate whether the recombined cells are maintained over time following illumination, KI mice that were illuminated by blue light at P1 were sacrificed and characterized after 8 weeks of age. Histological and genetic analyses demonstrated PA-Cre recombination in the whole body (Supplementary Fig. 5). The efficiency of PA-Cre recombination tended to be decreased compared with mice examined 1 week after illumination, except in the heart and thymus, but there was no significant difference compared with mice examined 2 weeks after illumination. These results indicate that many cells induced to recombine via blue light remain for at least 2 months after illumination.

Low leaky activation of PA-Cre under natural light conditions

One of the critical limitations of the Cre-loxP system being controlled by a tissue-specific promoter or Tet-on/off system in vivo is the leakage of Cre activity at unexpected locations and times. The PA-Cre system shows almost no leakage of Cre activity in vitro when transfected cells are maintained in a dark incubator [12]. However, it is unknown whether KI mice show leaks of Cre activation caused by stable expression over time under natural light. Therefore, we bred F_1 mice under natural light conditions and sequentially evaluated PA-Cre recombination activity without blue light illumination. Initially, we checked for Cre activity leaks on day E18.5 in F_1 embryos not exposed to natural light from the outside. Histological analysis revealed no leaks of Cre-mediated recombination in embryos just before birth (Fig. 2a). At 1 week (P7) after birth, pups also showed no leaks in any tissue (Fig. 2b). In contrast, half of the adult mice at 8 weeks of age showed leaks of PA-Cre-mediated recombination (Fig. 2b). Histological analysis detected some red fluorescent cells, especially in the heart (2.2 ± 2.6%), liver (2.2 ± 2.5%), and thymus (2.1 ± 3.1%). The highest leakage was observed in an individual showing



about 5–7% recombination in the heart, liver, and thymus by histological analysis. These cells were absent from the tissues of floxed reporter mice not expressing PA-Cre with and without blue light illumination, indicating that PA-Cre KI mice can show slight leaks of Cre activity when maintained under natural light conditions for 2 months or more. Genetic analysis by PCR confirmed the leaky phenotype in PA-Cre KI mice (Fig. 2c–e), suggesting there is a risk for PA-Cre activation leakage during long-term experiments. However, the average leakage of Cre activity was <3%, which is much lower than seen after blue light illumination for 1 h (Fig. 2d). Therefore, we conclude that the PA-Cre KI

◀ **Fig. 1 Optogenetic Cre activation in the whole body of PA-Cre KI mice. a** Schematics of the detection of PA-Cre-mediated recombination via fluorescence. In F_1 mice expressing split PA-Cre proteins and an EGFP protein from each *Rosa26* locus, blue light illumination activated PA-Cre, which removed floxed regions. Recombined cells express DsRed protein. **b** Preparation of the blue LED device with a heating pad and outline of the in vivo illumination experiments. Illumination for 30 min on the heating pad was repeated after 2 h, and all treated mice were kept under natural light until sacrifice. **c** Representative histology of PA-Cre KI and no KI in each tissue at P7. Scale bar = 200 µm. **d** Enlarged images in the liver and muscle at P7. Either GFP- or DsRed-expressing cells were detected in the liver section, whereas multinucleate cells in skeletal muscles expressed both GFP and DsRed protein. Scale bar = 50 µm. **e** Recombination efficiency in five areas of each tissue 1 week after whole-body illumination. Average data and standard deviations (error bars) were obtained from four pups. $*p < 0.05$; $**p < 0.01$. **f** Electrophoresis of the PCR products with primers F1 and R1, which are shown in (**a**). White arrowhead: wild allele; black arrowhead: recombined allele. B brain, H heart, Li liver, Sp spleen, T thymus, Sk skin, Mu muscle, K kidney, Lu lung.

strain is a good Cre model that has little noise under natural light conditions and that conditionally induces Cre activation via blue light.

Spatiotemporal Cre-mediated recombination via blue spotlight illumination

Conditional KO mice with the Tet-on/off and Cre-ER systems are widely used for various genetic analyses and human disease research. In contrast, the use of doxycycline (Dox) or tamoxifen as derivatives to induce these systems often results in toxicity, and systems in some tissues cannot be activated by basic administration strategies which can limit versatile conditional KO [1, 4]. Therefore, we used blue light illumination of the brain, liver, and muscle of F_1 mice at 8 weeks of age to investigate whether noninvasive and tissue-specific Cre activation could be achieved in adults (Fig. 3a). We illuminated the right side of the head, abdomen, and groin by a 600 mW/cm^2 blue spotlight for 1 h under anesthesia and dissected mice after 3 weeks (Fig. 3b). Macroscopic observation and histological analysis indicated significant PA-Cre recombination in the illuminated side of the liver and muscle (Fig. 3c, d). The recombination efficiency was 4.4 ± 3.7% ($p = 0.28$) in the brain, 23.8 ± 0.8% ($p < 0.01$) in the liver, and 61.0 ± 17.5% ($p < 0.01$) in the muscle (Fig. 3e). The opposite side of the same individual (not exposed to light) showed the same recombination efficiency as nonactivated controls (Fig. 3e). Genotyping analysis also revealed Cre recombination activity which confirmed histological findings (Fig. 3f). These results indicate that blue light illumination during adulthood, as well as whole-body illumination at P1, can specifically activate the PA-Cre locus. Therefore, we conclude that it is possible to easily and spatiotemporally control PA-Cre activity via blue spotlight.

A limitation of using blue spotlight is the short time available for illumination under anesthesia. To overcome this, we applied the wireless LED lighting system X-Base to continuously illuminate specific tissues of freely moving mice. X-Base consists of a wireless power supply station and small blue LEDs ~4.5 mm in diameter and height. We implanted the LEDs of 240 µW/cm^2 power under the skin of the head, abdomen, and groin of F_1 mice at 8 weeks of age (Fig. 4a). The implanted mice could move and live freely in their cage (Supplementary Videos 1 and 2). At autopsy, we observed high DsRed expression, indicating PA-Cre activation, especially in the cortex and cerebellum of the brain, as well as in the liver and muscle (Fig. 4b). Histological analysis demonstrated that recombination efficiency in the liver was similar to that achieved with spotlight illumination (23.2 ± 8.7% in the liver; $p < 0.01$), whereas efficiency in the brain and muscle was significantly improved (25.7 ± 6.2% in the brain; $p < 0.01$ and 81.2 ± 11.1% in the muscle; $p < 0.01$) (Fig. 4c, d). Genotyping also showed strong signals of shortened bands, representing a high recombination efficiency (Fig. 4e). The improvements in efficiency enabled the visualization of individual neurons such as Purkinje cells in the cerebellum and neuronal projections from the cerebral cortex to the corpus callosum or pons (Fig. 4f, g). These results indicate that long-term illumination of free-moving mice via the X-base system could improve the PA-Cre recombination efficiency, especially in the brain.

Discussion

In this study, we established a novel PA-Cre KI mouse and characterized the utility of the strain for spatiotemporal applications of the Cre-loxP recombination system. Main strategies for introducing the Cre gene into animals in vivo include direct introduction of the Cre construct via lentivirus, adenovirus, or adeno-associated virus infection, hydrodynamics-based transfection, and in utero electroporation [21–25].

The targeted locus of Cre-mediated recombination can be precisely controlled using the appropriate introduction method. However, surgical procedures such as intravenous injection and intraventricular administration require sophisticated skills and devices, and sometimes additional animals to compensate for the number of unsuccessful procedures. Producing injected animals for each experiment is another limitation. One method to circumvent these problems is the generation of Cre-transgenic animals with tissue-specific, chemically inducible promoters [1–3]. The use of high-quality transgenic strains allows continuous and reproducible experiments to be performed. In this study, therefore, we integrated a reliable PA-Cre recombinase

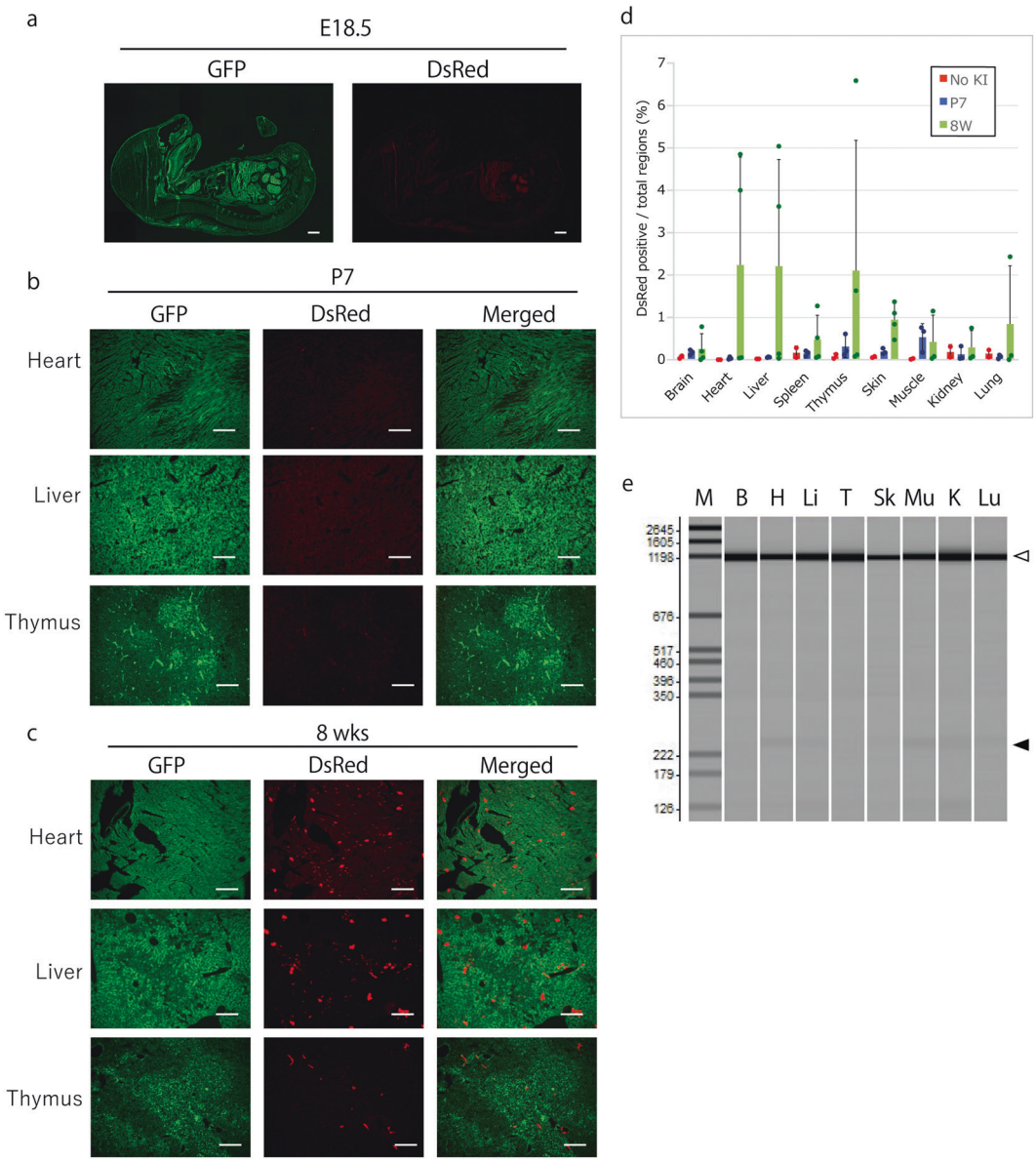


Fig. 2 Leaky activation of PA-Cre in KI mice under natural conditions. a–c Representative histology in each tissue at E18.5, P7, and 8 weeks without blue light illumination. **a** Scale bar: 1 mm. **b** Scale bar: 200 µm. **c** Several recombined cells with leaky Cre activation were detected in each tissue in 8-week-old mice. Scale bar: 200 µm. **d** Recombination efficiency in five areas of each tissue under natural conditions at P7 and 8 weeks (8 W). Average data and standard deviations (error bars) were obtained from four pups. **e** Electrophoresis of PCR products. White arrowhead: wild-type allele; black arrowhead: recombined allele. B brain, H heart, Li liver, T thymus, Sk skin, Mu muscle, K kidney, Lu lung.

using the Magnet system into the *Rosa26* safe harbor locus under the control of the CAG promoter, which was shown to be a strong and ubiquitous promoter [26, 27]. Strong and stable expression of the PA-Cre system enabled us to activate Cre and induce recombination by illumination for 1 h. Crossing the KI mice with various flox strains induced conditional KO mice that were spatiotemporally controlled by illumination. This strain is highly versatile and could be used to investigate carcinogenesis or function as a model of neurological disease.

One of the limitations of Cre KI is the nonspecific recombination that occurs following uncontrolled Cre activation. In particular, a number of transgenic animals with tissue- or cell-specific promoters have been reported to show leaks at locations other than the targeted locus [28, 29]. Although chemically inducible systems have been developed to overcome this problem, it remains difficult to completely control Cre activation at only the targeted site [30]. Indeed, Cre expression under the Tet-on system often results in leaks without Dox in vivo, so expression under the

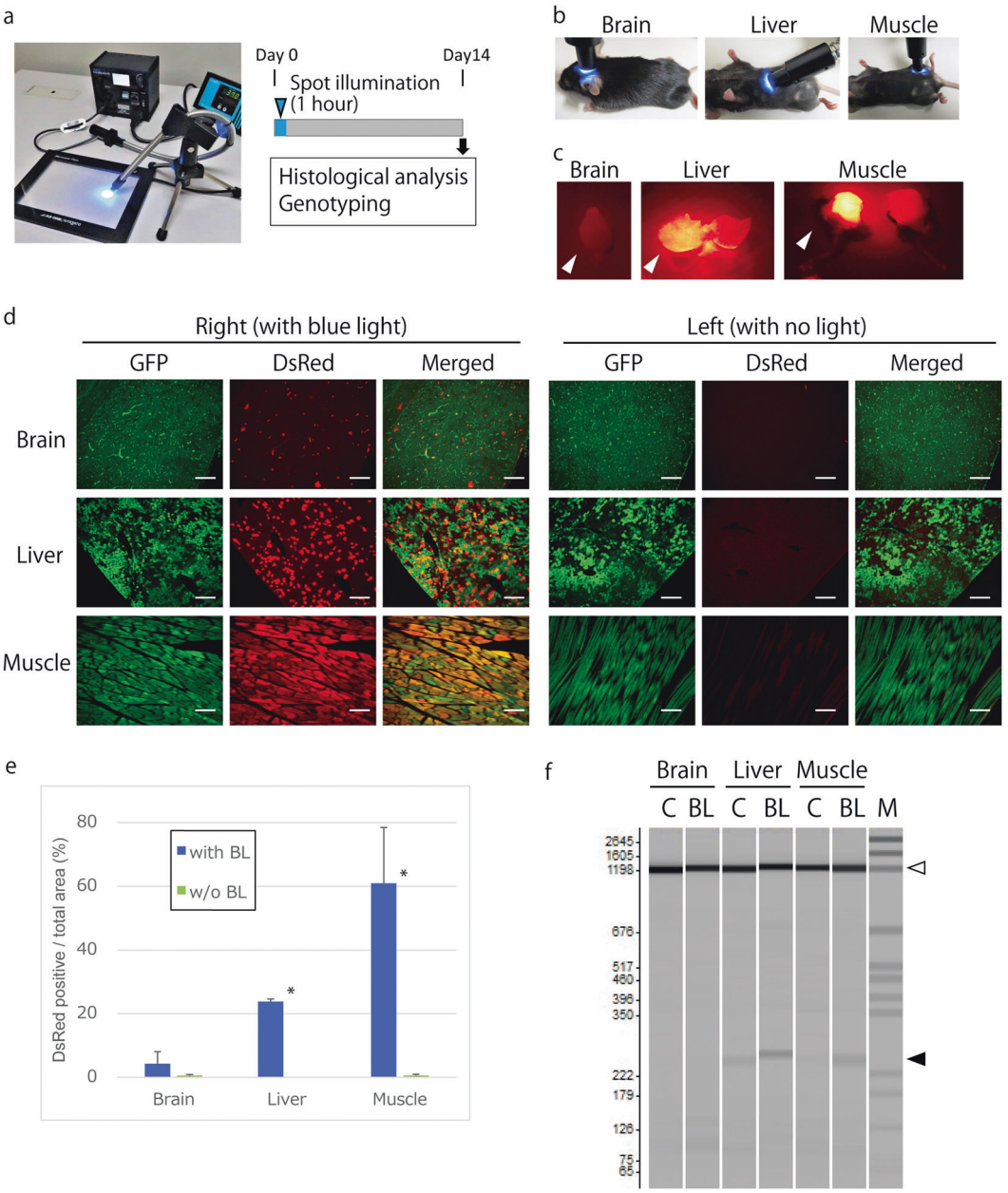


Fig. 3 Tissue-specific PA-Cre recombination by blue spotlight illumination. a, **b** Preparation of the spot LED device with a heating pad and outline of the in vivo illumination experiments. An anesthetized 8-week-old mouse was illuminated for 1 h on the heating pad as shown in (**b**), and all treated mice were kept under natural light conditions until sacrifice. **c** Macroscopic observation of the tissues with an LED device for the excitation of red fluorescent protein. Arrowhead: illuminated side. **d** Representative histology in each tissue with and without spotlight illumination. Scale bar: 200 µm. **e** Recombination efficiency of illuminated and nonilluminated sides in each tissue in F_1 mice. Average data and standard deviations (error bars) were obtained from three pups; $*p < 0.01$. **f** Electrophoresis of the PCR products. White arrowhead: wild-type allele; black arrowhead: recombined allele; C: nonilluminated control locus; BL: locus illuminated with a blue spotlight device.

Tet-off system should be suppressed continuously with Dox until the appropriate timing. In addition, the use of chemicals to control expression systems has the limitations of target tissue accessibility and host toxicity [31]. In this study, PA-Cre KI mice showed almost no Cre activity at a young age, suggesting almost no leaks of Cre activity in the absence of blue light illumination. However, some individuals showed slight leaks, probably from natural light during long-term maintenance. We maintained the mice under natural conditions to avoid adverse effects caused by the loss of the light/dark cycle. Thus, it may be possible to minimize the leakage of Cre activity by keeping mice in the dark or in the presence of filters that block blue light wavelength [32, 33]. Nevertheless, we demonstrated only

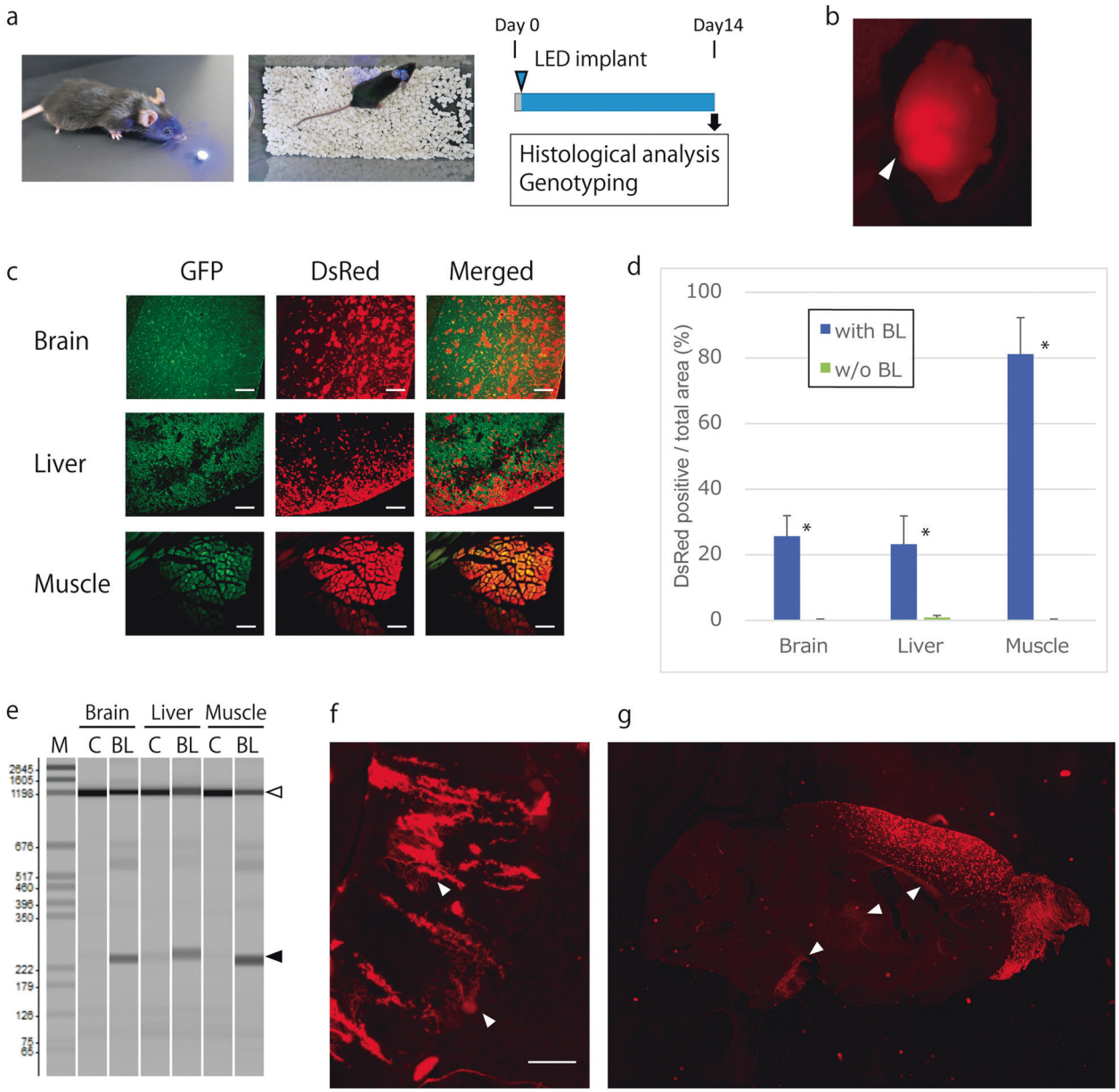


Fig. 4 Spatiotemporal PA-Cre activation in free-moving mice with wireless LED. a A tiny wireless LED device and free-moving mice in which the LED was transplanted at the head and groin. Outline of the illumination experiments is also shown. **b** Macroscopic observation of the brain for the excitation of red fluorescent protein 2 weeks after LED implantation. Arrowhead: locus illuminated by blue LED. **c** Representative histology in each tissue with LED illumination. Scale bar: 200 µm. **d** Recombination efficiency of illuminated and nonilluminated sides in each tissue in F_1 mice. Average data and standard deviations (error bars) were obtained from four pups. $^*p < 0.01$. **e** Electrophoresis of the PCR products. White arrowhead: wild-type allele; black arrowhead: recombined allele; C: nonilluminated control locus; BL: locus illuminated with a blue LED device. **f** Enlarged images of the neurons expressing DsRed in the brain. Scale bar: 50 µm. **g** Projection of the neuronal pathway through the corpus callosum and pons from the cortex.

low or no leak of Cre activation, suggesting that PA-Cre can be activated stably and safely at only the target site.

The great advantage of the PA-Cre system is the possibility of genetic manipulation with high spatiotemporal resolution using blue light. We succeeded in inducing Cre-mediated recombination at several targeted regions such as the liver, heart, and muscle by partial external illumination. Furthermore, we facilitated long-term illumination with blue light by embedding wireless LEDs in free-moving mice. As a result, we successfully induced Cre-mediated recombination at a significantly higher efficiency in the brain. By using this wireless device, multiple sites can be activated easily and simultaneously.

The PA-Cre recombination activity depends on the duration and intensity of blue light illumination in a cell line, which was previously reported [12]. PA-Cre quickly

forms a heterodimer upon blue light illumination, but the complex dissociates slowly over several hours after illumination stops (t1/2, 1.8 h), which enables efficient recombination to occur over short periods such as 30 s [15]. Nevertheless, continuous exposure to illumination for 1, 3, or 24 h could increase the recombination activity. In addition, as the intensity of the blue light was increased from 1 to 50 W/m^2, the recombination efficiency was increased more in a previous study [12]. Our KI mice showed that long-term illumination with wireless LEDs increased the recombination efficiency in the brain. Therefore, PA-Cre recombination activity could be increased by improving the intensity and duration of blue light illumination such as by the transplantation of multiple devices of higher intensities. Multiple transplantation could be possible in subcutaneous tissue, although it would be difficult in the brain because of the LED's size. Taken together, these results demonstrate the simple, spatiotemporal, and efficient activation of Cre according to the purpose of the in vivo study. Recently, a tiny wireless optoelectronic device that can be sealed in the mouse body has been reported [34]. The development of such devices will further facilitate the availability of KI mice.

During the characterization of PA-Cre recombination in KI mice, we detected variations in recombination activity in some tissues. The skin, which was expected to have the highest PA-Cre recombination efficiency because it receives the most blue light exposure, showed a surprisingly low efficiency compared with other tissues. Histopathological images of the skin after blue light exposure revealed that capillary cells in the extracellular matrix and subcutaneous muscle tissue under the hair follicles expressed DsRed, whereas the hair follicles still expressed GFP. These results indicate that hair follicles, which include pigment stem cells, hair follicle stem cells, and melanocytes, resist PA-Cre recombination because the melanin produced in these cells of a black coat C57BL/6 background absorbs blue light. This limitation could be resolved by knocking out the Tyr gene or backcrossing with albino strains such as Balb/c. Recombination in the brain with a wireless LED also showed patchy red fluorescence (Fig. 4g). The observations in Purkinje cells of the cerebellum support the fact that PA-Cre in neurons becomes active after blue light exposure, whereas it can also be expected that some cell types will be sensitive/resistant to this exposure.

In summary, the PA-Cre KI mouse established in this study is a powerful optogenetic model that can be used to precisely control gene expression by light. The strain will be widely available from Miibio Inc., Japan (email: info@miibio.com). Genetic manipulation by optogenetics can be applied not only to Cre recombinase but also to Flp recombinase and Cas9 and Cas12a nucleases [9, 11, 35, 36]. These technologies are excellent genetic modification tools for in vivo use, and the resultant KI mice have applications in in vivo genetic analysis.

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Compliance with ethical standards

Conflict of interest TT and TS are employees of KAC Co., Ltd.

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