Optogenetic regulation of embryo implantation in mice using photoactivatable CRISPR-Cas9

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Embryo implantation is achieved upon successful interaction between a fertilized egg and receptive endometrium and is mediated by spatiotemporal expression of implantation-associated molecules including leukemia inhibitory factor (LIF). Here we demonstrate, in mice, that LIF knockdown via a photoactivatable CRISPR-Cas9 gene editing system and illumination with a light-emitting diode can spatiotemporally disrupt fertility. This system enables dissection of spatiotemporal molecular mechanisms associated with embryo implantation and provides a therapeutic strategy for temporal control of reproductive functions in vivo.

photoactivatable CRISPR-Cas9 | optogenetic | implantation | leukemia inhibitory factor

Embryo implantation is a spatiotemporal event in that it occurs only when the uterus is receptive to blastocyst(s). The limited time span in which the uterus, particularly the uterine endometrium, is receptive is termed "window of implantation" (WOI) that, in mice, is days 4 and 5 (day 1 = vaginal plug) and, in humans, occurs during the midluteal phase (cycle days 20 to 24) (1). In mice, leukemia inhibitory factor (LIF) is expressed spatiotemporally around the periimplantation period in the uterine endometrium, particularly in the glandular epithelium. LIF levels peak on day 4 of pregnancy, and gene knockout mouse models demonstrated that LIF is essential for uterine receptivity and embryo implantation (2–4).

Photoactivatable Cas9 (paCas9) is a new molecular device that was recently developed by Nihongaki et al. (5). In this system, Cas9 nuclease activity can be controlled by a blue light-emitting diode (LED). The paCas9 consists of split Cas9 fragments and LED illumination-inducible dimerization domains termed Magnets (nMag and pMag) (5). The genome editing activity of paCas9 can be switched on and off simply by the presence and absence, respectively, of LED illumination, which can minimize off-target effects associated with stable expression of Cas9. Moreover, the LED paCas9-mediated genome editing system does not have off-target effects that can accompany use of chemical inducing agents such as doxycyline (6) and tamoxifen (7). In this study, we used the CRISPR-paCas9 system to develop a murine fertility regulatory model that enables optogenetic and spatiotemporal regulation of reproductive functions in vivo.

Results

paCas9 Combined with sgLif Inhibits LIF Expression and Embryo Implantation in Mouse Uteri. To generate an in vivo mouse model with optogenetic regulation of fertility by paCas9, we prepared single guide RNA targeting *Lif* (sg*Lif*) and designed experiments involving three treatment groups designated Tx-I (single guide RNA control [sgCtrl] + LED), Tx-II (sg*Lif* only), and Tx-III (sg*Lif* + LED; Fig. 1*A*), because LIF is spatiotemporally up-regulated during the WOI and plays essential roles in newly pregnant female mice (2–4). Female mice were given intraperitoneal (i.p.) administration of paCas9 in combination with sg*Lif* or sgCtrl at 2.0 d to 2.5 d postcoitum (dpc) followed by systemic LED illumination on 3.0 dpc to 3.5 dpc. The uteri were excised on 4.5 dpc and subjected to immunofluorescence staining and extraction of protein for immunoblot analysis. LIF was strongly expressed in the Tx-I or Tx-II group, but was dramatically decreased in Tx-III (Fig. 1*B*). Immunofluorescent staining revealed that LIF was strongly expressed in the endometrial glands and adjacent decidualizing stroma in pregnant uteri in Tx-I or Tx-II, whereas LIF expression was decreased or nearly absent in Tx-III (Fig. 1*C*). These results indicated that paCas9 with LED illumination successfully disrupted the uterine *Lif* gene in mice. Next, we collected uteri from mice in the three treatment groups at 7.5 dpc and examined whether Lif disruption affected fertility. Embryo implantation was significantly inhibited in the uteri of mice in Tx-III compared to Tx-I and Tx-II mice (Fig. 1 *D* and *E*). Expression of desmin, a marker of decidualization, was also reduced in uteri from Tx-III mice compared to Tx-I and Tx-II mice (Fig. 1*F*). Collectively, these results demonstrate the validity of our optogenetic method to regulate fertility in a mouse model.

Administration of Recombinant LIF Protein Rescues Blocking of Implantation Induced by paCas9-Mediated sgLif Knockdown Activated by LED Exposure. To confirm the bona fide on-target effect of paCas9 and sg*Lif* for embryo implantation, we examined whether blockade of implantation by light-inducible Lif knockdown can be rescued by supplementation with recombinant LIF protein (recLIF) (Fig. 2*A*), since LIF acts as a secretory protein to support embryo implantation. Mice were subjected to the same treatment as those in the Tx-III group, but were given either phosphate-buffered saline (PBS) or a transvaginal injection of recLIF (10^5 U/10 μL) into the uterus prior to LED illumination at 3.5 dpc. The treated uteri collected at 7.5 dpc showed a significant increase in the number of implantation sites for mice supplemented with recLIF compared to those that received PBS (Fig. 2 *B* and *C*). The LIF-dependent blockade of implantation together with reversal of these effects by LIF supplementation demonstrates the specificity and validity of our system.

Discussion

This is a demonstration of optogenetic genome editing using photoactivatable Cas9 in living mice and an application of this system for regulation of mouse embryo implantation. The advantages of this system are 1) rapid and spatiotemporal control of Cas9 activity by LED illumination, 2) minimization of off-target effects through pinpoint activation of Cas9, 3) targeting

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The authors declare no competing interest.

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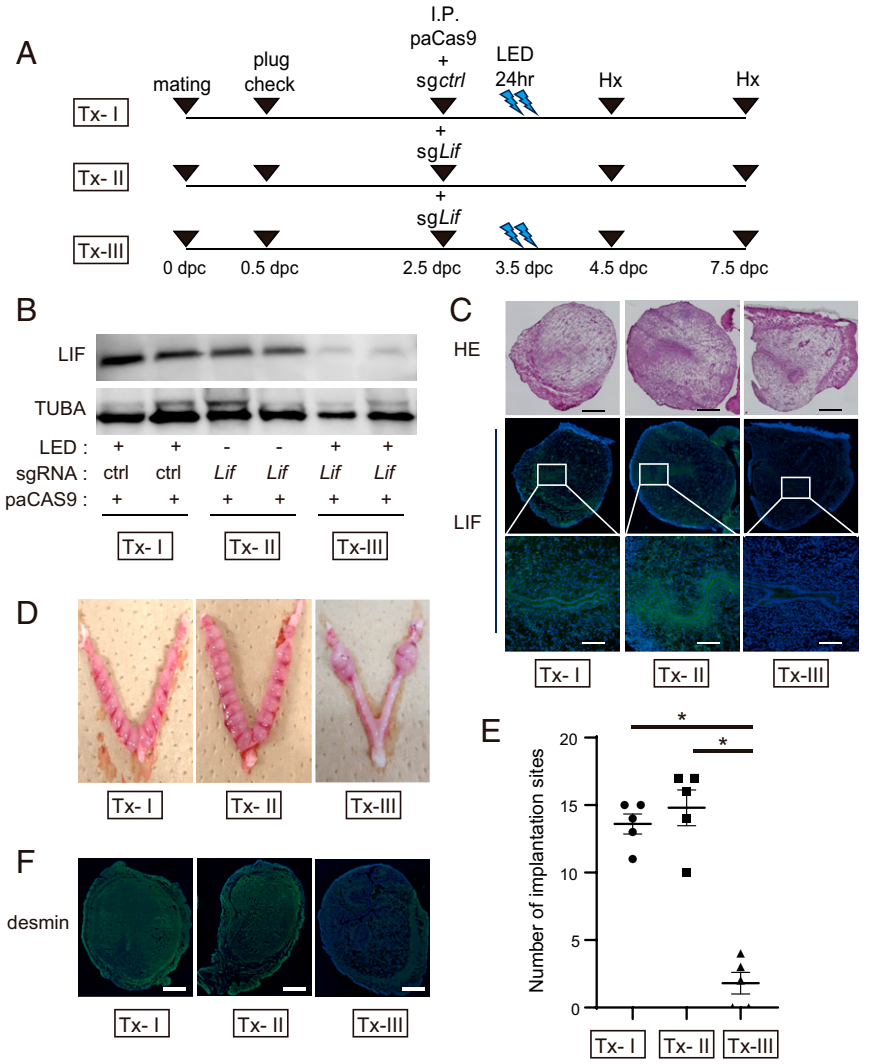

Fig. 1. LED illumination inhibits LIF expression and embryo implantation in uteri of mice treated with paCas9 combined with sg*Lif*. (*A*) Experimental protocol for paCas9-induced *Lif* deletion. Treatment groups are designated Tx-I (sgCtrl + LED), Tx-II (sg*Lif* only), or Tx-III (sg*Lif* + LED). Hx, hysterectomy. (*B*) Immunoblot analysis of LIF expression and TUBA as an internal control in 4.5-dpc uteri treated as indicated. (*C*) (*Upper*) H&E and (*Middle* and *Lower*) immunofluorescence staining images of LIF (green) and DAPI (blue) in 4.5-dpc uteri from mice treated as indicated in *A* (Scale bars, 1.0 mm [*Upper*] and 200 μm [*Lower*].) (*D*) Macroscopic appearance of representative 7.5-dpc uteri from mice treated as indicated. (*E*) Number of implantation sites in 7.5-dpc uteri in the indicated treatment groups. Implantation number and the mean ± SEM from the independent experiments are indicated by dots and horizontal lines, respectively. $*P < 0.0001$. (*F*) Immunofluorescence of desmin (green) and DAPI (blue) in 7.5-dpc uteri treated as indicated (Scale bars, 1.0 mm.)

of spatiotemporal reproductive events such as embryo implantation, 4) low invasiveness of LED and avoidance of negative effects associated with chemical agents to induce expression, and 5) simple genome editing procedures involving i.p. injection and LED illumination. The combined advantages of this method contribute to a valuable research tool for revealing and dissecting spatiotemporal molecular mechanisms that will allow elucidation of the spatiotemporal role of implantation-associated molecule(s). In addition to the impact on basic research, demonstration of optogenetic regulation of embryo implantation will provide insights into the development of optogenetic treatment(s) for infertility and contraception.

Methods

Reagents. Antibodies used in this study include rat polyclonal anti-LIF (BioLegend, Inc.), mouse monoclonal anti-desmin (DAKO), rabbit polyclonal anti-α-Tubulin (TUBA) (Abcam), Alexa Fluor 488-labeled donkey anti-rabbit IgG and donkey anti-rat IgG (Thermo Fisher Scientific), horseradish peroxidase-conjugated goat anti-rabbit IgG Ab (Santa Cruz Biotechnology), and anti-rat IgG (Vector Laboratories, Inc.). The recLIF was obtained from Thermo Fisher Scientific.

paCas9 Plasmid and Single Guide RNA. The paCas9 vector was modified from the paCas9-2 vector (5) through connection of pMag and modified nMag genes by a P2A sequence. CRISPR direct software (8) was used to design two different sg*Lif* sequences: 5′-ggagccgtttcccaacaacg-3′ and 5′-gcagaggaacgccctcgatc-3′. The sgCtrl sequence was 5′-gggaaaccttgttttacattca-3′.

Mouse Model. All experiments using Institute of Cancer Research (ICR) mice were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Keio University School of Medicine (approval nos. 16066-1 and 160660-2). After mating, paCas9 (60 μg) plasmids together with two sgLifs (30 μg each) or sgCtrl (60 μg) were injected intraperitoneally with in vivo-jetPEI reagent in a total volume of 1 mL (Polyplus transfection SA) at 2 dpc to 2.5 dpc. The entire body of the mouse was illuminated with an LED light source (470 ± 20 nm, 1.5 W/m^2, CCS Inc.).

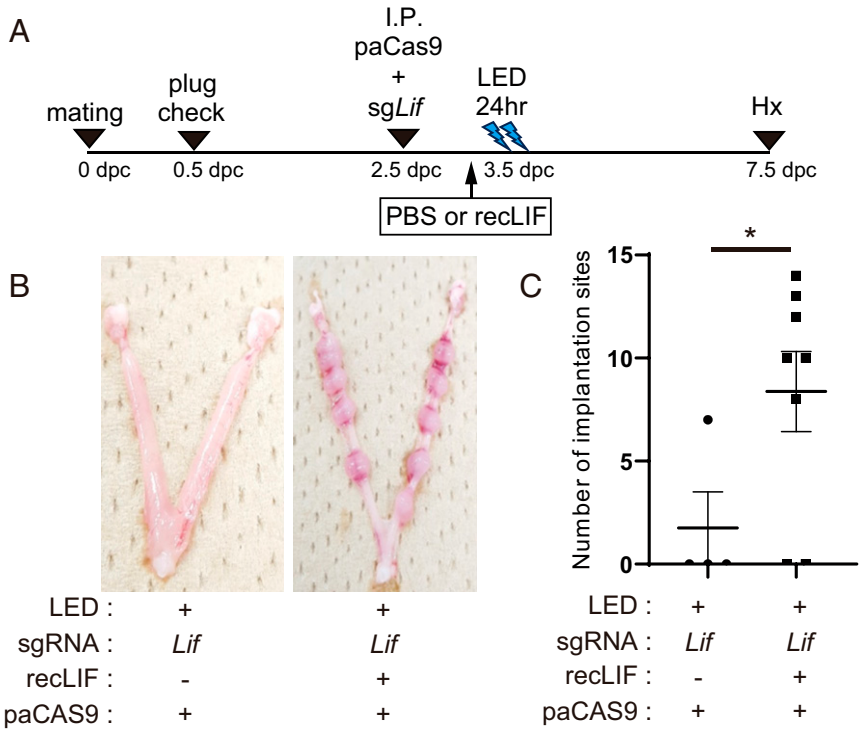

Fig. 2. Attenuation of LED-induced paCas9 and sg*Lif*-mediated blockade of embryo implantation by administration of recombinant LIF protein (recLIF). (*A*) Protocol for rescue experiments with Tx-III treated mice supplemented with recLIF. (*B*) Macroscopic appearance of representative 7.5-dpc uteri treated as per the Tx-III with or without recLIF supplementation. (*C*) Number of implantation sites in 7.5-dpc uteri shown in *B*. Implantation number and the mean ± SEM from the independent experiments are shown by dots and horizontal lines, respectively. $*P = 0.032$.

Immunofluorescent Staining. Uterine tissue samples were fixed in 4% paraformaldehyde and permeabilized. After blocking, tissue sections were incubated with primary antibodies followed by incubation with secondary antibodies. Nuclei were counterstained with DAPI (Thermo Fisher Scientific).

Immunoblotting. Uterine protein extract samples were separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane, which was incubated with primary antibodies. After incubation with secondary antibodies, signals were detected using ImmunoStar LD (Wako).

Statistics. Statistical significance was set at $P < 0.05$ and determined using two-tailed and unpaired Welch's *t* tests or one-way ANOVA analysis of variance with Tukey's post hoc test.

Data Availability. All study data are included in the article.

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