—Original—

Generation of CRISPR/Cas9-mediated bicistronic knock-in *ins1-cre* driver mice

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Abstract: In the present study, we generated novel *cre* driver mice for gene manipulation in pancreatic β cells. Using the CRISPR/Cas9 system, stop codon sequences of *Ins1* were targeted for insertion of *cre*, including *2A* sequences. A founder of C57BL/6J-*Ins1*[em1 (cre) Utr] strain was produced from an oocyte injected with *pX330* containing the sequences encoding gRNA and Cas9 and a DNA donor plasmid carrying *2A-cre*. (R26GRR x C57BL/6J-*Ins1*[em1 (cre) Utr]) F_1 mice were histologically characterized for cre-loxP recombination in the embryonic and adult stages; cre-loxP recombination was observed in all pancreatic islets examined in which almost all insulin-positive cells showed tdsRed fluorescence, suggesting β cell-specific recombination. Furthermore, there were no significant differences in results of glucose tolerance test among genotypes (homo/hetero/wild). Taken together, these observations indicated that C57BL/6J-*Ins1*[em1 (cre) Utr] is useful for studies of glucose metabolism and the strategy of bicistronic *cre* knock-in using the CRISPR/Cas9 system could be useful for production of *cre* driver mice.

Key words: bicistronic, cre-loxP, CRISPR/Cas9, pancreatic β cells, mouse

Introduction

Although knockout mouse strains are invaluable for investigating gene function *in vivo*, roughly one third of all genes cause embryonic lethality in homozygous knockout mice (https://www.jax.org/research-and-faculty/tools/knockout-mouse-project/high-throughput-production). It is not possible to utilize the traditional knockout approach to examine the functions of genes that are lethal in the embryonic stage but may be involved in distinct roles during postnatal life. Using traditional knockout mice, it is also difficult to determine tissue-specific functions of genes that are expressed in multiple tissues. Such difficulties can be resolved by production of conditional knockout mice using the *cre-loxP* system, in which conditional knockouts are pro-

duced by crossing floxed mice with *cre* driver mice. To improve tissue- or cell-restricted expression of the cre enzyme, there are two major approaches to the generation of bacterial artificial chromosome (BAC)-carrying transgenic *cre* mice through zygotic microinjection and knock-in *cre* mice through targeted embryonic stem (ES) cells. However, there are also disadvantages to both approaches. As BAC cre constructs are randomly integrated into the mouse genome, there is a danger of disrupting endogenous genes. Although the *cre* gene is integrated into the targeted site through homologous recombination, the target gene is usually disrupted by *cre* gene insertion in knock-in mice.

The development of genome engineering will markedly alter the methods used for production of transgenic animals. The CRISPR/Cas9 system is a very convenient method for generating specific mutations in animals. This system is composed of a single guide RNA (sgRNA) and Cas9 protein which cause site-specific DNA double-strand breaks (DSB), leading to indel mutations by non-homologous end joining (NHEJ). In contrast, when a large amount of homologous DNA donor is present in Cas9/sgRNA-mediated DNA break, site-specific knock-in is generated by homologous direct repair (HDR). Indeed, we reported that simple production of albino C57BL/6J mice was possible using C57BL/6J zygote microinjection with the CRISPR/Cas9 system composed of a plasmid carrying a single guide RNA for *Tyr* and single-stranded DNA (G291T in *Tyr*) donor [11]. Furthermore, reporter gene knock-in mice can also be generated relatively easily by zygote co-microinjection of CRISPR/Cas9 components and reporter gene DNA, compared with conventional production using ES cells through homologous recombination [15].

Recently, we developed a *cre* driver mouse strain, C57BL/6N-*Tg (Ins1-cre) 25Utr/Rbrc*, carrying the *cre* gene in the BAC of insulin 1 (*Ins1*) to establish pancreatic β-cell-specific *cre-loxP* recombination [4]. Although the BAC *Ins1* transgene was located in a position far distal to the centromere of chromosome 15, it is unclear whether an endogenous gene is damaged by insertion of the BAC transgene in this strain. In fact, homozygous mutant mice have not yet been obtained from intercrosses of the C57BL/6N-*Tg (Ins1-cre) 25Utr/Rbrc* strain. To avoid the disadvantages of BAC transgenic and conventional knock-in mice, we generated novel bicistronic *Ins1-cre* knock-in mice by CRISPR/Cas9-mediated homologous recombination in mouse zygotes without disruption of the endogenous *Ins1* gene.

Materials and Methods

Construction of pX330 for Ins1 and donor plasmid

The CRISPR target sequence (5′-CTGGAGAAC-TACTGCAACTAAGG-3′) was selected for integration of the 2A-cre sequence just before the stop codon of *Ins1*. The *pX330* plasmid, carrying both gRNA and Cas9 expression units, was a gift from Dr. Feng Zhang (Addgene plasmid 42230) [2]. Ins1-CRISPR-F (5′-caccCTG-GAGAACTACTGCAACTA-3′) and Ins1-CRISPR-R (5′-aaacTAGTTGCAGTAGTTCTCCAG-3′) were annealed and inserted into the entry site of *pX330* as described previously [11]. This plasmid was designated as *pX330-Ins1*. The cleavage activity of *pX330-Ins1* was confirmed by the traffic reporter system using the *p2color* vector containing the CRISPR target (Supplementary Fig. 1). Transfection to HEK293T cells and fluorescence observations were performed as described [11].

The donor plasmid *pIns1/2A-cre* contained the P2A sequence and nuclear translocation signal (NLS)-cre. The 2.0-kb 5′-arm (from 1.4 kb upstream of exon 1 to just before the stop codon of *Ins1*) and the 1.7-kb 3′-arm (from the stop codon to 1.1 kb downstream of exon 2) were cloned into this vector. The strategy of bicistronic cre expression in pancreatic cells is shown in Fig. 1A. The *pX330-Ins* (RDB13945), *pIns1/2A-cre* (RDB13946), *p2color-Ins1* (RDB13947) and *p2color* (RDB13948) were deposited in the RIKEN Bio-Resource Center (DNA Bank, Tsukuba, Japan) for distribution to the scientific community.

Animals

C57BL/6N-*Gt (ROSA) 26Sor^tm1 (CAG-EGFP,tdsRed) Utr/Rbrc*, R26GRR [5], were obtained from the RIKEN BioResource Center (Tsukuba, Japan) through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan. The R26GRR cre reporter mice show bright green fluorescence in non-cre-recombined cells and markedly stronger bright red fluorescence in cre-recombined cells. C57BL/6J were purchased from Charles River Laboratories International, Inc. (Yokohama, Japan). Mice were kept in plastic cages under pathogen-free conditions in a room maintained at 23.5°C ± 2.5°C and 52.5% ± 12.5% relative humidity under a 14-h light:10-h dark cycle. Mice had free access to commercial chow (MF diet;
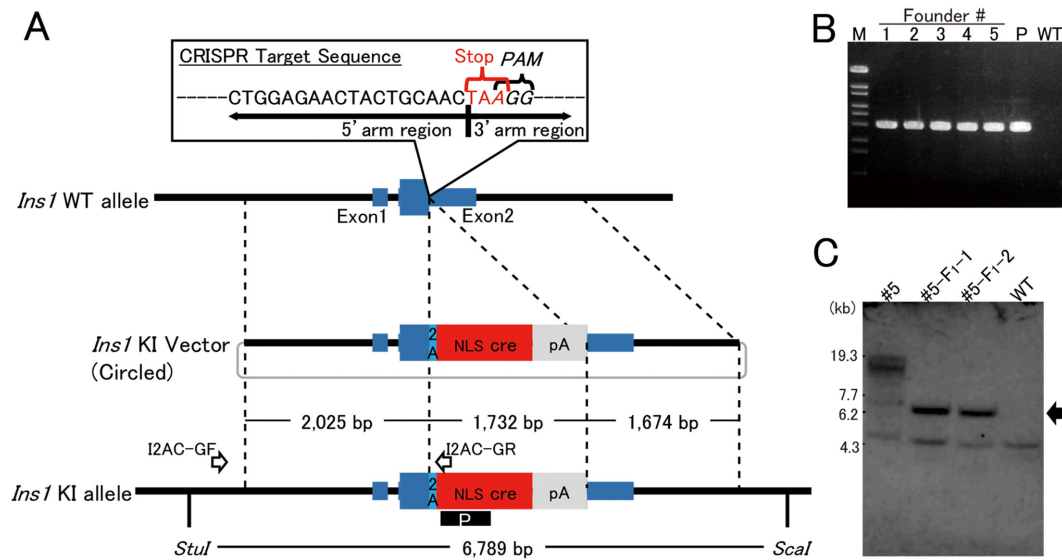
Fig. 1. Strategy of bicistronic *cre* expression in pancreatic beta cells. A: To integrate the 2A-cre sequence just before the stop codon of *Ins1*, the 23-nt sequence (5′-CTGGAGAACTACTGCAACTAAGG-3′) containing both PAM and stop codon was chosen as the CRISPR target. The 3′-end of the region of 5′-homology arm (2.0 kb) is the final coding sequence of *Ins1*. The 5′-end of the region of 3′-homology arm is the stop codon of *Ins1*. The arrows labeled I2AC-GF and I2AC-GR indicate the primers for detecting the knock-in allele. The black box including the white letter P indicates the probe used for Southern blotting. B: PCR products, amplified with the founder genome DNA as the template and the primers I2AC-GF and I2AC-GR, of the appropriate size (2,631 bp) were detected. M: Marker 6 (Nippongene). C: Southern blotting analysis with founder #5 and its offspring (#5-F$_1$-1 and #5-F$_1$-2). In #5, the knock-in band (arrow) and longer random integration band were detected. In contrast, the KI band and no random integration band were detected in offspring. Lower weight nonspecific bands were detected in all samples.

Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered water. All mouse experiments were approved by the University of Tsukuba Animal Experiment Committee.

Microinjection

DNA vectors (*pX330-Ins1* and *pIns1/2A-cre*) for microinjection were isolated with FastGene Gel/PCR Extraction Kit (Nippon genetics, Tokyo, Japan) and diluted to 5 ng/μl by deionized distilled water and mix. The DNA solution was filtrated by MILLEX-GV® 0.22 μm Filter unit (Merk Millipore, Darmstadt, Germany). The DNA vectors were microinjected into the male pronuclei of fertilized oocytes which were harvested from superovulated mated C57BL/6J females. Survived one-cell embryos were transferred into the oviduct of pseudopregnant ICR females.

Genomic DNA analyses

The genomic DNA were purified from the tail with PI-200 (KURABO INDUSTREIS LTD, Osaka, Japan) according to manufacture's protocol. Genomic PCR was performed with PrimeSTAR GXL DNA Polymerase® (TAKARA Bio, Shiga, Japan) and the primers (Supplementary Table 1). PCR products of off-target candidate were sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit and 3500 genetic analyzer (Thermo Fisher Scientific, Massachusetts, U.S.).

For Southern blotting, 10 μg genomic DNA was double-digested by *Stu*I and *Sca*I (New England Biolabs, Massachusetts, U.S.) and electrophoresed in 0.8% agarose gel. The DNA in gel was transferred to hybond n+ membrane (GE Healthcare Life Sciences, Chicago, U.S.). The DIG labeling probe for *cre* was synthesized with PCR DIG Probe Synthesis Kit (Roche, Basel, Switzerland) and primers (Supplementary Table 1), then hybridized to DNA transferred membrane at 45°C. Hybridization and detection steps were performed with DIG DNA Labeling and Detection Kit (Roche, Basel, Switzerland) according to manufacture's protocol.

Stereomicroscopic findings

After anesthesia with a mixture of medetomidine,

midazolam, and butorphanol [8], animals were fixed by perfusion with cold PBS and then with 4% paraformaldehyde (PFA) in cold PBS. EGFP and tdsRed fluorescence in 36 tissues were examined by fluorescence stereomicroscopy (M205FA; Leica, Wetzlar, Germany) provided with internal light sources and appropriate filter sets (excitation and emission: 470 ± 40 nm and 525 ± 50 nm and 545 ± 30 nm and 620 ± 60 nm band-pass filters for EGFP and tdsRed, respectively).

Immunohistochemical findings

For immunohistological demonstration of insulin and glucagon in pancreatic islets of Langerhans tissue, fixed samples were equilibrated in sucrose by placing in 50-ml tubes containing graded concentrations of sucrose (10%, 20%, and 30% in PBS). Samples were embedded in Tissue-Tek OCT (Fisher, Pittsburgh, PA) and frozen in liquid nitrogen. Frozen tissue blocks were brought to $-20°C$ and sections 14 μm thick were cut and mounted on amino silane-coated slides. Tissue sections were incubated with guinea pig anti-insulin antibody (Dako, Carpinteria, CA) or mouse anti-glucagon antibody (Sigma-Aldrich, St. Louis, MO) for 1 h at room temperature. The antigens were visualized using appropriate secondary antibodies conjugated with Alexa 647 and then sections were stained with diamidino-2-phenylinodole (DAPI) to detect nuclei (Invitrogen, Carlsbad, CA). Fluorescence was examined by fluorescence microscopy (BZ-X710; Keyence, Osaka, Japan) with internal light sources and appropriate filter sets (band-pass filter excitation and emission: 360 ± 40 nm and 460 ± 50 nm for DAPI, 470 ± 40 nm and 525 ± 50 nm for EGFP, 560 ± 40 nm and 630 ± 75 nm for tdsRed, and 620 ± 60 nm and 700 ± 75 nm for Alexa 647). Image data were analyzed with a BZ-X analyzer (Keyence).

Glucose tolerance test

Glucose tolerance tests were performed in 16 h fasted mice after intraperitoneal injection of glucose (2 g/kg body weight) [9]. Blood samples were collected from the tail veins. Blood glucose values were measured immediately before and 15, 30, 60, and 120 mins after glucose injection by One Touch Ultra (Johnson and Johnson, U.S.). All results are reported as means ± SD for equivalent groups and compared with the independent *t* test. In all analyses, $P<0.05$ was taken to indicate statistical significance.

Results

The *pX330-Ins1* vector and donor plasmid *pIns1/2A-cre* were co-injected into the pronuclei of fertilized oocytes obtained from C57BL/6J mice to obtain integration of the knock-in vector into the *Ins1* allele target site. After birth, developed weanlings were genotyped by PCR designed to amplify an integrated knock-in fragment of 2,631 bp in length (Fig. 1B). Five founder mice were obtained from 373 injected oocytes, and the *pX330-Ins1* construct was not detected in the tail genome of founders by PCR (data not shown). The efficiency of knock-in mouse production is shown in Supplementary Table 2. The inheritance of the knock-in construct was identified in one (#5) of the five founders, i.e., mouse #5, although Southern blotting analysis indicated that it carried a randomly integrated *Ins1/2A-cre* construct (s) in addition to the knock-in construct. The extra *Ins1/2A-cre* construct (s) was segregated from the knock-in construct in the progeny derived by backcrossing with C57BL/6J (Fig. 1C). The heritability was also confirmed by PCR analysis (Supplementary Fig. 2). The knock-in mice were designated as C57BL/6J-*Ins1*$^{em1 (cre) Utr}$. Moreover, the ORF of *Ins1* was connected to 2A and *NLS cre* sequence correctly in this strain (Supplementary Fig. 3).

We then investigated the off-target effects in founder #5. Cong *et al.* [2] reported that single-base mismatch up to 11 bp 5′ of the PAM sequence (5′-NGG-3′) completely abolished genomic cleavage by *Streptococcus pyogenes* Cas9, which was used in the present study. Therefore, we examined off-target sites that were completely consistent with 12 bases at the 3′ end and PAM (5′-CTACTGCAAACTANGG-3′ or 5′-CCNTAGTTG-CAGGTAG-3′). Fifteen off-target candidates were isolated from the whole mouse genome sequence with CRISPRdirect (https://crispr.dbcls.jp/) (Supplementary Table 3), and these 15 off-target candidates were confirmed by direct sequencing analysis. No mutations were found in any of the off-targets, indicating that little or no Cas9-mediated off-target mutation occurred in C57BL/6J-*Ins1*$^{em1 (cre) Utr}$. Addition, we obtained 23 offspring from natural mating between #5 and wild-type and the knock-in allele were found in 12 of them (12/23, 52.2%), suggesting that founder #5 carried hemizygous, not mosaical, knock-in allele. However, the other founders (#1, #2, #3 and #4) were mosaics, because there were no their offspring carrying the knock-in allele.

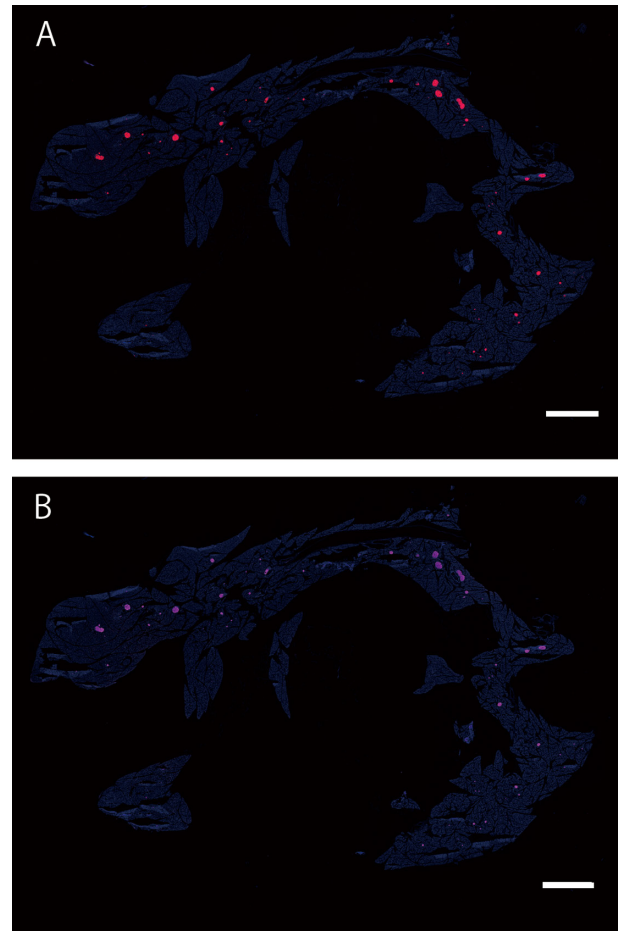To evaluate the site of *cre* expression in the fetal stage
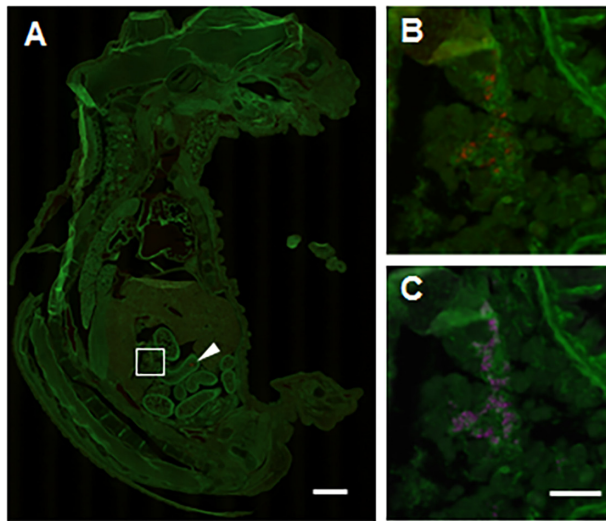
Fig.2. Histological analysis of *cre-loxP* recombination in the fetal stage of (R26GRR × C57BL/6J-*Ins1*[em1 (cre) Utr]) F_1 mice. A: tdsRed signals (cre-loxP recombination) in the whole body of a fetus at E16.5. Specific recombination signals were detected in the fetal pancreas (surrounded by a square) (B and C). The arrowhead indicates nonspecific signal in the intestine. B: tdsRed signals in the pancreas of a fetus at E16.5. C: Insulin signals in the pancreas of a fetus at E16.5. Each experiment (*n*=4). Scale bar: 1 mm (low-power field) and 100 μm (high-power field).
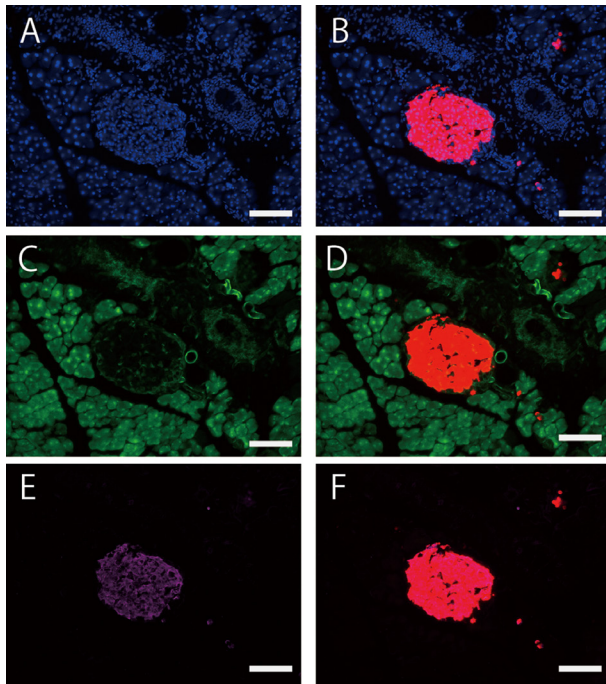

Fig.3. *cre-loxP* recombination of islets in a wide range of regions in the adult pancreas of (R26GRR × C57BL/6J-*Ins1*[em1 (cre) Utr]) F_1 mice. A: tdsRed signals (Cre-loxP recombination), B: Insulin signals (using anti-insulin + 2nd antibody conjugated with Alexa647). All tdsRed-positive islets were insulin-positive. Each experiment (*n*=3). Scale bar: 1.5 mm

after expressing endogenous *Ins1*, heterozygous C57BL/6J-*Ins1*[em1 (cre) Utr] males were crossed with homozygous R26GRR females. In (R26GRR × C57BL/6J-*Ins1*[em1 (cre) Utr]) F_1 mice (n=3) on embryonic day (E) 16.5, red fluorescence was observed in the tissues surrounded by a rectangular frame and in the intestinal cavity as indicated with an arrow in Fig. 2A. Immunohistochemistry using anti-insulin antibody clearly demonstrated the co-localization of red fluorescence with insulin (Alexa647) signals, indicating that the staining-positive tissue in the abdomen was the pancreas. However, the red signals in the intestinal cavity appeared to be nonspecific because there was not staining with DAPI or for insulin (data not shown). Examination of whole embryos indicated no red signals in other tissues. These results suggested that *cre* in C57BL/6J-*Ins1*[em1 (cre) Utr] was expressed dominantly in the fetal pancreas at E16.5, but not in other tissues.

To determine the recombination site of *cre-loxP* in the adult stage, we dissected adult (R26GRR × C57BL/6J-*Ins1*[em1 (cre) Utr]) F_1 males (n=3) and females (n=3) after perfusion with 4% PFA and divided into 36 kinds of tissues. Each tissue was cut into coronal, transverse, or

sagittal sections, which were then examined by fluorescence stereomicroscopy. As shown in Supplementary Fig. 4, red fluorescence indicating the *cre-loxP* recombination signal was observed in the pancreas, but not in other tissues. These data were consistent with the results in fetuses. In mice, insulin is synthesized within the β-cells of the islets of Langerhans in the pancreas. As the localization and efficiency of *cre-loxP* recombination are the most important characteristics in *cre* driver mice, fluorescence-microscopy was performed using cryosections of the pancreas from adult (R26GRR × C57BL/6J-*Ins1*[em1 (cre) Utr]) F_1 mice. More than 40 islets in each mouse (n=3) were examined by Alexa647 fluorescence for insulin and tdsRed fluorescence for *cre-loxP* recombination. All individuals showed complete recombina-

Fig. 4. Co-localization of insulin-positive islets with tdsRed-positive islets in the adult pancreas of (R26GRR × C57BL/6J-*Ins1*$^{em1\ (cre)\ Utr}$) F_1 mice. A: DAPI, B: DAPI/tdsRed (*cre-loxP* recombination), C: EGFP (non *cre-loxP* recombination), D: EGFP/tdsRed, E: Insulin (using anti-insulin + 2nd antibody conjugated with Alexa647), F: Insulin/tdsRed. Each experiment (*n*=3). Scale bar: 100 μm
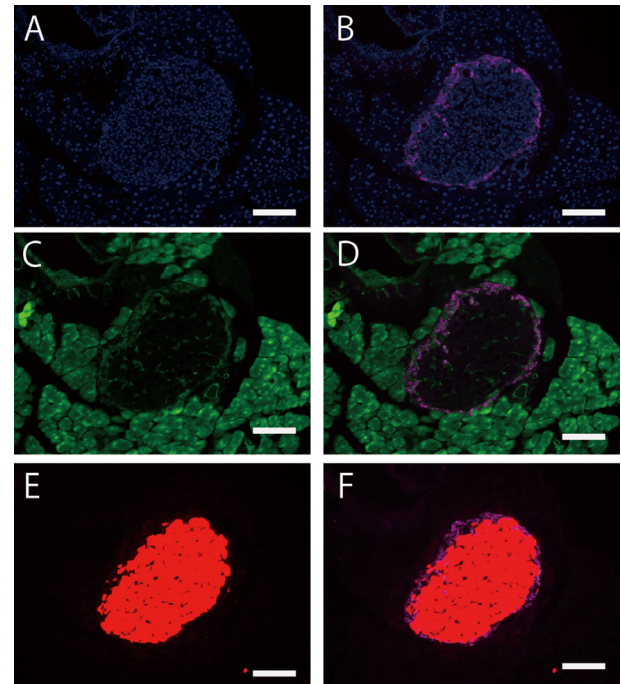
Fig. 5. De-localization of glucagon-positive signals with tdsRed-positive signal in adult pancreatic islets of (R26GRR × C57BL/6J-*Ins1*$^{em1\ (cre)\ Utr}$) F_1 mice. A: DAPI, B: DAPI/Glucagon (using anti-glucagon + 2nd antibody conjugated with Alexa647), C: EGFP (no *cre-loxP* recombination), D: EGFP/Glucagon, E: tdsRed (*cre-loxP* recombination), F: Glucagon/tdsRed. Each experiment (*n*=3). Scale bar: 100 μm

tion in the insulin-positive islets examined (Fig. 3 and Supplementary Table 4).

Next, we examined whether *cre-loxP* recombination was limited to the β-cells of the pancreatic islets of Langerhans. On higher magnification observation, tdsRed-positive cells were well overlaid with insulin-positive cells in the pancreatic islets, but not with glucagon-positive cells (Figs. 4 and 5). Quantitative analysis (n=3) indicated that *cre-loxP* recombination had occurred in approximately 98% of insulin-positive cells (Supplementary Table 5), suggesting that *cre* gene expression is spatially regulated in exactly the same way as the endogenous *Ins1* gene in C57BL/6J-*Ins1*$^{em1\ (cre)\ Utr}$ mice.

To examine whether *Ins1* transcript fused with 2A-*cre* affects glucose metabolism, we performed glucose tolerance tests in fasted 8-week-old heterozygous C57BL/6J-*Ins1*$^{em1\ (cre)\ Utr}$ mice. Compared with wild-type males and females, C57BL/6J-*Ins1*$^{em1\ (cre)\ Utr}$ males and females exhibited no significant differences in glucose clearance at any time point examined after glucose injection, respectively. Finally, we investigated whether homozygous

mice were generated from intercrosses of heterozygous mutants in the C57BL/6J-*Ins1*$^{em1\ (cre)\ Utr}$ strain. As expected, homozygous males and females were obtained, and they showed no significant differences between genotypes of each sex in the glucose tolerance test (Fig. 6). C57BL/6J-*Ins1*$^{em1\ (cre)\ Utr}$ is a highly useful cre driver mouse strain with induction of β-cell-specific *cre-loxP* recombination for studying glucose metabolism.

Discussion

Several *cre* driver mouse strains have been developed for investigating gene functions in pancreatic β-cells. Transgenic mice carrying the rat insulin promoter fused to *cre* (RIP-cre) have been widely used for *cre-loxP* recombination in pancreatic β-cells. However, *cre* was also shown to be expressed in the brain in addition to the pancreas in three different RIP-*cre* mouse strains, i.e., *Tg (Ins2-cre) 25Mgn* [12], *Tg (Ins2-cre) 1Herr* [6], and *Tg (Ins2-cre/Esr) 1Dam* [3]. To obtain exclusive *cre* expression in pancreatic β-cells, Wicksteed *et al.* [14]
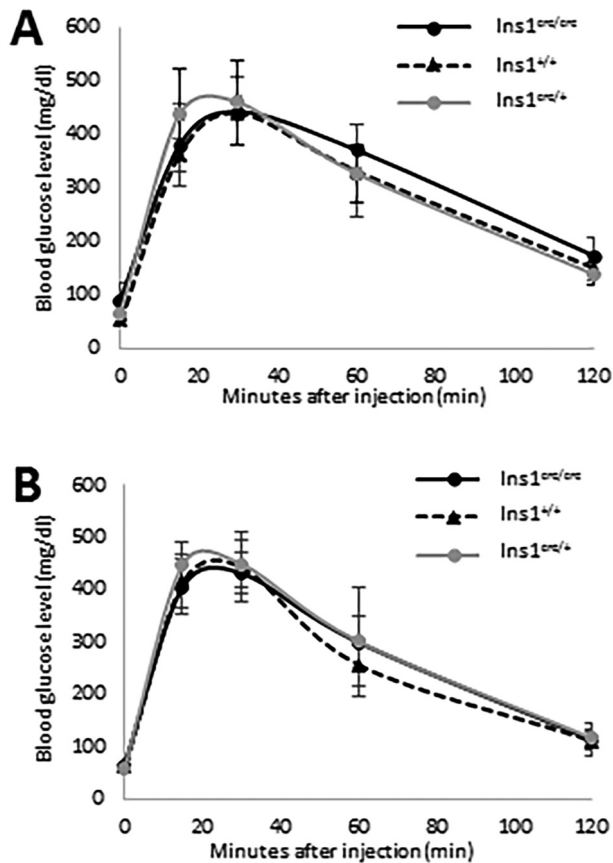
Fig. 6. Glucose tolerance test of C57BL/6J-*Ins1*$^{em1 \ (cre) \ Utr}$ mice. Males (A) and females (B) were tested at 8 weeks old. *Ins1*$^{+/+}$ (male: *n*=3, female: *n*=9), *Ins1*$^{cre/+}$ (male: *n*=7, female: *n*=7), and *Ins1*$^{cre/cre}$ (male: *n*=7, female: *n*=6) indicate wild-type, heterozygous, and homozygous C57BL/6J-*Ins1*$^{em1 \ (cre) \ Utr}$, respectively.

and Hasegawa *et al.* [4] developed *Tg (Ins1-cre/ERT) 1Lphi* and C57BL/6N-*Tg (Ins1-cre) 25Utr/Rbrc* mouse strains, respectively, which lacked *cre-loxP* recombination in the brain. Similar to both of these transgenic lines carrying *cre* fused to the *Ins1* promoter, the results of the present study showed that C57BL/6J-*Ins1*$^{em1 \ (cre) \ Utr}$ mice have highly specific cre recombinase activity in pancreatic β-cells and lack cre recombinase activity in the brain. The most important difference between C57BL/6J-*Ins1*$^{em1 \ (cre) \ Utr}$ and other β-cell-specific *cre* driver mice is clarity of the integration site of *cre* into the genome. Although Thorens *et al.* [13] reported the generation of *Ins1*^{cre} knock-in mice through targeted ES cells with conventional homologous recombination techniques, exon 2 encoding the whole of proinsulin was replaced with an exogenous construct encoding cre recombinase, which resulted in loss of *Ins1* function. In

contrast, *cre* transcripts are expressed in a bicistronic manner of *Ins1* in C57BL/6J-*Ins1*$^{em1 \ (cre) \ Utr}$ mice, suggesting no loss of function of *Ins1*. This is the first report of the development of bicistronic *Ins1* cre driver mice.

The bicistronic knock-in strategy is an effective method for generating endogenous gene-dependent cre recombinase activity without losing one copy of the target allele. The internal ribosomal entry site and self-cleaving 2A peptides have been frequently used for multicistronic gene expression. Four different 2A peptides derived from foot-and-mouth disease virus (F2A), equine rhinitis A virus (E2A), porcine teschovirus-1 (P2A), and Thosea asigna virus (T2A) in addition to an internal ribosomal entry site (IRES) were widely used in biomedical research increasing the cleavage efficiency and maintaining the expression of more than one gene. In the present study, a 2A peptide derived from porcine teschovirus-1 was chosen for bicistronic cre expression, because the viral 2A peptide maintains more reliable expression levels of the appended downstream cistron than IRES [1]. Kim *et al.* [7] reported that P2A has high cleavage efficiency in human cell lines, as well as in zebrafish and mice. C57BL/6J-*Ins1*$^{em1 \ (cre) \ Utr}$ mice showed cre recombinase activity in almost all cells of the pancreatic islets synthesizing insulin. Therefore, our results suggest that P2A peptide is functional in bicistronic constructs containing *cre* in the pancreatic β-cells of living mice.

From the results of the present study, it is unclear whether insulin protein is translated from the *Ins1* knock-in allele, because C57BL/6J-*Ins1*$^{em1 \ (cre) \ Utr}$ mice also possess an *Ins2* allele. Further, we should investigate the synthesis and secretion of insulin derived from the *Ins1* knock-in allele in *Ins2*-deficient C57BL/6J-*Ins1*$^{em1 \ (cre) \ Utr}$ mice.

Bicistronic knock-in mice have been generated from chimeras with genetic contributions from both the recipient embryo and the ES cells targeted with the knock-in vector. Although reliable, this method is laborious, costly, and time consuming. Recently, we [10, 11] and many other groups have adopted the CRISPR/Cas9 technique for gene modification of mouse because of its high degree of flexibility and accuracy in cutting and pasting DNA. As the founder of the C57BL/6J-*Ins1*$^{em1 \ (cre) \ Utr}$ line carried randomly integrated transgene (s) in addition to the knock-in allele, Southern blotting analysis using a restriction enzyme with a recognition site outside of the knock-in construct is necessary for genotyping of

CRISPR/Cas9-mediated knock-in mice, similar to conventional knock-in mice. However, the transgene could be easily segregated from the knock-in *Ins1* allele by backcrossing. To our knowledge, C57BL/6J-*Ins1*$^{em1\ (cre)\ Utr}$ is the first bicistronic knock-in cre driver mouse strain generated by the CRISPR/Cas9 system.

In conclusion, we generated a novel CRISPR/Cas9-mediated bicistronic knock-in *cre* mouse strain, C57BL/6J-*Ins1*$^{em1\ (cre)\ Utr}$. The cre activity was observed in insulin-synthesized cells in the fetal pancreas at E16.5. In adults, cre activity was detected in almost all insulin-synthesizing cells in the pancreatic islets. However, there was no cre activity in other tissues. Moreover, the knock-in *cre* mice showed no abnormalities in the glucose tolerance test. These results suggest that C57BL/6J-*Ins1*$^{em1\ (cre)\ Utr}$ is a useful *cre* driver mouse strain for gene modification in pancreatic β-cells. Utilization of the CRISPR/Cas9 system could be useful for generating bicistronic knock-in *cre* driver mice. The C57BL/6J-*Ins1*$^{em1\ (cre)\ Utr}$ mouse strain (RBRC09525) is available from the RIKEN BioResource Center.

Disclosure/Conflict of Interest Statement

The authors declare there are no conflicts of interest.

Acknowledgments

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