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# Novel ROSA26 Cre-reporter Knock-in C57BL/6N Mice Exhibiting Green Emission before and Red Emission after Cre-mediated Recombination

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**Abstract:** The Cre/loxP system is a strategy for controlling temporal and/or spatial gene expression through genome alteration in mice. As successful Cre/loxP genome alteration depends on Cre-driver mice, Cre-reporter mice are essential for validation of Cre gene expression *in vivo*. In most Cre-reporter mouse strains, although the presence of reporter product indicates the expression of Cre recombinase, it has remained unclear whether a lack of reporter signal indicates either no Cre recombinase expression or insufficient reporter gene promoter activity. We produced a novel ROSA26 knock-in Cre-reporter C57BL/6N strain exhibiting green emission before and red after Cre-mediated recombination, designated as strain R26GRR. Ubiquitous green fluorescence and no red fluorescence were observed in R26GRR mice. To investigate the activation of tdsRed, *EGFP*-excised R26GRR, R26RR, mice were produced through the crossing of C57BL/6N mice with R26GRR/Ayu1-Cre F$_1$ mice. R26RR mice showed extraordinarily strong red fluorescence in almost all tissues examined, suggesting ubiquitous activation of the second reporter in all tissues after Cre/loxP recombination. Moreover, endothelial cell lineage and pancreatic islet-specific expression of red fluorescence were detected in R26GRR/Tie2-Cre F$_1$ mice and R26GRR /Ins1-Cre F$_1$ mice, respectively. These results indicated that R26GRR mice are a useful novel Cre-reporter mouse strain. In addition, R26GRR mice with a pure C57BL/6N background represent a valuable source of green-to-red photoconvertible cells following Cre/loxP recombination for application in transplantation studies. The R26GRR mouse strain will be available from RIKEN BioResource Center (http://www.brc.riken.jp/lab/animal/en/).
**Key words:** *CAG* promoter, Cre-reporter mouse, EGFP, *Rosa26*, tdsRed

## Introduction

Cre protein of bacteriophage P1, which is a 38-kDa protein [1], causes DNA recombination at *loxP* sites, each of which is 34 bp in length and comprised of two 13-bp inverted repeats flanking an asymmetric 8-bp core sequence, located within the mouse genome. Such Cre/ loxP recombination events induce the accurate excision, inversion, insertion, and translocation of genomic DNA at the locus of interest, thus facilitating our understanding of gene function.

The Cre/loxP system is utilized for temporal and spatial control of gene expression in mice. Successful expression control of predetermined genes through Cre-

mediated genome modification is dependent on strict expression of the *cre* gene in Cre-driver mice. A number of Cre-reporter mouse strains have been developed to validate the expression of Cre recombinase. It is necessary for Cre-reporter mice to strongly and ubiquitously express the reporter gene after Cre recombination. The *ROSA26* locus has been widely used to activate universal gene expression from the *ROSA26* promoter. Soriano [21] generated ROSA26-reporter (R26R) mice by knock-in of the floxed transcriptional stopper element flanking a splicing acceptor at the 5′-end and *lacZ* gene at the 3′-end into the *ROSA26* locus. The endogenous *ROSA26* promoter was able to ubiquitously drive expression of the exogenous *lacZ* gene through Cre-mediated excision of the floxed stopper element. Although R26R is one of the most commonly used Cre-reporter mouse strains, recent studies suggested that the endogenous *ROSA26* promoter is not suitable for ubiquitous Cre-dependent genome alteration *in vivo* [3, 10, 12, 25]. In most previously reported Cre-reporter mouse strains, including R26R, although the presence of reporter product indicated expression of Cre recombinase, it remained unclear whether a lack of reporter signal indicated either no Cre recombinase expression or insufficient promoter activity of the reporter gene.

Recently, Cre-reporter mice have been used not only for detection of Cre recombinase, but also as a useful cell source for Cre-based fate mapping studies. However, the reporter enzyme is visualized in an invasive manner in R26R mice. Fluorescent reporters, such as green fluorescent protein (GFP) and its variants, have advantages for detecting and isolating living cells showing Cre activity in a noninvasive manner. Enhanced green fluorescence protein (EGFP), a variant of GFP, is a robust and reliable reporter for live cell imaging. In comparison with EGFP, red fluorescent proteins (RFPs) provide better tissue penetration, allowing deeper noninvasive detection [8]. In addition, RFPs show less luminescent background interference than GFP, improving the specificity of fluorescence labeling. Therefore, RFPs would be convenient for use as Cre reporters in mice.

Here, we describe the development of a novel Cre-reporter C57BL/6N mouse strain by knock-in of the floxed *EGFP*-tandem version of dimeric *DsRed*, *tdsRed*, under the control of the *CAG* promoter into *ROSA26*. This strain, referred to as R26GRR, showed bright green fluorescence in non-Cre-recombined cells and markedly stronger bright red fluorescence in Cre-recombined cells.

## Materials and Methods

### Construction of a binary Cre-reporter gene

The core cassette of the targeting vector to generate double Cre-reporter mice included the *CAG* promoter [16], *loxP* sites flanking the coding region for *EGFP*(Clontech, Palo Alto, CA, USA) containing rabbit β-globin poly (A) signal, and *FLAG*-tagged *tdsRed*-SV40 poly (A). The cassette was ligated into the *FRT-PGK-gb2-neo* expression cassette comprised of eukaryotic and prokaryotic promoters and neomycin resistance gene flanked by *FRT* sequences (Gene Bridges, Heidelberg, Germany) for positive selection. The binary Cre-reporter cassette including the *FRT-PGK-gb2-neo* fusion gene was inserted into the *Asc*I site of the ROSA26 targeting vector *pmROSA26-1* comprised of a 1.1-kb 5′ homology arm of *ROSA26*, 4.5-kb 3′ homology arm of *ROSA26*, and PGK-diphtheria toxin A (*DTA*) gene for negative selection.

### Embryonic stem cells targeting ROSA26

The targeting vector was linearized with *Kpn*I and electroporated into B6N-22[Utr] ES cells derived from C57BL/6N [24]. Embryonic stem cells (ES cells) were cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEF) with the cell culture method described previously [24]. Neomycin-resistant clones were selected with G418 and screened by PCR for homologous recombination at the *ROSA26* locus (sense primer 5′-CTCAGAGAGCCTCGGCTAGG-TAGGGGATCG-3′ and antisense primer 5′-ACGT-CAATGGAAAGTCCCTATTGGCGTTAC-3′, which amplified a 1.3-kb product for the targeted *ROSA26* locus). Southern blotting analysis was performed using standard techniques. *Eco*RI digestion and Southern blotting using the 5′ probe were performed to confirm proper recombination of the short arm (the targeted and wild-type *ROSA26* loci produced products of 5.7 kb and 15.6 kb, respectively), while *Msc*I digestion and Southern blotting using the 3′ probe were performed to confirm proper recombination of the long arm (the targeted and wild-type *ROSA26* loci produced products of 10.0 kb and 15.7 kb, respectively). Further, *Eco*RI digestion and Southern blotting using the Neo probe were performed to reconfirm the long arm of the targeted allele (the targeted and wild-type *ROSA26* loci produced a product of 16.1 kb and no product, respectively). PCR-amplified 5′ probes (5′-GGGATGCTTCTGCTTCTGAG-3′ and

5′-GCCTTATCTGGAATGGGACA-3′), 3′ probes (5′-GGGATGCTTCTGCTTCTGAG-3′ and 5′-GCCT-TATCTGGAATGGGACA-3′), and *neo* probes (5′-AAG-TATCCATCATGGCTGATGC-3′ and 5′-TAGCCAAC-GCTATGTCCTGATA-3′) were labeled with DIG using DIG DNA Labeling Mix (Roche Applied Science, Penzberg, Germany). Hybridization and detection were performed using an DIG Wash and Block Buffer Set (Roche Applied Science, Mannheim, Germany).

To examine Cre-mediated excision, the *CAG-cre* fusion gene was electroporated into the targeted ES cells. After 72 h of cultivation, cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. After washing with PBS, cells were incubated with primary antibody against FLAG (F3165; Sigma, St. Louis., MO, USA) and then with secondary antibody (Alexa 647-conjugated donkey anti-mouse IgG, A-31571; Life Technologies, Gaithersburg, MD, USA). Fluorescence was observed by fluorescence stereomicroscopy (BZ-900; Keyence, Osaka, Japan) with appropriate filter sets (excitation and emission: $470 \pm 20$ nm and $535 \pm 25$ nm, $560 \pm 20$ nm and $630 \pm 30$ nm, and $620 \pm 30$ nm and $700 \pm 37.5$ nm band-pass filters for EGFP, tdsRed, and Alexa 647, respectively).

*Animals*

All mouse strain-related experiments were performed under the approval of the University of Tsukuba Animal Experiment Committee. To generate binary Cre-reporter mice, the targeted ES cells were aggregated with CD-1 embryos as described previously [24]. Chimeric males were crossed with C57BL/6N females to examine germ line transmission of the targeted allele. C57BL/6N and CD-1 (ICR) mice were purchased from Charles River Laboratories Japan (Atsugi, Japan). Ayu1-Cre mice (kind gifts from Dr. Ken-ichi Yamamura, Kumamoto University) and Tie2-Cre (kind gifts from Dr. Masashi Yanagisawa, University of Texas Southwestern Medical Center) [7] were backcrossed into the C57BL/6 background for more than seven generations. The C57BL/6N-*Tg (Ins1-cre) 25Utr*/Rbrc (RBRC03934, Ins1-Cre) mouse strain was provided by RIKEN BioResource Center (Tsukuba, Japan) through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan. Genotypes were confirmed by PCR using the following primers: 5′-GGACAT-GTTCAGGGATCGCCAGGCGT-3′ and 5′-GCATAAC-CAGTGAAACAGCATTGCTG-3′ for Ayu1-Cre mice;

5′-TAAGATACAGCCTTTCCCAT-3′ and 5′-GCATA-ACCAGTGAAACAGCATTGCTG-3′ for Tie2-Cre mice; and 5′-AGGCCATCTGGTCCCTTATTAAGAC-3′ and 5′-CTAATCGCCATCTTCCAGCAGG-3′ for Ins1-Cre mice. Animals were kept in plastic cages under specific pathogen-free conditions in a room maintained at $23.5°C \pm 2.5°C$ and $52.5\% \pm 12.5\%$ relative humidity under a 14-h light:10-h dark cycle. Mice had free access to commercial chow (MF diet; Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered water.

*Fluorescence stereomicroscopic findings*

For fluorescence imaging during embryonic development and newborn stage, pregnant mice were euthanized by $CO_2$ inhalation. For fluorescence imaging at the adult stage, mice were anesthetized by isoflurane inhalation, perfused with PBS, and then perfused with 4% paraformaldehyde. EGFP and tdsRed fluorescence were observed by fluorescence stereomicroscopy (M205FA; Leica, Wetzlar, Germany) provided with internal light sources and appropriate filter sets (excitation and emission: $470 \pm 20$ nm and $525 \pm 25$ nm and $545 \pm 15$ nm and $620 \pm 30$ nm band-pass filters for EGFP and tdsRed, respectively).

*Fluorescence microscopic findings*

For histological analysis, mice were anesthetized by isoflurane inhalation, perfused with PBS, and then perfused with 4% paraformaldehyde. Fixed samples were equilibrated in sucrose by placing the samples in 50-ml tubes with graded concentrations of sucrose (10%, 20%, and 30% in PBS). Samples were embedded in Tissue-Tek OCT (Fisher, Pittsburgh, PA, USA) and frozen in liquid nitrogen. Frozen tissue blocks were brought to −20°C and sections 10 $\mu$m thick were cut and placed on amino silane-coated slides. Slides were dried at room temperature (RT) overnight, and then either probed with antibodies or stored at −80°C. Fluorescence was observed by fluorescence microscopy (DMLB; Leica) with internal light sources and appropriate filter sets (excitation and emission: $480 \pm 40$ nm and $527 \pm 30$ nm and $546 \pm 12$ nm and $600 \pm 40$ nm band-pass filters for EGFP and tdsRed, respectively). EGFP in high-magnification views was observed by immunohistochemical analysis using rabbit anti-GFP (MBL International, Nagoya, Japan).

## Results

### Strategy of double-color reporter for Cre/loxP recombination

Ubiquitous expression of exogenous genes at high levels in mice could be expected by using the *CAG* promoter targeting the *ROSA26* locus on chromosome 6. Therefore, to generate double-reporter mice for Cre/loxP recombination, we designed a color-convertible Cre-reporter knock-in *ROSA26*$^{CAG-EGFP/tdsRed}$ allele (Fig. 1C). The first reporter prior to Cre-excision used EGFP as the most reliable fluorescent protein. With emission maxima at 509 and 587 nm, respectively, EGFP and tdsRed are suited for almost crossover-free dual-color labeling upon simultaneous excitation [2]. Thus, we determined the utilization of tdsRed, a very bright red fluorescent protein, as the second reporter after Cre excision. The core cassette of the targeting vector, referred to as GRR, consisted of the *CAG* promoter, *loxP*-flanked *EGFP* with transcriptional stop sequences, *FLAG*-tagged *tdsRed* with transcriptional stop sequences, and the *PGK-gb2-neo* gene. The fusion *PGK-gb2-neo* cassette was flanked by *FRP* sites and was positioned downstream of *tdsRed* in reverse orientation relative to *ROSA26* transcription. To construct the R26GRR targeting vector, the GRR DNA fragment was inserted into the *Asc*I site of restriction enzyme site-modified *pROSA26-1* containing a 1.1-kb 5′ homology arm of *ROSA26*, 4.1-kb 3′ homology arm of *ROSA26*, and *PGK-DTA* (Fig. 1B). When *ROSA26* is successfully targeted by the R26GRR vector, *EGFP* is expressed from the targeted *ROSA26* locus, leading to green fluorescence. When Cre recombinase is present, the floxed *EGFP* gene is excised, evoking transcription of *FLAG*-tagged *tdsRed* and thus leading to red fluorescence.

### Generation of R26GRR mice

The R26GRR targeting vector was linearized and electroporated into the B6N-22$^{Utr}$ ES cells, which were established from C57BL/6N blastocysts in our laboratory [24]. Eighty-six G418-resistant ES clones were assessed by PCR with one primer outside the 5′ homology arm and one primer specific to the introduced R26GRR. The amplified 1.3-kb DNA fragment was observed in three ES clones. Southern blotting analysis revealed homologous insertion of the R26GRR targeting vector into the *ROSA26* locus in two (clones #9 and #20) of three PCR-positive clones (data not shown).

To examine the properties of the mutated *ROSA26* allele, we carried out fluorescence imaging of the targeted ES cells with and without the *CAG*-driven Cre recombinase gene (*CAG-cre*). On observation of the targeted R26GRR ES cells, both colonies (clones #9 and #20) appeared bright green (Fig. 2A, B), indicating the expression of EGFP. We introduced the *CAG-cre* gene into the targeted R26GRR ES cells and observed fluorescence 72 h after electroporation. Without selection for the positive clone carrying *CAG-cre*, the majority and a minority of colonies showed green fluorescence (Fig. 2E) and red fluorescence (Fig. 2F), respectively. Although we did not perform Southern blotting analysis of the red ES colonies, the appearance of red fluorescence indicated expression of tdsRed following Cre-mediated *EGFP* excision. Furthermore, red ES colonies were detected with Alexa647-labeled antibody against the FLAG-tag peptide sequence, because tdsRed was tagged with N-terminal FLAG (Fig. 2D).

Each ES cell clone was aggregated with 100 CD-1 embryos at the 8-cell stage, and chimeric embryos were transferred into the uteri of pseudopregnant females. Five and four chimeric males were generated from clones #9 and #20, respectively. Germ line transmission of the R26GRR mutation in the *ROSA26* locus was obtained from clone #20. To reconfirm homologous recombination of the *ROSA26* locus with R26GRR, *Eco*RI- or *Msc*I-digested genomic DNA from wild-type mice and the heterozygous and homozygous mutant mice was examined by Southern blotting analysis with 5′, 3′, and Neo probes (Fig. 1E–G). Homozygous mutant mice were obtained at the expected Mendelian ratio from heterozygous pairs. We could not find any phenotypic abnormalities or infertility in heterozygous or homozygous R26GRR mice.

### Constitutive expression of constitutively activated EGFP in mice until before Cre/loxP recombination

We examined whether the first reporter was constitutively expressed from the *ROSA26*$^{CAG-EGFP/tdsRed}$ allele during embryonic and postnatal development. The fertilized oocytes (Suppl. Fig. 1A), 2-cell embryos (Suppl. Fig. 1B), 4–8-cell embryos (Suppl. Fig. 1C), morulae, and blastocysts (Suppl. Fig. 1D), which were derived from homozygous R26GRR females crossed with wild-type males, constitutively emitted bright green fluorescence. After implantation, heterozygous R26GRR mice maintained widespread emission of green fluorescence
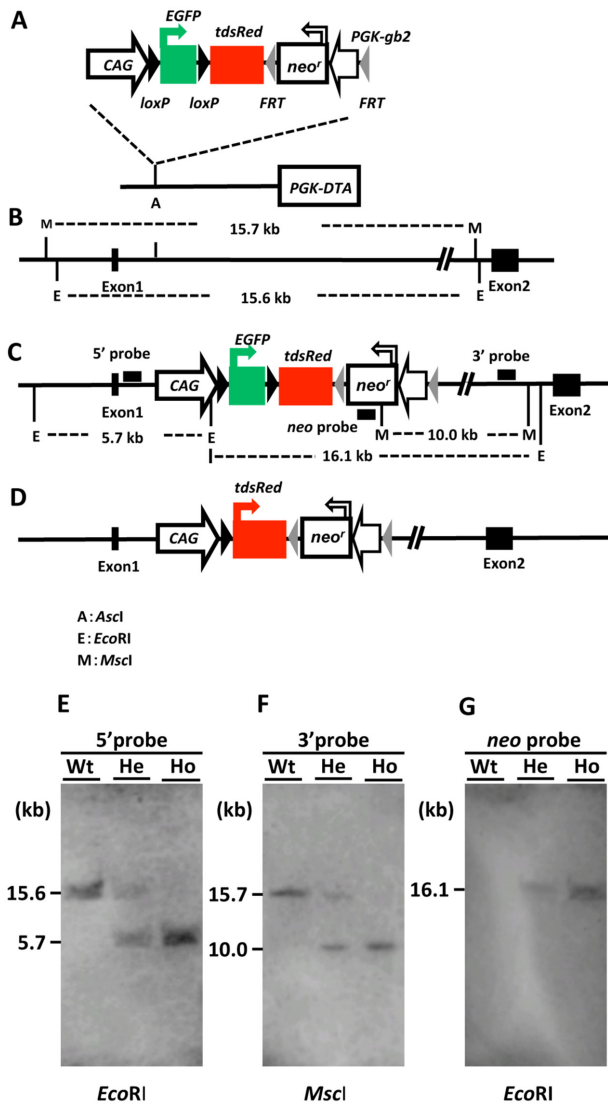
**Fig. 1.** Generation of the *ROSA26* locus with insertion of a novel Cre reporter. Schematic illustration showing the gene targeting strategy to insert the double Cre reporter into the intron between exons 1 and 2 of the *ROSA26* locus (A–D). Construction of the targeting vector (A). Wild-type *ROSA26* (B). R26GRR: Targeted *ROSA26* locus until Cre-mediated recombination (C). R26RR: Targeted *ROSA26* locus after Cre-mediated recombination (D). Southern blotting analysis was performed to confirm successful homologous recombination in *ROSA26* targeting with the double Cre reporter (E–G). Wt, wild-type mouse; He, heterozygous R26GRR mouse; Ho, homozygous R26GRR mouse. *Eco*RI digestion and Southern blotting using the 5′ probe (E). *Msc*I-digestion and Southern blotting using the 3′ probe (F). *Eco*RI digestion and Southern blotting using the *Neo* probe (G).



**Fig. 2.** Fluorescence of ES cells targeted with the R26GRR vector. All colonies of the targeted ES cells before Cre-mediated excision showed green fluorescence (A–B). Clone #9 (A), Clone #20 (B). To examine Cre-mediated *EGFP* excision, the *CAG-cre* fusion gene was electroporated into the targeted ES cells (clone #20) (C–F). Propagated ES cell colonies at 72 h after electroporation were stained with anti-FLAG M2 antibody (Sigma) and then secondarily stained with Alexa Fluor® 647 goat anti-mouse IgM (A-21235; Invitrogen). Nuclei were counterstained with DAPI. Fluorescence was observed by fluorescence microscopy (BZ-9000; Keyence). DAPI staining (C). FLAG staining (D). EGFP fluorescence (E). tdsRed fluorescence. Scale bar, 300 $\mu$m (F).

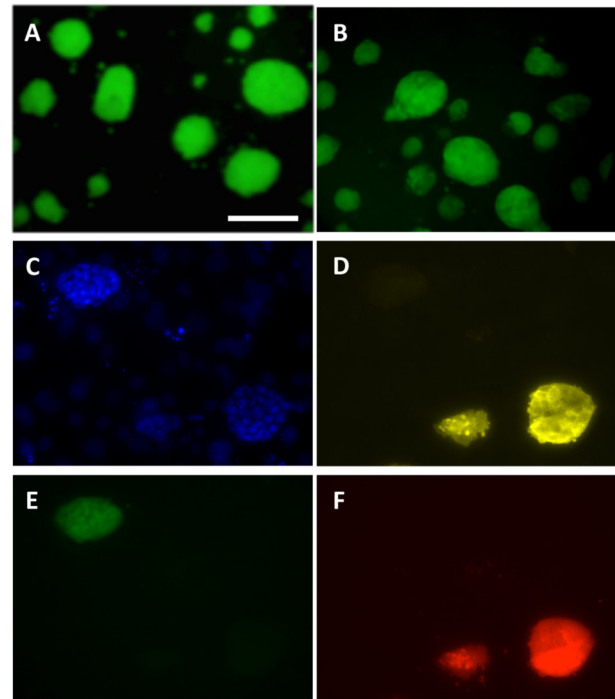throughout the embryos at 7.5 days post coitus (dpc) (Suppl. Fig. 1E) and 9.5 dpc (Suppl. Fig. 1F). Further, we investigated neonatal fluorescence of EGFP and td-sRed in heterozygous R26GRR mice. Wild-type neonates showed no fluorescence (Fig. 3A–C). With appropriate excitation, heterozygous R26GRR neonates showed bright green fluorescence throughout the body (Fig. 3E), but no red fluorescence was observed (Fig. 3F). Moreover, we examined whether constitutive expression of EGFP was maintained in the adult stage. Immediately after perfusion fixation with 4% paraformaldehyde, a variety of organs from heterozygous adults were dissected and examined. The fluorescence of EGFP was detected in all organs examined, and the brightness level varied among different organs (Suppl. Fig. 2 and Fig. 4). The pancreas showed the highest level of green fluorescence, while the lowest level of fluorescence was detected in the spleen. These observations would have
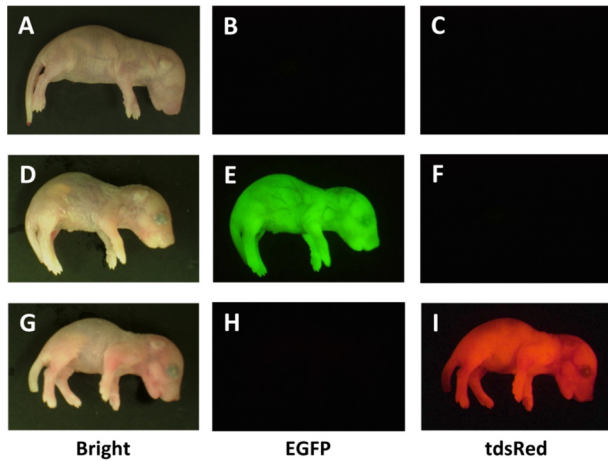
**Fig. 3.** EGFP and tdsRed expression in R26GRR and R26RR neonates. There was no fluorescence in the wild-type neonate (A–C). R26GRR neonates showed green fluorescence throughout the body, but no red fluorescence (D–F). R26RR neonates showed red fluorescence throughout the body, but no green fluorescence (G–I).
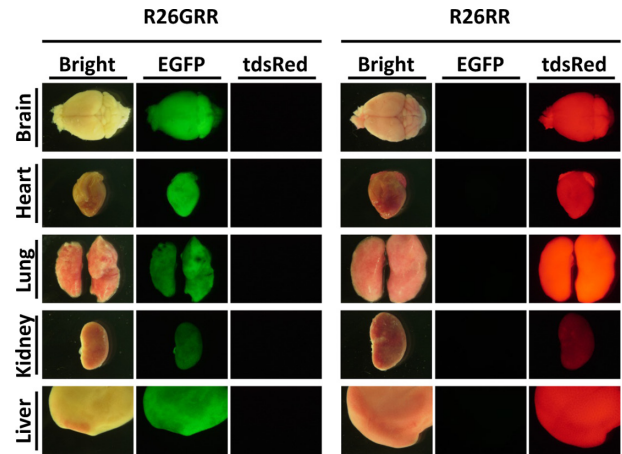


**Fig. 4.** EGFP and tdsRed expression in the organs of R26GRR and R26RR adults. Under anesthesia, mice were perfused with cold PBS and 4% paraformaldehyde solution, and the organs were dissected. The brain, heart, lungs, kidney, and liver of R26GRR adults showed green fluorescence, but not red fluorescence. In contrast to R26GRR adults, R26RR adults showed red fluorescence, but not green fluorescence.

been due to the abundance of erythrocytes in the spleen. There was no expression of red fluorescence derived from the second reporter in the organs examined. Representative images are shown in Fig. 4. Further, frozen tissue sections of the brain (neocortex, dentate gyrus, and cerebellum), cardiac ventricle, lung, kidney cortex, and liver of heterozygous R26GRR adults were examined for expression of the first and second reporters. We confirmed ubiquitous expression of EGFP and non-expression of tdsRed in almost all cell types (Fig. 5). These results suggest that the *CAG* promoter in the *ROSA26* locus is capable of constitutively activating the first reporter gene but incapable of activating the second reporter gene in nearly ubiquitous nucleated cells in R26GRR mice until Cre excision. The R26GRR mice would also be a useful source for green cells with a pure C57BL/6N genetic background.

*Extraordinarily robust red fluorescence constitutively activated from tdsRed in mice following Cre/loxP recombination*

We next examined whether the second reporter, *tdsRed*, was activated from the targeted allele in ubiquitous cells in Cre-reporter mice after *EGFP* excision. Ayu1-Cre transgenic mice express Cre recombinase in multiple tissues, including the germ line [15]. We crossed homozygous R26GRR females with Ayu1-Cre males to obtain R26GRR/Ayu1-Cre F$_1$ progeny. The *EGFP*-excised
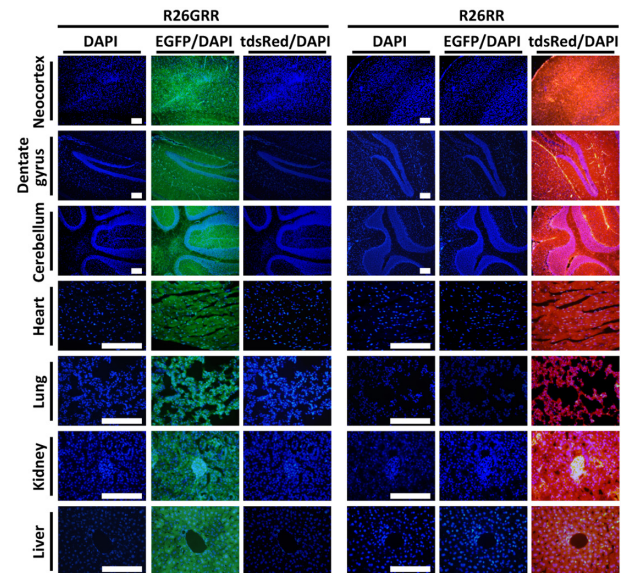


**Fig. 5.** EGFP and tdsRed expression in tissues of R26GRR and R26RR adults. Although DAPI and tdsRed were observed directly, EGFP was detected immunohistochemically with GFP antibody and FITC-labeled second antibody in fixed tissue sections. EGFP expression in nearly all cells was confirmed in the neocortex, dentate gyrus, olfactory bulb, cerebellum, heart, lung, kidney, and liver in R26GRR mice. Similarly, ubiquitous and strong tdsRed expression was detected in fix sections from R26RR mice. However, dual-emission of EGFP and tdsRed was not observed in R26GRR and R26RR mice. Scale bar, 100 $\mu$m (neocortex, dentate gyrus, cerebellum), 20 $\mu$m (heart, lung, kidney, liver).

R26GRR, R26RR, mice were produced by crossing R26GRR/Ayu1-Cre F$_1$ mice with C57BL/6N mice. EGFP excision and lack of Ayu1-Cre were confirmed by PCR analyses (data not shown). On bright field observation, skin color was apparently different between heterozygous R26RR (Fig. 3G, reddish skin) and wild-type neonates (Fig. 3A). With appropriate irradiation, heterozygous R26RR neonates emitted strong red fluorescence (Fig. 3I), but not green fluorescence (Fig. 3H). The R26RR neonates grew to adults without obvious abnormalities. Wild-type DsRed has apparent toxicity in ES cells and mice [4]. However, tdsRed did not have a deleterious effect on embryonic development and postnatal growth, similar to DsRedT3 [26]. Surprisingly, extraordinarily robust red fluorescence was detected in all organs of heterozygous R26RR adults (Suppl. Fig. 2), but green fluorescence was undetectable (Fig. 4). Red fluorescence in R26RR mice was markedly brighter in all tissues and organs compared with green fluorescence in R26GRR mice. Further, in frozen sections of R26RR, almost all cells were labeled with tdsRed (Fig. 5). These results suggested that the second reporter, *tdsRed*, was constitutively activated by *CAG* promoter activity in nearly ubiquitous cells in R26RR mice after Cre-mediated *EGFP* excision. The R26RR mice with excised *EGFP* would be a useful source of red cells with the C57BL/6N genetic background.

*Tissue-specific activation of tdsRed in green mice*

We finally examined whether the red second reporter, tdsRed, was focally expressed in specific tissue of the green organ in R26GRR mice following tissue-specific Cre/loxP recombination. Mouse insulin 1 (*Ins1*) promoter has been used to express the exogenous gene in β-cells of the pancreatic islets in mice [19]. Transgenic mice carrying the *Ins1* bacterial artificial chromosome including Cre, Ins1-Cre mice, were phenotypically normal and showed specific expression of Cre recombinase in pancreatic β-cells (data not shown). To investigate pancreatic islet-specific emission of tdsRed, homozygous R26GRR mice were crossed with hemizygous Ins1-Cre mice to obtain R26GRR/Ins1-Cre F$_1$ progeny. Pancreatic islets were clearly labeled with strong red fluorescence, and other pancreatic tissues were labeled with green fluorescence in R26GRR/Ins1-Cre mice. On histological analysis, although we did not observe colocalization between insulin signal and red fluorescence, the appearance of pancreatic islet-specific red fluorescence

suggested Cre/loxP recombination in pancreatic β-cells (Fig. 6).

We also investigated endothelial cell lineage-specific Cre/loxP recombination in R26GRR mice. The regulatory region of the receptor tyrosine kinase *Tek* (*Tie2*) gene has been well characterized for vascular endothelial cell lineage-specific transgene expression [18]. Tie2-Cre mice expressed Cre recombinase under the control of the *Tie* promoter/enhancer, which has been shown to provide uniform expression in endothelial cells during embryogenesis and in the adult stage [7]. To inspect vascular endothelial-specific emission of tdsRed, homozygous R26GRR mice were crossed with hemizygous Tie2-Cre mice to obtain R26GRR/Tie2-Cre F$_1$ progeny. The major axial vessels form in the mouse fetus at 9.5 dpc, and vascular networks then begin to extend throughout the body [18]. Vascular formation with red bright fluorescence was clearly observed in the green R26GRR/Tie2-Cre fetuses at 9.5 dpc, suggesting successful tissue-specific Cre/loxP recombination. In frozen sections of liver tissue from R26GRR/Tie2-Cre F$_1$ adults, the central vein and liver sinusoids expressed tdsRed, and abundant hepatocytes were positive for EGFP expression. These results are shown in Fig. 7. Taken together, the tissue-specific expression of Cre could be successfully detected by using R26GRR mice in which it is possible to discriminate between recombined and non-recombined cells based on different colored fluorescent proteins.

## Discussion

Gene function in the mouse has been determined by analyzing the phenotypes of traditional constitutive knockout strains. Further, the conditional knockout approach is a powerful method for spatial and temporal dissection of complex gene function in the mouse. Recently, the European Conditional Mouse Mutagenesis (EUCOMM) and the National Institute of Health Knock Out Mouse (KOMP) programs reported that more than 9000 conditional targeted alleles, the *loxP* and *FRT* sites of which were located to minimize possible interference with the splice site of the critical exon, have been systematically generated in C57BL/6N ES cells [20]. Therefore, there is increasing interest in the development of Cre-driver and Cre-reporter C57BL/6N lines for use in biological and basic medical sciences. We considered that accurate evaluation of the Cre expression pattern in Cre-driver mice would be achieved using Cre-reporter
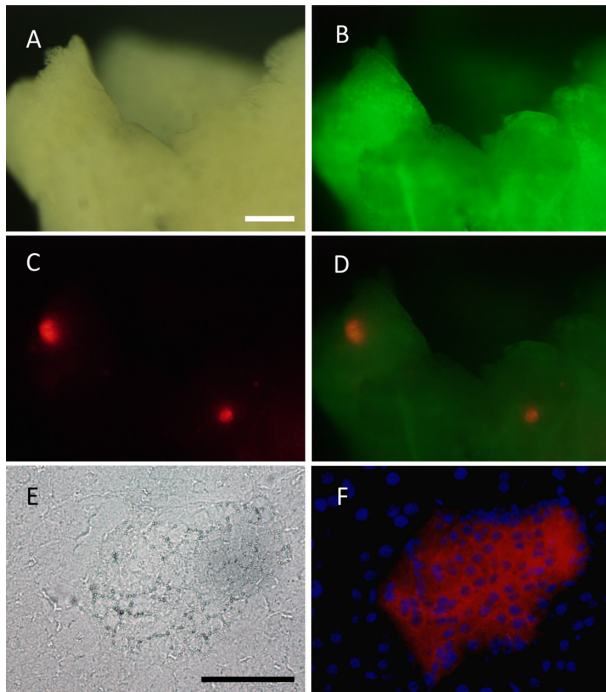
Fig. 6.  Double-color imaging of EGFP and tdsRed fluorescence in the pancreas of R26GRR/Ins1-Cre $F_1$ mice at the adult stage. Under anesthesia, mice were perfused with cold PBS and 4% paraformaldehyde solution, and the pancreas was then dissected. Bright field (A), EGFP emission (B), tdsRed emission (C), and EGFP/tdsRed emission (D) of the pancreases were examined by fluorescence stereomicroscopy. The pancreatic islets were histologically observed by bright field microscopy (E) and fluorescent microscopy with red and blue fluorescent filters for tdsRed and DAPI (F), resprctively. Scale bar, 100 $\mu$m.
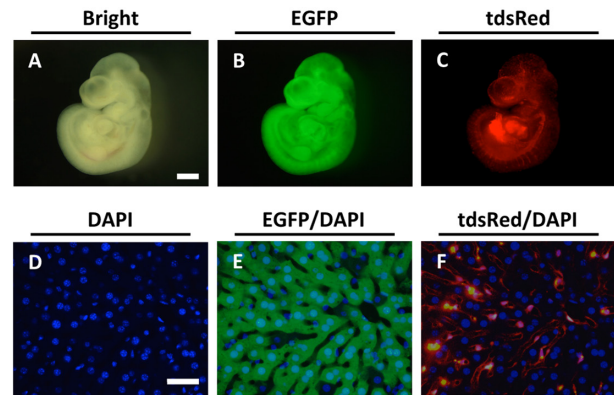


Fig. 7.  Double-color imaging of EGFP and tdsRed fluorescence in R26GRR/Tie2-Cre $F_1$ mice. Fluorescence microscopy revealed EGFP expression throughout the embryo at 9.5 dpc (B). In contrast to EGFP, tdsRed fluorescence was localized in the vascular network, including the dorsal aorta, umbilical artery, cerebral artery, and intersomitic vessels (C). Bright field (A). Scale bar, 100 $\mu$m. Transverse frozen sections of the adult liver revealed DAPI (D), EGFP/DAPI (E) and tdsRed/DAPI (F) emission in hepatocytes and blood vessels, such as the central vein and sinusoid, respectively. DAPI staining (D). Scale bar, 20 $\mu$m.

strains capable of monitoring the promoter activity of the reporter before Cre/loxP recombination. In the present study, we generated and characterized a novel Cre-reporter mouse strain, R26GRR, which showed bright green fluorescence in non-recombined cells and remarkably stronger bright red fluorescence in Cre-recombined cells.

To simultaneously monitor promoter activity of reporter gene and Cre/loxP recombination, several approaches using two types of reporter have been applied in the development of Cre-reporter mice. Lobe *et al.* [9] reported the generation of Z/AP transgenic mice carrying the floxed *ßgeo* gene distally fused to the human alkaline phosphatase (AKP) gene under the control of the *CAG* promoter. The exogenous genes allowed extensive expression of the *lacZ* gene as the first reporter until Cre-mediated recombination with expression of the *AKP* gene as the second reporter after Cre-mediated *ßgeo* excision.

Further, Novok *et al.* [17] generated a Z/EG transgenic mouse line in which the second reporter was the *EGFP* gene in place of the *AKP* gene, because detection of the second marker in Z/AP mice required invasive treatment of the tissue. In both Z/AP and Z/EG mouse lines, widespread expression of the second makers, i.e., AKP and EGFP, respectively, was observed after universal Cre-mediated *ßgeo* excision, but the staining pattern of the first marker, i.e., β-galactosidase, was different from those of the second markers following universal deletion of *ßgeo* in several tissues. It was speculated that *lacZ* sequences derived from prokaryotes may be prone to gene silencing compared with the *AKP* and *EGFP* genes derived from eukaryotes. In the present study, we used *EGFP* derived from *Aequorea victoria* as the first marker and *tdsRed* derived from *Discosoma* sp. reef coral as the second marker. There were no discrepancies in sites of visualization between EGFP and tdsRed fluorescence in R26GRR mice and R26RR mice, respectively.

Recently Hartwish *et al.* [5] reported the development of a dual-Cre-reporter transgenic mouse line bearing the floxed *mCherry* gene, a color variant of *DsRed*, with fusion of the *EGFP* gene at the 3′-end of the first reporter gene under the control of the *CAG* promoter. The dual-Cre-reporter mice carrying eukaryotic fluorescent protein genes were produced by the conventional meth-

od of pronucleus DNA injection. Multiple copies of the exogenous Cre-reporter unit were integrated randomly. Although there was no description of the problems with regard to the first and second reporter expression in the report, it is possible that the random integration and multiple copies would result in transgene-silencing position effects and unpredictable results of Cre-mediated excision, respectively. Therefore, a single copy number of the double Cre-reporter apparatus should be positioned in a reliable chromosome site to eliminate potential silencing effects. The optimal orientation for transgene expression involves integration of the transgene into the *ROSA26* locus through homologous recombination in ES cells. The endogenous *ROSA26* promoter is effective for ubiquitous gene expression [21, 27], but results only in moderate ubiquitous expression and is unsuitable for achieving high expression levels [11, 22]. To obtain reliable fluorescent reporter signals, the reporter gene was driven by inserting the *CAG* promoter 5′ adjacent to the floxed first reporter gene in the *ROSA26* locus in R26GRR mice. The presence of bright green fluorescence and extremely bright red fluorescence in R26GRR mice would be dependent on *CAG* promoter activity in combination with the genomic features of the *ROSA26* locus.

Muzumdar *et al.* [13] generated double reporter mice for monitoring of reporter gene promoter activity and Cre/loxP recombination. Similar to R26GRR mice, their double reporter apparatus was driven by the *CAG* promoter in the *ROSA26* locus and expressed membrane-targeted tdTomato (mT) prior to Cre excision and membrane-targeted EGFP (mG) following Cre excision. They showed that reporter expression was nearly ubiquitous, allowing visualization of strong fluorescent signals in all tissues examined. However, there were discrepancies in features between R26GRR mice and the mT/mG mice generated by Muzumdar *et al.* [13]. First, the ordering of double reporter genes is different between the two strains, and therefore red and green fluorescence occurred after Cre excision in R26GRR mice and mT/mG mice, respectively. Red fluorescence produces less background interference and shows higher tissue penetration than green fluorescence. Thus, precise detection of Cre-recombined cells could be achieved with the robust red fluorescent signal. Second, the double reporters of R26GRR and mT/mG are expressed in the cell body and cell membrane, respectively. It seems that cytoplasmic GFP is considerably different with regard

to the appearance of certain projection neurons in comparison with membrane-targeted GFP [6]. Finally, the mT/mG mouse strain was produced using R1 ES cells, which were established from (129/Sv × 129/Sv-CP) F$_1$ blastocysts [14]. Some strains of 129 mice showed a high incidence of spontaneous testicular teratomas, although the incidence differed depending on the parental lineage [23]. In contrast, the R26GRR mouse strain was produced using B6N-22 ES cells, which were established from C57BL/6N blastocysts [24]. The C57BL/6 mouse is the best-known inbred mouse strain, and has been widely used as a genetic background for congenic and mutant mice. Moreover, the International Knockout Mouse Consortium has commenced a project to mutate all protein-coding genes in mice using a combination of gene trapping and gene targeting in C57BL/6N mouse ES cells. As described above, more than 9000 conditional targeted alleles have already been produced in C57BL/6N ES cells by EU COMM and KOMP [20]. By crossing of R26GRR mice with the C57BL/6N genetic background, it would be possible to generate Cre-driver mouse strains with precise information regarding Cre/loxP recombination sites in conditional knockout C57BL/6N strains supplied by EU COMM and KOMP. Moreover, R26GRR C57BL/6N carrying stage-specific Cre expression genes would be useful for fate mapping studies in combination with transplantation, because this strain is a source of green fluorescent cells that are convertible to red fluorescence at specific stages of development.

To our knowledge, this is the first report of the production of double Cre-reporter mice with a pure genetic background of C57BL/6N for simultaneously monitoring promoter activity of reporter gene and Cre/loxP recombination. The R26GRR mouse strain, RBRC04874 C57BL/6N-*Gt (ROSA) 26Sor<tm1*(*CAG-EGFP,-tdsRed*)*Utr>*/Rbrc, will be available from the RIKEN BioResource Center.

## References

1. Abremski, K., Hoess, R., and Sternberg, N. 1983. Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. *Cell* 32: 1301–1311. [Medline] [CrossRef]

2. Bevis, B.J. and Glick, B.S. 2002. Rapidly maturing variants of the Discosoma red fluorescent protein (DsRed). *Nat Biotechnol.* 20: 83–87. [Medline] [CrossRef]

3. Chen, C.M., Krohn, J., Bhattacharya, S., and Davies, B. 2011. A comparison of exogenous promoter activity at the ROSA26 locus using a PhiC31 integrasemediated cassette exchange approach in mouse ES cells. *PLoS ONE* 6: e23376. [Medline] [CrossRef]

4. Hadjantonakis, A.K., Macmaster, S., and Nagy, A. 2002. Embryonic stem cells and mice expressing different GFP variants for multiple non-invasive reporter usage within a single animal. *BMC Biotechnol* 2: 11. [Medline] [CrossRef]

5. Hartwich, H., Satheesh, S.V., and Nothwang, H.G. 2012. A pink mouse reports the switch from red to green fluorescence upon Cre-mediated recombination. *BMC Res. Notes* 5: 296. [Medline] [CrossRef]

6. Ito, K., Okada, R., Tanaka, N., and Awasaki, T. 2003. Caution observations on preparing and interpreting brain images using molecular biology-based stainingtechniques. *Microsc. Res. Tech.* 62: 170–186. [Medline] [CrossRef]

7. Kisanuki, Y.Y., Hammer, R.E., Miyazaki, J., Williams, S.C., Richardson, J.A., and Yanagisawa, M. 2001. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev. Biol.* 230: 230–242. [Medline] [CrossRef]

8. Lenz, P. 1999. Fluorescence measurement in thick tissue layers by linear or nonlinear long-wavelength excitation. *Appl. Opt.* 38: 3662–3669. [Medline] [CrossRef]

9. Lobe, C.G., Koop, E.K., Kreppner, W., Lomeli, H., Gertsenstein, M., and Nagy, A. 1999. Z/AP, a double reporter for Cre-mediated recombination. *Dev. Biol.* 208: 281–292. [Medline] [CrossRef]

10. Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R., Lein, E.S., and Zeng, H. 2010. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat. Neurosci.* 13: 133–140. [Medline] [CrossRef]

11. Mao, X., Fujiwara, Y., and Orkin, S.H. 1999. Improved reporter strain for monitoring Crerecombinase-mediated DNA excisions in mice. *Proc. Natl. Acad. Sci. USA* 96: 5037–5042. [Medline] [CrossRef]

12. Miyoshi, G., Hjerling-Leffler, J., Karayannis, T., Sousa, V.H., Butt, S.J., Battiste, J., Johnson, J.E., Machold, R.P., and Fishell, G. 2010. Genetic fate mapping reveals that the caudal ganglionic eminence produces a large and diverse population of superficial cortical interneurons. *J. Neurosci.* 30: 1582–1594. [Medline] [CrossRef]

13. Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. 2007. A global double-fluorescent Cre reporter mouse. *Genesis* 45: 593–605. [Medline] [CrossRef]

14. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl.*

15. Niwa, H., Araki, K., Kimura, S., Taniguchi, S., Wakasugi, S., and Yamamura, K. 1993. An efficient gene-trap method using poly A trap vectors and characterization of gene-trap events. *J. Biochem.* 113: 343–349. [Medline]

16. Niwa, H., Yamamura, K., and Miyazaki, J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108: 193–199. [Medline] [CrossRef]

17. Novak, A., Guo C., Yang, W., Nagy, A., and Lobe, G.C. 2000. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 28: 147–155. [Medline] [CrossRef]

18. Schlaeger, T.M., Qin, Y., Fujiwara, Y., Magram, J., and Sato, T.N. 1995. Vascular endothelial cell lineage-specific promoter in transgenic mice. *Development* 121: 1089–1098. [Medline]

19. Sekiguchi, Y., Owada, J., Oishi, H., Katsumata, T., Ikeda, K., Kudo, T., and Takahashi, S. 2012. Noninvasive monitoring of b-cell mass and fetal b-cell genesis in mice using bioluminescence imaging. *Exp. Anim.* 61: 445–451. [Medline] [CrossRef]

20. Skarnes, W. C., Rosen, B., West, A. P., Koutsourakis, M., Bushell, W., Iyer, V., Mujica, A. O., Thomas, M., Harrow, J., Cox, T., Jackson, D., Severi, J., Biggs, P., Fu, J., Nefedov, M., de Jong, P. J., Stewart, A.F., and Bradley, A. 2011. A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* 474: 337–342. [Medline] [CrossRef]

21. Soriano, P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* 21: 70–71. [Medline] [CrossRef]

22. Srinivas, S., Watanabe, T., Lin, C.S., William, C., Tanabe, Y., Jessell, T., and Costantini, F. 2001. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.* 1: 4. [Medline] [CrossRef]

23. Stevens, L.C. 1973. A new inbred strain (129-terSv) with a high incidence of spontaneous congenital teratomas. *J. Natl. Cancer Inst.* 50: 235–242. [Medline]

24. Tanimoto, Y., Iijima, S., Hasegawa, Y., Suzuki, Y., Daitoku, Y., Mizuno, S., Ishige, T., Kudo, T., Takahashi, S., Kunita, S., Sugiyama, F., Yagami, K. 2008. Embryonic stem cells derived from C57BL/6J and C57BL/6N mice. *Comp. Med.* 58: 347–352. [Medline]

25. Tchorz, J.S., Suply, T., Ksiazek, I., Giachino, C., Cloëtta, D., Danzer, C.P., Doll, T., Isken, A., Lemaistre, M., Taylor, V., Bettler, B., Kinzel, B., and Mueller, M. 2012. A Modified RMCE-Compatible Rosa26 Locus for the Expression of Transgenes from Exogenous Promoters. *PLoS ONE* 7: e30011. [Medline] [CrossRef]

26. Vintersten, K., Monetti, C., Gertsenstein, M., Zhang, P., Laszlo, L., Biechele, S., and Nagy, A. 2004. Mouse in red: Red fluorescent protein expression in mouse ES cells, embryos, and adult animals. *Genesis* 40: 241–246. [Medline] [CrossRef]

27. Zambrowicz, B.P., Imamoto, A., Fiering, S., Herzenberg, L.A., Kerr, W.G., and Soriano, P. 1997. Disruption of overlapping transcripts in the ROSAßgeo 26 gene trap strain leads to widespread expression of β galactosidase in mouse embryos and hematopoietic cells. *Proc. Natl. Acad. Sci. USA* 94: 3789–3794. [Medline] [CrossRef]