



## Original

## Tamoxifen feeding method is suitable for efficient conditional knockout

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**Abstract:** The Cre-driver system is used to generate conditional knockout mice. Tamoxifen inducible Cre-driver mice can be used for spatiotemporal knockout by administration of the drug. A major tamoxifen administration is performed by intraperitoneal administration or oral administration. However, these forced administrations may be damaging to mice. Herein, we have demonstrated an improved method of administering tamoxifen with powdered food to mice. A mouse line expressing the tamoxifen-inducible *Cre* gene was used ubiquitously in this experiment to evaluate the efficiency of Cre recombination in the whole body. Our method also achieved efficient recombination without causing injury to mice. The X-gal staining intensity of the feeding method was equivalent to that of the intraperitoneal administration method. Furthermore, this method can be used for recombination before birth, or during the fetal period. We recommend researchers to employ this feeding method to administer tamoxifen to minimize the risk of injury to mice.

**Key words:** conditional knockout, Cre, feeding method, tamoxifen

### Introduction

Gene disruption has become commonplace owing to the development of the genome editing technology; however, conditional knockout of genes in a tissue-specific and time-specific manner remains challenging. The Cre-driver mouse line plays an important role in this technology. In 1995, Littlewood *et al.* showed that a mutation in the hormone-binding domain of the mouse estrogen receptor results in its inability to bind to 17 $\beta$ -estradiol, and enhances binding to 4-hydroxytamoxifen, an anti-estrogen drug [1]. In 1996, Zhang *et al.* showed that the binding of Cre to the tamoxifen-binding domain activates inducible site-directed recombination [2]. In 1995, Metzger *et al.* discovered a fusion protein Cre-ER, in which Cre recombinase was linked to the ligand-binding domain (LBD) of the human estrogen receptor [3]. In 1996, Feil *et al.* reported a new protein Cre-ER<sup>T</sup>, consist-

ing of the mutated LBD of the human estrogen receptor [4]. In 1997, they reported another protein Cre-ER<sup>T2</sup> containing the G400V/M543A/L544A triple mutation of the human estrogen receptor LBD as being more sensitive to 4-hydroxytamoxifen induction than Cre-ER<sup>T</sup> [5]. The promoter-dependently expressed CreER<sup>T2</sup> localizes in the cytoplasm until exposed to tamoxifen. Tamoxifen drives CreER<sup>T2</sup> to the cell nucleus, causing recombination between *loxP* sites. The administration of tamoxifen is generally carried out by dissolving tamoxifen in a food oil followed by intraperitoneal administration (*i.p.*) using a needle, or oral administration using a gastric tube.

We have developed an “exchangeable gene trap method” capable of replacing a reporter gene with arbitrary genes in the publish database EGTC [<http://egtc.jp>] [6–8]. The promoter trap method was an efficient mechanism to generate various Cre-driver mice by replacing the reporter gene of the exchangeable trap clone with

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Supplementary Tables: refer to J-STAGE: <https://www.jstage.jst.go.jp/browse/expanim>



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the *Cre* gene [7]. We have created a Cre-driver mouse library by replacing the reporter gene of the exchangeable gene trap clone with the tamoxifen-inducible *Cre* gene. The recombination site of the tamoxifen-inducible Cre-driver mouse in each line was evaluated by *i.p.* of tamoxifen to the offspring obtained by mating each line with the ROSA26 reporter mouse, followed by X-gal staining [9, 10]. However, the Cre recombination efficiency was low. Additionally, the mortality rate of certain mouse lines was high and could not be analyzed. Furthermore, adhesions of the organs and oil remaining in the abdominal cavity hindered dissection.

Andersson *et al.* have proposed a method of feeding tamoxifen-supplemented food as an alternative to the conventional method of *i.p.* to reduce the risk of injury to mice [11]. They used this feeding method to generate a heart-specific conditional knockout. In this study, to test the feeding method, we used a mouse line that expresses *Cre-mER<sup>T2</sup>* ubiquitously and evaluated the recombination efficiency in the whole body. We found that tamoxifen administration conditions are able to induce good recombination in fetuses, during the perinatal period, and in adults.

## Materials and Methods

### Animal care

Animal care and experiments were conducted in accordance with the guidelines for animal and recombinant DNA experiments of Kumamoto University, Japan, and approved by the Ethics Committee of the Center for Animal Resources and Development (CARD), Kumamoto University Japan.

### Cre-driver mice to study tamoxifen administration conditions

Tamoxifen administration conditions were examined in Ayu21-B165CAG-Cre-mER<sup>T2</sup> mice, B6.Cg-Gt(Ayu21-B165\*CAGCre-mER<sup>T2</sup>)1Card (CARD ID: 2561, Kumamoto University). The Ayu21-B165 embryonic stem (ES) cell line was obtained using the gene trap method, followed by the insertion of a trap vector pU-21B into *BY730561* gene (EST) [6–8]. The Cre-mER<sup>T2</sup>, in which Cre recombinase is fused with the LBD of a mouse estrogen receptor, contains the G404V/M547A/L548A triple mutation relative to human ER<sup>T2</sup> [5]. The construct (*lox66-CAG promoter-Cre-mER<sup>T2</sup>-pA-FRT-PGK-Puro-FRT-loxP*) was then knocked in at this trap vector insertion position using the Cre/mutant *lox* system [6, 7].

### Mice for tamoxifen induction

To detect Cre recombination, the Cre-driver mice were

crossed with ROSA26 reporter mice (R26R) [10]. The offspring (B165CAG-Cre-mER<sup>T2+/-</sup>; R26R<sup>+/-</sup>, Cre/R26R) were used for tamoxifen induction. Additionally, the feeding method conditions were applied to pregnant ICR mice (Charles River Laboratories Japan, Yokohama, Japan) obtained via *in vitro* fertilization of Cre-driver mice (heterozygotes) and ROSA26 reporter mice (homozygotes).

The Ayu21-W241SACre mice, B6.Cg-Gt(Ayu21-W241\*SACre)1Card (CARD ID: 2040, Kumamoto university), were crossed with ROSA26 reporter mice, and the resulting offspring (W241SACre<sup>+/-</sup>; ROSA26R<sup>+/-</sup>, W241SACre/R26R) were used as an index for determining the level of X-gal staining.

### RT-PCR analysis

Total RNA was extracted from the organs (brain, thymus, heart, lung, stomach, intestine, liver, spleen, pancreas, kidney, muscle, testis, ovary, and uterus) of Cre/R26R mice using ISOGEN (Nippon Gene, Tokyo, Japan) and was purified using RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was synthesized using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA, USA). The negative control was synthesized without reverse transcriptase. PCR was performed using the primers, Cre3; 5'-AATGCTTCTGTCCGTTTGCC-3' and Cre4; 5'-CTACACCAGAGACGGAAATC-3' for *Cre-mER<sup>T2</sup>*, and *Gapdh*-1; 5'-GGAAAGCTGTGGCGTGATG-3' and *Gapdh*-2, 5'-CTGTTGCTGTAGCCGTATTC-3' for *Gapdh*.

### Tamoxifen feeding method

Tamoxifen (T5648, Sigma-Aldrich, St. Louis, MO, USA) was administered to Cre/R26R mice. A standard diet pellet (CE-2, CLEA Japan Inc., Tokyo, Japan) was discontinued approximately 4 days before commencing tamoxifen administration. Then, a Y-type feeder (KN-675-8, Natsume Seisakusho, Tokyo, Japan) contained the powder of the standard diet was placed in a mouse cage. The feeder was removed the day before the start of tamoxifen administration and mice were fasted. On the day of tamoxifen administration, powdered food (50 g per feeder) containing tamoxifen was mixed using a mortar and a pestle, transferred to a plastic bag, and mixed well by shaking. The concentration of the powder containing tamoxifen was 0.5 mg, 1.0 mg and 2.0 mg tamoxifen/g feed.

The tamoxifen-containing powdered food was weighed and fed to the mice. As a negative control, Cre/R26R was fed powdered food without tamoxifen. On the last day of administration, the amount of remaining food was measured. Food intake was calculated by subtract-

ing the amount of food remaining at the end of administration from the amount of food at the start of administration.

### Intraperitoneal administration of tamoxifen

The stock solution of tamoxifen was 20 mg/ml. One tenth of the total dose of ethanol and tamoxifen were added in a tube and vortexed. Corn oil (C8267, Sigma-Aldrich) was added to the tube and the solution was mixed for approximately 30 min using a micro-tube mixer (MT-360, TOMY, Tokyo, Japan). Cre/R26R mice were injected via *i.p.* with 75 mg/kg tamoxifen for 5 consecutive days. As a negative control, corn oil was injected via *i.p.* to Cre/R26R mice.

### X-gal staining

To identify the Cre recombination cells, the organs (ear (skin), eye, brain, lung, heart, thymus, spleen, pancreas, liver, stomach, small intestine, kidney, genital organ, and thigh muscle) of Cre/R26R mice were analyzed by X-gal staining. The brain, lung, heart, liver, kidney, and thigh muscles were sliced by a blade (S35, Feather, Osaka, Japan) to allow the stain to penetrate. Organs were fixed for 30 min in fixing solution [1% formaldehyde, 0.2% glutaraldehyde, and 0.02% NP40 in phosphate-buffered saline (PBS)]. For washing, the organs were treated with 1% triton X-100 in PBS for 10 min, followed by two treatment cycles with PBS for 10 min, with shaking. Thereafter, the organs were incubated overnight for 20 h in a staining solution [5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.5 mM MgCl<sub>2</sub>, and 0.5% X-gal in PBS]. After staining, the organs were washed three times in PBS and then post-fixed in 10% formalin solution. The organs were photographed using a digital single-lens reflex camera (E-620, Olympus, Tokyo, Japan).

### Transparency

A clearing treatment was performed for detailed analysis of the X-gal stained organs and then scored. The organs were transferred to a container containing 50% glycerol and shaken gently for approximately 2 h. Subsequently, the organs were treated with 70% glycerol.

### Scoring

The W241SACre mice exhibits ubiquitous and strong staining in the offspring obtained by crossing with the ROSA26 reporter mice. Therefore, various organs of this mouse were used as a standard with a maximum score. The degree of X-gal staining of various organ sites of the examined mice was evaluated on a scale of 0 (no staining) to 5.

The 17 dissected tissues were divided into 34 sites and scored. The score of the individual mouse was obtained by subtracting the score of the negative control, that is, the background from the score of the sample. The 17 tissues (34 sites) were testis (outside seminiferous tubule, inside seminiferous tubule), uterus, ovary, oviduct, kidney (outside medulla layer, inner medulla layer, cortex, renal pelvis), eye (optic nerve, cornea, eye muscle), pancreas, spleen, thymus, small intestine, stomach (forestomach, glandular stomach), liver, lung (bronchiole, alveolar), heart (heart, auricle), skeletal muscle, brain (cerebral cortex, cerebellum, hippocampus, thalamus, midbrain, hypothalamus, medulla, pons, olfactory bulb), and skin. Scoring was performed while observing organs under a stereomicroscope (SZX12, Olympus).

## Results

### Evaluation of Cre recombination activity by tamoxifen feeding method

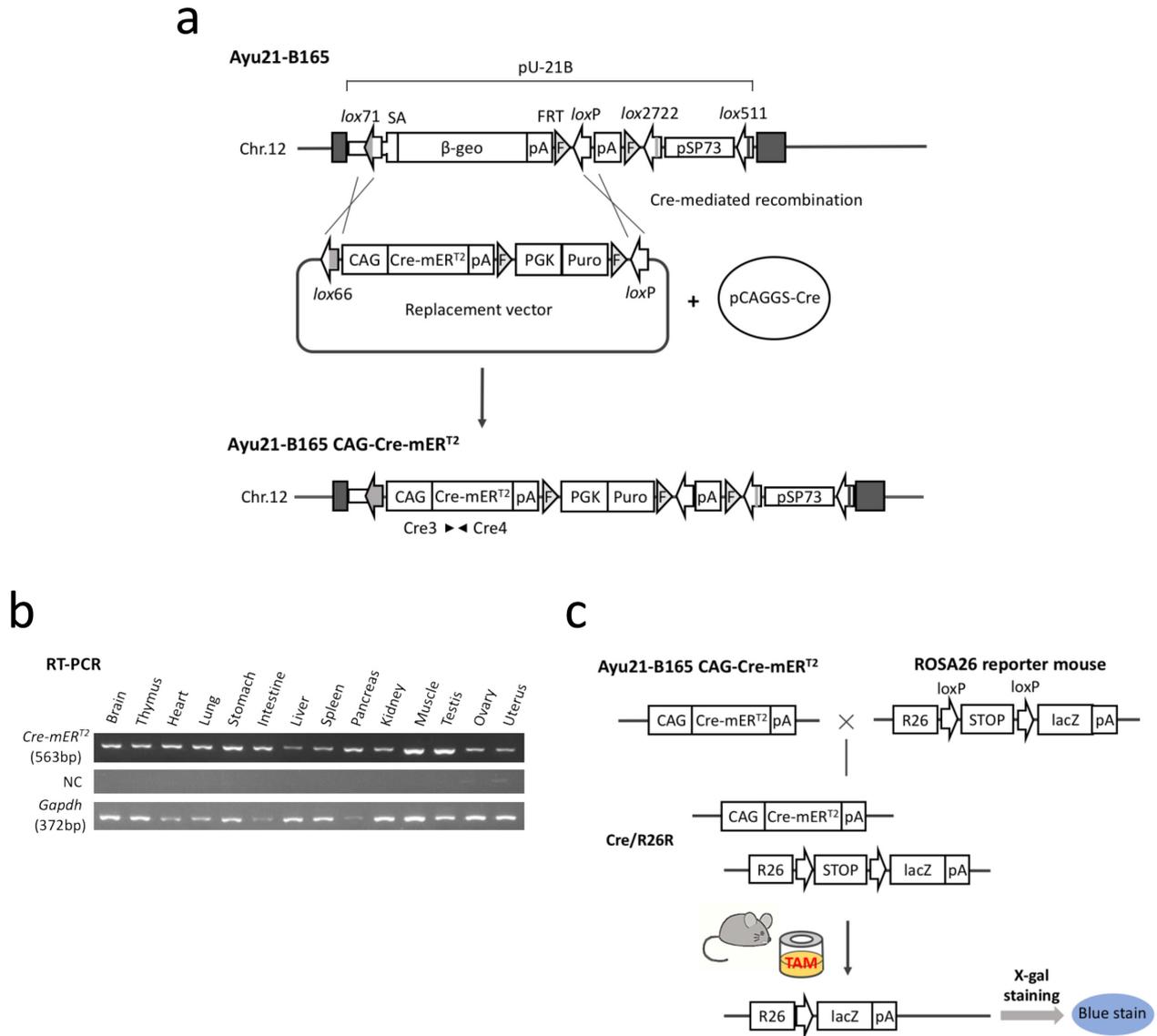
We examined tamoxifen feeding conditions using Cre-driver mice that ubiquitously express *Cre-mER<sup>T2</sup>* (Figs. 1a and b). With the recombination of Cre-mER<sup>T2</sup> at the R26R locus, following the translocation into the nucleus by tamoxifen administration, cells expressed the *lacZ* gene and were stained blue by X-gal staining (Fig. 1c). To evaluate Cre recombination efficiency, the degree of staining was graded for each organ and the total score per mouse was calculated. The method is shown in Fig. 2.

### Contrivance to increased food intake

As the amount of tamoxifen entering the body depends on the food intake of the mouse in the feeding method, we examined the device for administering food, the housing condition of the mice before administration, the presence or absence of acclimation to the Y-type feeder (4 days), and the presence or absence of fasting (1 day).

The food intake under each condition is summarized in Table 1. The amount of food intake was determined by subtracting the amount of food remaining on day 5 from the amount of food on day 1. For housing groups, the total food intake was divided by the number of mice to determine food intake per animal.

The average food intake of the individually housed mice was 6.31 g (n=2) and that of grouped-housed mice under conditions without acclimatization and fasting was 3.70 g (n=10). Therefore, we selected individual housing. However, in the condition with acclimatization and without fasting, there was not much difference between the food intakes of individually housed mice (average 10.33 g, n=3) and grouped-housed mice (average 10.29 g, n=3).



**Fig. 1.** Strategy for examining the effect of tamoxifen feeding. (a) Establishment of a tamoxifen-inducible Cre-driver mouse. The trap vector pU-21B was inserted into the *BY730561* gene (EST, 660bp). The construct (*lox66*-CAG promoter-*Cre-mER<sup>T2</sup>*-pA-FRT-PGK promoter-Puro-FRT-*loxP*) was knocked in the trap vector of Ayu21-B165 ES cell using the Cre/mutant *lox* system. SA, splice acceptor;  $\beta$ -geo,  $\beta$ -galactosidase/neomycin-resistance fusion gene; pA, polyadenylation signal; pSP73, plasmid sequence; *Cre-mER<sup>T2</sup>*, Cre recombinase fused with the LBD of a mouse ER containing the G404V/M547A/L548A triple mutation; Puro, puromycin resistance marker; pCAGGS-Cre, Cre expression vector. (b) RT-PCR analysis. *Cre-mER<sup>T2</sup>* is expressed in various organs of Ayu21-B165CAG-*Cre-mER<sup>T2</sup>* mice. PCR was performed with primer set Cre3 and Cre4. NC, no-RT negative control. (c) Detection of tamoxifen-induced Cre activity. After removal of the *loxP*-flanked STOP cassette by Cre-mediated recombination, the *lacZ* reporter was expressed in cells. The X-gal staining was performed for each organ. R26, ROSA26 promoter.

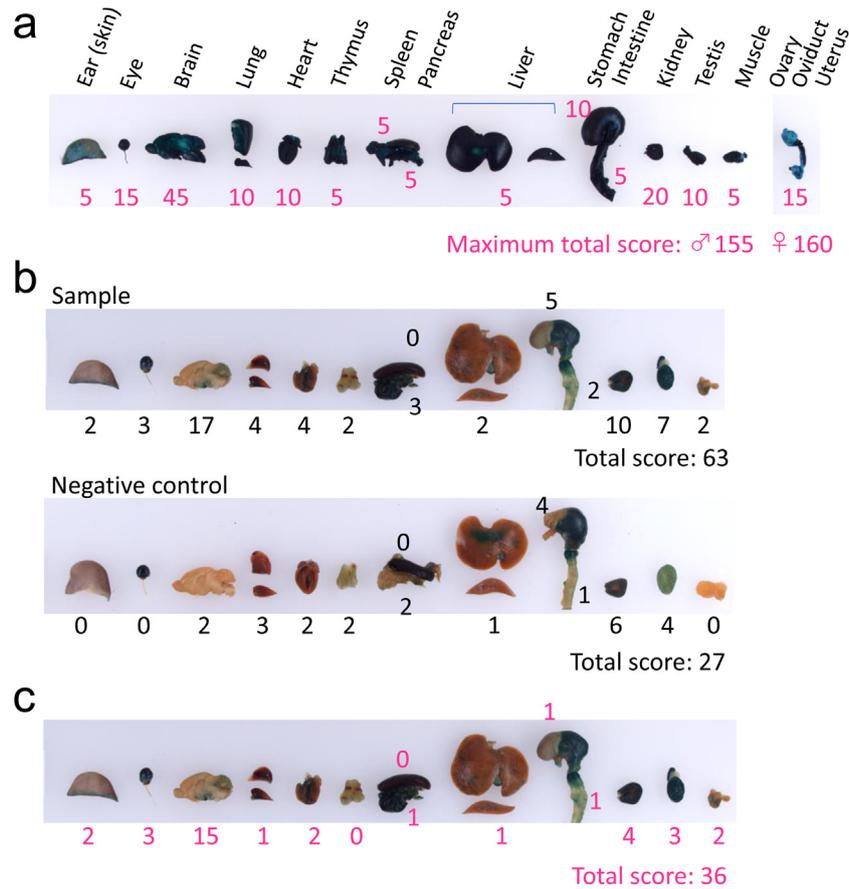
Next, we compared rearing condition with or without acclimation (4 days). In the condition of individual housing and fasting, the average food intake with acclimation was 10.33 g (n=3), and average of food intake without acclimation was 6.31 g (n=2). In the condition of group housing and without fasting, average of food intake with acclimation was 10.29 g (n=3) and average of food intake without acclimation was 3.70 g (n=10). Both results showed that acclimation is better.

We also compared rearing conditions with or without fasting (1 day). The average food intake of the individu-

ally housed, non-acclimated, fasted mice was 10.42 g (n=3) and that of the non-fasted mice was 6.31 g (n=2). Therefore, fasted mice showed higher food intake than non-fasted mice. Based on these results, individual housing, acclimation (4 days), and fasting (1 day) conditions were used to increase food intake.

#### Examination of administration period

Tamoxifen was administered for a period of 5 days, 7 days, and 4 weeks. The photographs of various X-gal stained organs of the individual with the maximum score



**Fig. 2.** Scoring method: (a) Stained images of various organs in the W241SACre/R26R mouse. The staining intensities were taken as the maximum score. The 34 sites in 17 mouse tissues were used to evaluate Cre recombinase efficiency. (b) Stained images of a sample mouse (Cre/R26R, tamoxifen treated) and a negative control mouse (Cre/R26R, non-tamoxifen-treated). (c) The final sample score was obtained by subtracting the negative control score from the sample score shown in (b).

**Table 1.** Invention to increase feed intake

Mouse No.	Condition			Food amount			
	Housing	Acclimation	Fasting	Start (g)	End (g)	Intake (g)	Average (g)*
87	Individual	No	No	24.62	16.45	8.17	6.31*
91	Individual	No	No	25	20.55	4.45	
225	Group	No	No	49.89	33.03	16.86	5.62*
226	Group	No	No				
227	Group	No	No				
79	Group	No	No	49.63	43.37	6.26	2.09*
80	Group	No	No				
81	Group	No	No				
1	Group	No	No	49.94	38.47	11.47	5.74*
2	Group	No	No				
1	Group	No	No	50	47.6	2.4	1.20*
2	Group	No	No				
4856	Individual	Yes	No	50.12	38.66	11.46	10.33*
4857	Individual	Yes	No	50.46	38.23	12.23	
4858	Individual	Yes	No	50.96	43.66	7.3	
356	Group	Yes	No	49.8	18.93	30.87	10.29*
357	Group	Yes	No				
358	Group	Yes	No				
360	Individual	No	Yes	24.87	14.22	10.65	10.42*
361	Individual	No	Yes	24.83	13.64	11.19	
365	Individual	No	Yes	24.83	15.41	9.42	

\*Food amount represented by gTAM feed/mouse/5 days.

obtained under each condition are shown in Fig. 3. The results for all individuals are shown in Supplementary Table 1. For 7 days and 4 weeks, the amount converted to 5 days was described as food intake. The total scores of individuals dosed for 5 days ( $n=2$ ) were 22 and 35, for 7 days ( $n=3$ ) were 28, 34, and 35, and that for 4 weeks ( $n=3$ ) were 26 and 61. Mouse lines administered tamoxifen for 4 weeks showed the highest scores; however, individual differences were considerable. In addition, there was no difference in the results between 5 days and 7 days. Hence, a 5-day administration period was considered optimal.

### Period from the end of administration to X-gal staining

The period from the end of tamoxifen administration to X-gal staining ranged from 2 to 9 days. The results are shown in Figs. 3, 4, Supplementary Tables 2 and 3. The total scores of individuals ( $n=2$ ) stained with X-gal 2 and 9 days after tamoxifen administration with 0.5 mg tamoxifen/g feed were 22 and 35, and 52 and 58, respectively. The signal was stronger for the 9-day period than that for the 2-day period. Therefore, a 9-day period from the end of tamoxifen administration to X-gal staining was considered.

### Examination of dosage

Different amounts of tamoxifen per gram of powdered food (0.5, 1.0, and 2.0 mg) were tested. The results are shown in Fig. 4 and Supplementary Table 2. The total scores of individuals ( $n=2$ ) fed with 0.5 mg tamoxifen/g feed were 52 and 58, 1.0 mg tamoxifen/g feed ( $n=3$ ) were 63, 74, and 78, and 2.0 mg tamoxifen/g feed ( $n=3$ ) were 64, 85, and 101. The highest total score was obtained when 2.0 mg tamoxifen/g feed. The photograph of the individual from whom the maximum total score was calculated under each condition is shown in Fig. 4.

### Comparison between the feeding method and the intraperitoneal administration method

The Cre activity was induced under optimal conditions of feeding, and the X-gal staining results of the feeding method were compared with those of the *i.p.* method. The results are shown in Fig. 5. The total scores of individuals ( $n=3$ ) collected from the feeding method were 72, 74, and 75. Conversely, the total scores of individuals ( $n=3$ ) collected via *i.p.* were 60, 71, and 72. The efficiency of Cre-activity induction by the feeding method was equivalent to that of *i.p.* method.

### Administration of tamoxifen to pregnant mice using the feeding method

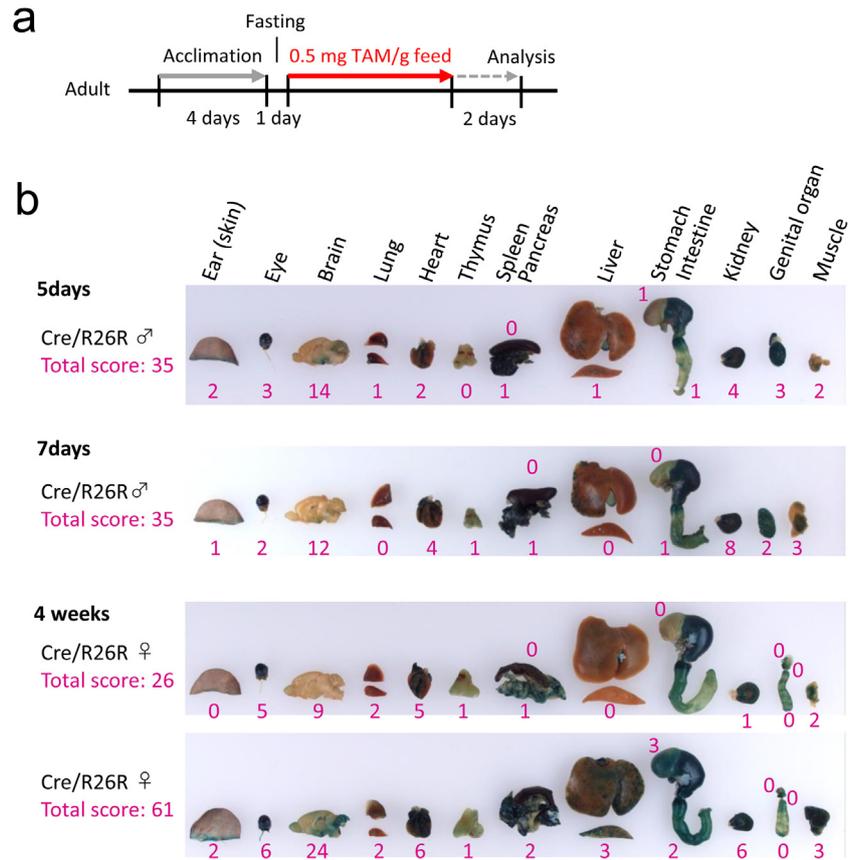
We next examined whether the feeding method conditions examined in adult mice can be used in other populations of mice. First, tamoxifen was administered to pregnant mice under the same conditions as for adult. The schematic is shown in Fig. 6a. A cesarean section was then performed on the day after the expected date of delivery; however, no offspring survived. Therefore, the concentration of tamoxifen was reduced to 0.5 mg/g feed, and the administration period was reduced to 48 h. Moreover, pregnant mice were not fasted to reduce the burden. X-gal staining was performed at 4 weeks of age after sufficient enlargement of each organ. The new schematic is shown in Fig. 6b. Consequently, all offspring were viable and Cre recombination was induced in whole body. The total scores of male individuals ( $n=3$ ) subjected to X-gal staining were 47, 63, and 71, and the total scores of female individuals were 58, 62, and 77. Next, the concentration of tamoxifen was increased to 1.0 mg/g feed. The total score of the individuals subjected to X-gal staining was 38, 54, and 74. However, because delayed delivery was observed at 1.0 mg tamoxifen/g feed, it was determined that 0.5 mg tamoxifen/g feed was suitable to administer to pregnant mice (Supplementary Table 4). The photograph of the individual with the maximum score in each condition is shown in Fig. 6c. In addition, the results of all the individuals are shown in Supplementary Table 4.

### Fetal application

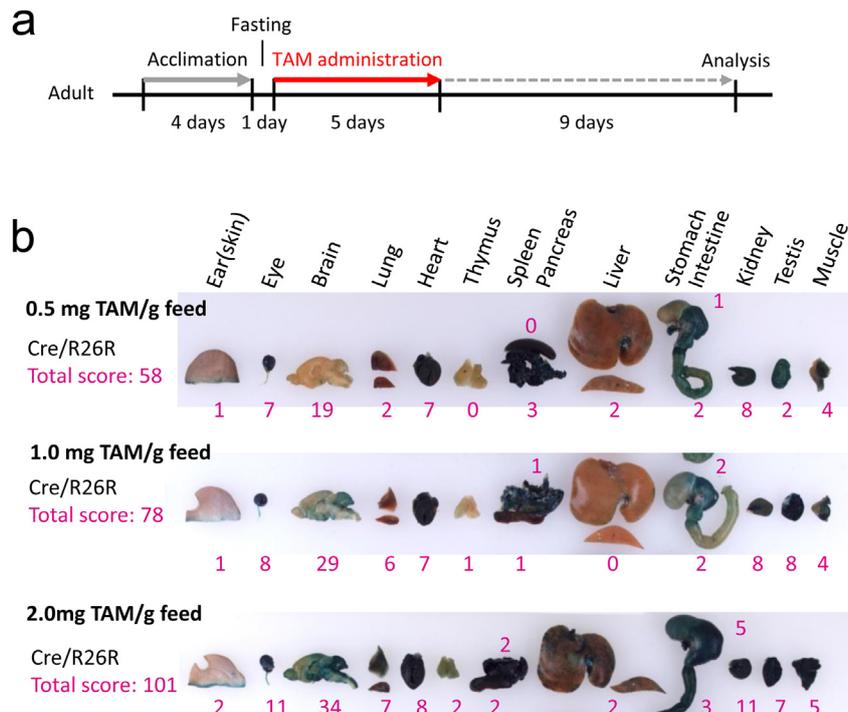
The induction of Cre recombination was attempted in fetuses of E7.5 and E12.5 by feeding pregnant mice with tamoxifen-containing food. The administration period was limited to 24 h to account for the toxicity of tamoxifen and to efficiently induce recombination. X-gal staining was performed at 4 weeks of age after sufficient enlargement of each organ. The dosing schedules for E7.5 and E12.5 are shown in Fig. 7a and 7b. After a 24 h administration of tamoxifen, no offspring of E7.5 and E12.5 died after birth. Moreover, systemic recombination was detected in E7.5; although E12.5 showed unstained tissues, they achieved recombination induction. The photograph of the individual with the maximum score in each condition is shown in Fig. 7c. In addition, the results of all individuals are shown in Supplementary Table 4.

## Discussion

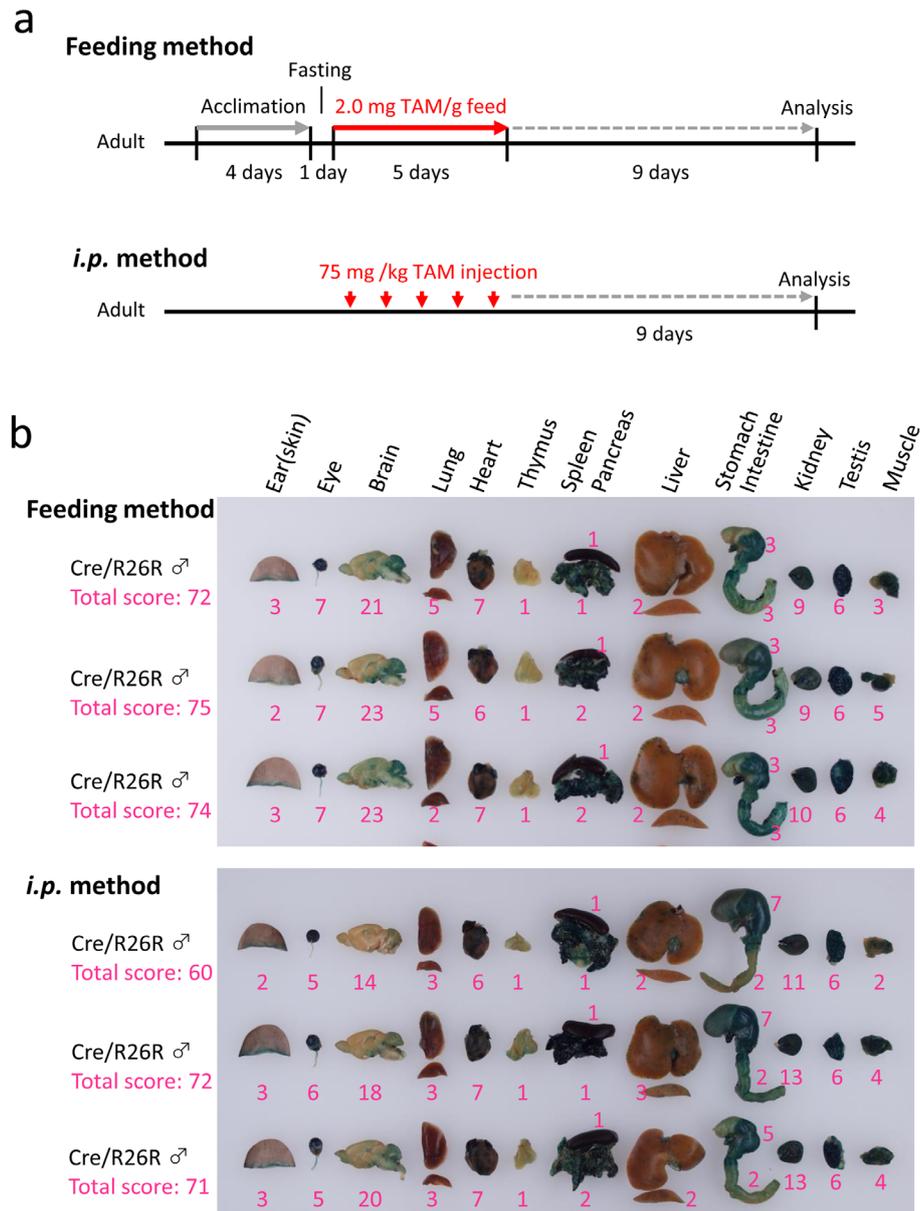
In this study, we demonstrated an efficient feeding method for to generate conditional knockout mice. Mice



**Fig. 3.** Examination of administration period in Cre/R26R mice: (a) Dosing schedule of tamoxifen feeding method for adult mice. (b) Results of X-gal staining of individuals with the maximum score for each condition. Individuals treated with tamoxifen for 4 weeks similarly showed low scores.



**Fig. 4.** Examination of tamoxifen concentration: (a) Dosing schedule of tamoxifen feeding method for adult mice. (b) Results of X-gal staining of individuals that obtained the maximum score for each condition.



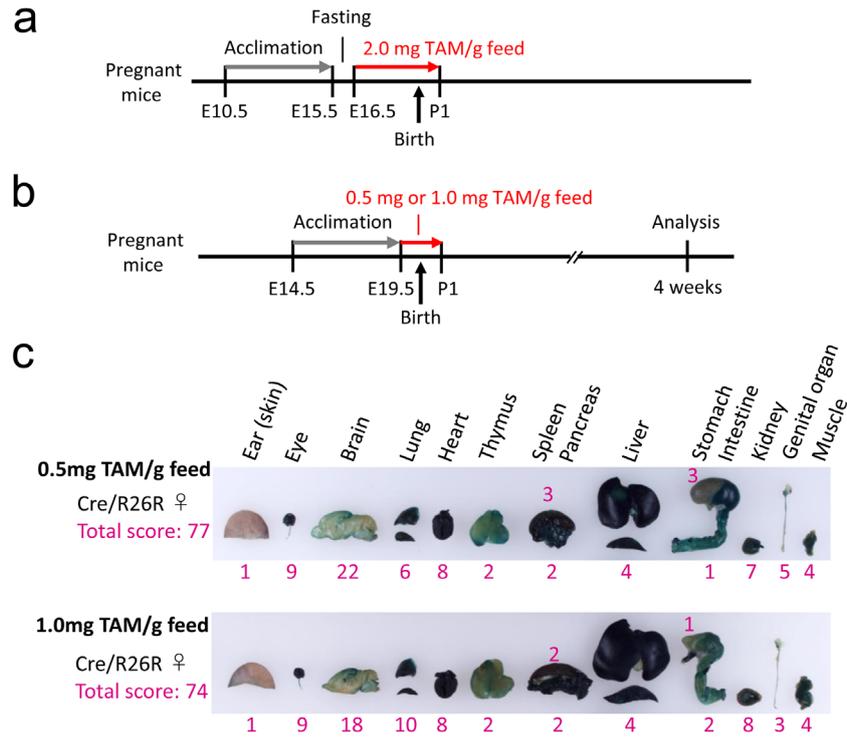
**Fig. 5.** Comparison between tamoxifen feeding method and *i.p.* method: (a) Dosing schedule of tamoxifen feeding method and *i.p.* method for adult mice. (b) Results of X-gal staining for tamoxifen feeding method (upper) and *i.p.* method (lower).

usually eat solid food; however, they can be acclimated to powdered food before commencing administration. Our results showed that the amount of food intake increases by fasting mice the day before the start of administration. Moreover, at a high dose of tamoxifen, Cre-induced recombination occurred efficiently. Because the degree of staining did not change between administration for 5 and 7 days, it is considered that administration for 5 days was a sufficient food intake for induction of Cre.

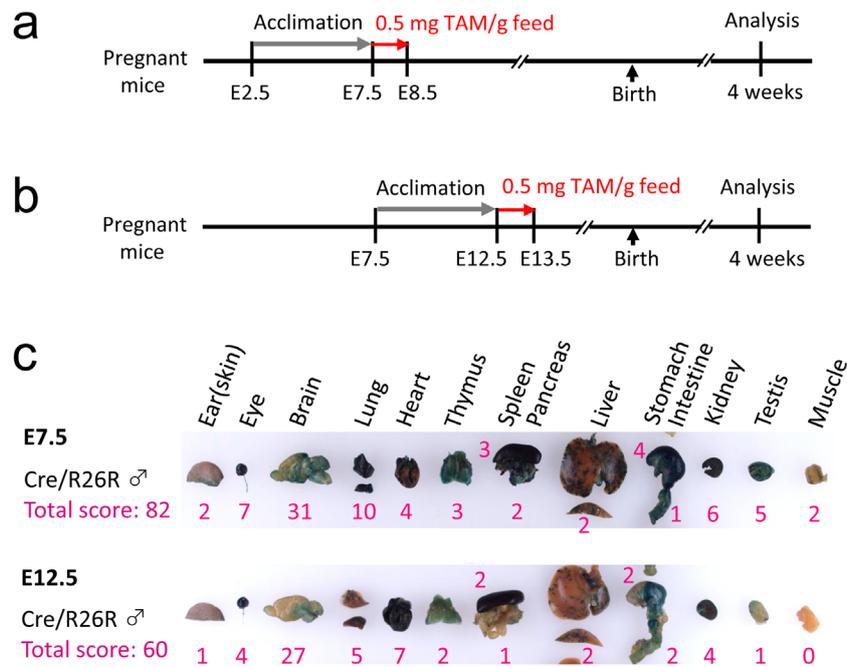
With regard to tamoxifen concentration, as the tamoxifen amount per g of powdered feed increased, the amount of tamoxifen entering the body increased even at small consumption. Therefore, by increasing the

amount of tamoxifen, Cre induction is proposed to occur more easily. However, if the tamoxifen concentration is increased beyond 2.0 mg/g feed, the risk of tamoxifen toxicity may increase [12], or change in the taste of the food may reduce its intake. Therefore, 2.0 mg tamoxifen/g feed should be used as the upper limit in this study.

The X-gal staining patterns were compared between an individual who was administered under optimal conditions of the feeding method and an individual who was administered intraperitoneally. It was found that the feeding method and the *i.p.* method had equal induction efficiency for Cre recombination in the whole body. In the brain, the feeding method showed higher induction



**Fig. 6.** Administration of tamoxifen to pregnant mice using the feeding method: (a) Tamoxifen was administered to pregnant mice under the same conditions as for adults. (b) Tamoxifen was administered to pregnant mice under milder conditions than that for adults. The concentration of tamoxifen was reduced to 0.5 mg/g feed, and the dosing period was reduced to 48 h. Moreover, fasting was not performed. X-gal staining was performed at 4 weeks of age. (c) Results of X-gal staining of individuals that obtained the maximum score for each condition.



**Fig. 7.** Examination of fetal application: Dosing schedule of tamoxifen feeding method for (a) E7.5 and (b) E12.5. (c) Results of X-gal staining of individuals that obtained the maximum score for each condition.

efficiency than the *i.p.* method. The advantage of the feeding method was also a less stressful method for mice, as no animal weakened or died. At the time of dissection, there is also the advantage that the oil does not exit the abdominal cavity of the mouse, making the operation easier and less stressful for the experimenters.

Using the feeding condition in adults, the offspring of pregnant mice that were fed with tamoxifen could not survive, regardless of whether they possessed the Cre-driver gene or not—indicating the toxicity of tamoxifen. By improving the feeding method, it has become possible to efficiently induce Cre recombination during the fetal period. The offspring were healthy, although the toxicity of tamoxifen during the fetal period could not be avoided because the number of births from the tamoxifen-treated pregnant animals was lower than that of untreated mice (Supplementary Table 5).

We have optimized the conditions of the feeding method in which tamoxifen was mixed with powdered food to replace *i.p.*, which had a high mortality rate and prevented efficient analysis. We plan to use the feeding method to evaluate the Cre recombination in Cre-driver mice generated from EGTC clones. Although the above conditions are currently optimized, it may be necessary to optimize them according to the studied Cre-driver mouse line as the staining pattern may differ accordingly.

### Conflict of Interests

The authors declare that there are no competing financial interests.

### Acknowledgments

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