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Generation of *c-Fos* knockout rats, and observation of their phenotype

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Abstract: *c-Fos* is a useful marker gene of neuron activation for neuroscience and physiology research. The mechanism and function of neural networks have been elucidated using *c-Fos* reporter knock-in (KI) mice, but the small size of the mice makes it difficult to perform surgical procedures on specific brain regions. On the other hand, there is a large amount of accumulated data on behavioral studies using rats. Thus, the generation of *c-Fos* reporter rat is expected, but it is difficult to generate gene-modified rats. Furthermore, *c-Fos* gene abnormality is expected to be severe in rats, as shown in homozygous of *c-Fos* knockout (KO) mouse, but such analysis has rarely been performed and is not certain. This study generated *c-Fos*-deficient rats using CRISPR/Cas, with 1067 bp deletion including exon 1 of the *c-Fos* gene. Homozygous *c-Fos* KO rats had growth latency and the same tooth and bone abnormality as homozygous *c-Fos* KO mice but not heterozygous *c-Fos* KO rats. Therefore, the *c-Fos* gene in rats is expected to have the same function as that in mice, and the generation of *c-Fos* reporter KI rats is further anticipated.
Key words: bone, *c-Fos*, gene modification, knockout rat

Introduction

Fos (c-Fos) [1, 2] is one of the Fos family proteins, including Fra1 [3], Fra2 [4], and FosB [5], and works as a part of the compartment of activator protein-1 (AP-1), a dimeric transcription complex regulating gene expression [6–8]. More than 30 years ago, *c-Fos* was identified as a homolog of v-fos, FBJ murine osteosarcoma virus proviral DNA, from a murine cell line [1]. In 1992, two *c-Fos* knockout (KO) mice were established, and these strains had severe phenotypes, including reduced body size, a lack of teeth, and abnormal bone development [9, 10]. *c-Fos* KO mice have been used as model mice for osteopetrosis because *c-Fos* deficiency disturbed the differentiation of osteoclasts responsible for bone resorption [11, 12]. In contrast, Morgan *et al.* demonstrated that *c-Fos* expression is rapidly and transiently induced by the provoking of a voltage-dependent calcium influx with PC12 cells, which can differentiate neuron-like cells [13]. In addition, they revealed that the *c-Fos* expression of neuron is a short period after stimulation *in vivo* [14]. Therefore, c-Fos is widely used as a marker of neural activation in neuroscience and physiology.

c-Fos reporter mice, which can indicate *c-Fos* expression as reporters, such as fluorescent protein, luciferase, and LacZ using the Cre/loxP system, are useful experi-

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mental animals for neural network research in neuroscience and physiology [15–17]. In particular, Guenthner *et al.* established a novel method, targeted recombination in active populations (TRAP), combined with *c-Fos* reporter knock-in (KI) mice [18]. The TRAP system allows marking neural cells activated by a certain stimulus *in vivo*. Due to these *c-Fos* reporter mice, it is unnecessary to sacrifice the experimental animal immediately after the experiment to detect *c-Fos* expression with immunostaining or *in situ* hybridization.

In neurophysiology research, rats have been used as a major experimental animal compared with mice because rats have strong advantages. First is the large size of their body, which has a sufficient quantity of tissue or blood for molecular analysis, and surgery on specific brain regions for neurophysiology experiments. Second is the accumulation of valuable knowledge about physiology studies, including behavioral tests, or pharmacological and nutritional studies. Third are the breeding advantages like those of mice, such as easy handling and breeding, short life cycle, and high fertility. On the basis of these advantages, the establishment of *c-Fos* reporter rats would be useful for physiological studies.

To generate KI rats with a reporter gene inserted under the promoter of the endogenous *c-Fos* gene, one allele of *c-Fos* is inevitably lost in the generation of these rats. However, there are few *in vivo* studies about *c-Fos* deficiency in rats. There is a strong concern that a c-Fos defect in rats would disturb the physiological experiment because *c-Fos*-deficient mice have severe phenotypes described above [9, 10]. In this study, we generated a *c-Fos* KO rat strain with the CRISPR/Cas system, and observed the phenotype of *c-Fos* KO rats, focusing on growth and bone development as shown in *c-Fos* KO mice.

Materials and Methods

Ethics

The experimental protocol was approved by the Institutional Animal Care and Use Committee and the Safety Committee for Recombinant DNA Experiment at Tottori University.

Animals

Slc:Wistar and Slc:SD rats were purchased from Japan SLC (Japan) and kept under the standard conditions (room temperature: 23°C, light cycle: 12 h light/12 h dark, light from 7:00 AM). *c-Fos* KO rats were fed powder diets (CE-2, powder; CLEA Japan, Inc., Tokyo, Japan) after weaning.

CRISPR/Cas system

The rat genome sequence assembly Rnor_6.0 was used. The target sequence of guide RNA (gRNA) was designed by CRISPRdirect ("https://crispr.dbcls.jp") [19]. The 1,000 bp DNA sequence from 500 bp upstream of the 1st ATG was input in CRISPRdirect, and two target sequences were selected for gRNAs. The target sequences are shown in Fig. 1a. crRNAs containing the target sequences were synthesized (Alt-R® CRISPR-Cas9 crRNA; Integrated DNA Technologies, Inc., Coralvill, IA, USA). Ribonucleoprotein (RNP) was made of 12.5 ng/μl of each crRNA, 25 ng/μl Alt-R® CRISPR-Cas9 tracrRNA (1072532; Integrated DNA Technologies), and 20 ng/μl Alt-R® S.p. Cas9 Nuclease V3 (1081058; Integrated DNA Technologies) in sterilized water (W1503; Sigma-Aldrich Co., Burlington, MA, USA) and filtered with Ultrafree-MC (UFC30GV25; Merck KGaA, Darmstadt, Germany).

Generation of *c-Fos* KO rats

Thirteen- to 20-week-old female Slc:SD rats were superovulated with pregnant mare serum gonadotropin (PMSG; 150 IU/kg body weight, intraperitoneally, Serotropin; ASKA Animal Health Co., Ltd., Tokyo, Japan) at 11:00 AM on the day of metestrus, and human chorionic gonadotropin (hCG; 75 IU/kg body weight, intraperitoneally, Puberogen; Novartis Animal Health K.K., Tokyo, Japan) injection was administered 48 h after PMSG administration. After hCG injection, the female rats were crossed with sexually matured male Slc:SD rats. The next day, mating was confirmed by the vaginal plug and sperm in their vagina, and zygotes were collected from the oviduct after sacrificing by cervical dislocation under 5% isoflurane anesthesia. RNP was microinjected into the cytoplasm and pronuclei of zygotes, and injected zygotes were cultured *in vitro* with KSOM-R [20]. Injected zygotes were transferred into the oviduct of pseudo-pregnant Slc:Wistar rats, which were mated with vasectomized Slc:Wistar rats. *c-Fos* KO rats were deposited at the National BioResource Project-Rat (NBRP-Rat) [21], and the NBRP-Rat no. is 0968.

Genotyping

The genome of rats was purified from the tail, and PCR genotyping was performed with KOD FX Neo (KFX-201; Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. The PCR genotyping primers 5′-gactcgctaactagagcctgggagg and 5′-cctgaattccgcagctcagcctttc were used to detect the *c-Fos* Wild-type (WT) allele, 5′-ggcgagctgttcccgtcaatccc and 5′-gtccagaatcgctactcacctgctctac were used to detect the *c-Fos* KO allele.

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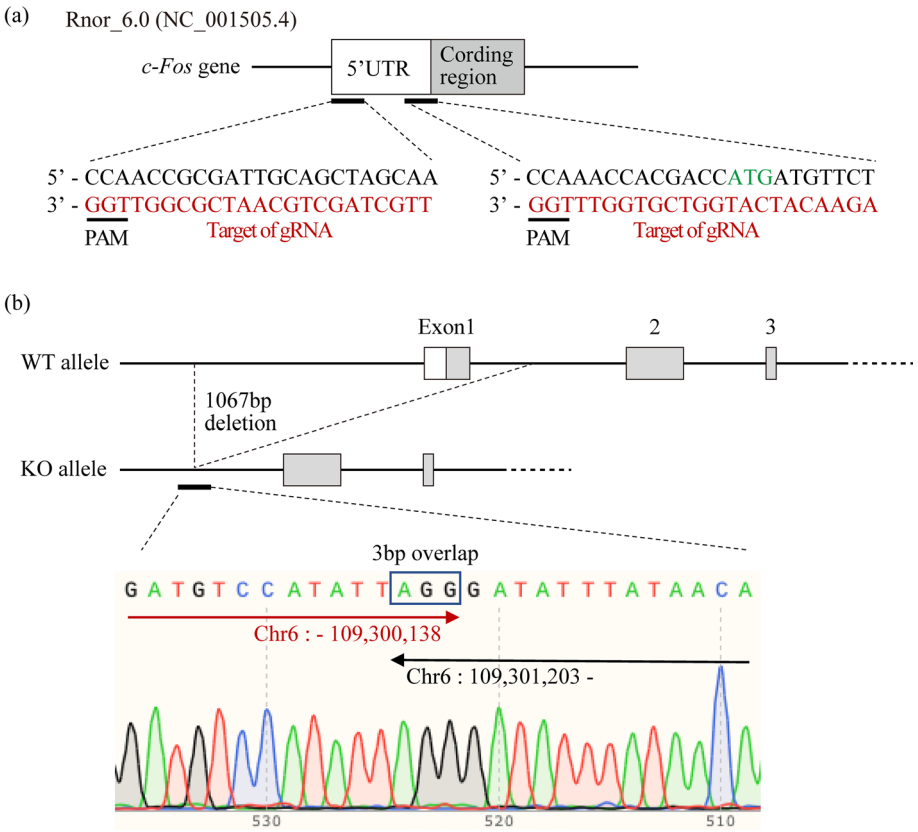

Fig. 1. Generation of *c-Fos* KO rats. (a) CRISPR/Cas design for *c-Fos* KO rats. Two gRNA were used for the generation of *c-Fos* KO rats. The red characters show the target sequence of gRNA, and the green characters show the first ATG of the *c-Fos* gene. (b) DNA sequence analysis of *c-Fos* KO rats. The genome of *c-Fos* KO rats was amplified with the sense primer 5′-ggcgagctgttcccgtcaatccc and the antisense primer 5′-gtccagaatcgctactcacctgctctac. The PCR product was sequenced with the antiprimer.

The PCR product was purified with Wizard® SV Gel and PCR Clean-Up System (A9281; Promega Co., Madison, WI, USA) and sequenced with Value Read DNA sequence (Eurofins Genomics Co., Ltd., Tokyo, Japan).

Histological analysis

Under anesthesia with 3.5% isoflurane inhalation, 4-week-old *c-Fos* KO rats were perfused with saline, followed by Mildform 10N (131-10317; FUJIFILM Wako Pure Chemical, Osaka, Japan). Tissue samples were decalcified in 10% EDTA (pH 7) for 1 week. Decalcified tissues were embedded in paraffin. Paraffin-embedded tissues were sliced into 5-μm-thick sections and analyzed using hematoxylin and eosin (H&E) staining. Safranin O (Waldeck GmbH & Co., Havixbecker, KG)-Fast Green (FUJIFILM Wako Pure Chemical) staining was performed to observe the morphology of cartilage tissues. The images were captured by Nanozoomer S60 (Hamamatsu Photonics, Hamamatsu, Japan). The quantification of the epiphysis and medullary cavity area with the images was performed using ImageJ 1.53k software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All error bars show the standard deviation. Normality and equal variances were calculated by the Shapiro-Wilk test and Levene tests using R [22]. The Wilcoxon rank-sum test was performed using the "exactRankTests" package.

Results

Generation of *c-Fos* KO rats

The generation of *c-Fos* KO rats is shown in Fig. 1. The CRISPR/Cas system was used to generate *c-Fos* KO rats, and two target sequences of gRNA were designed (Fig. 1a). The most effective condition of RNP was examined for the modification of the *c-Fos* gene, and 55 founder rats were generated. Among all *c-Fos* founder rats, founders KO7 and KO12 had no incisors, and founder KO22 lacked the lower incisors. Founder KO12 was bred with female Slc:SD rats, and an incisor-less rat again was found from mating between offspring. The DNA sequence of the toothless offspring revealed that they had a 1067 bp deletion, including exon 1 (Fig. 1b).

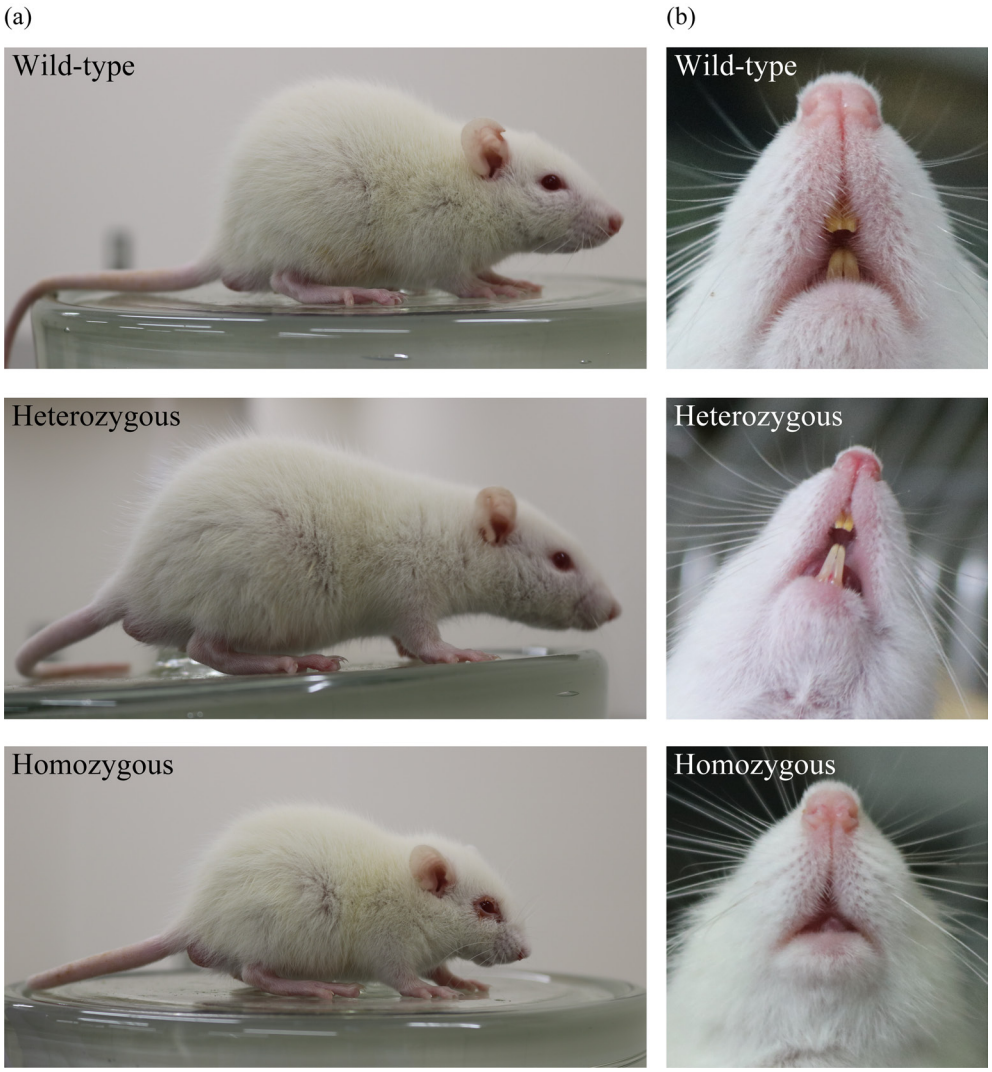

Fig. 2. Appearance of 4-week-old *c-Fos* KO rats. (a) Whole-body appearance of *c-Fos* KO rats. (b) Teeth of *c-Fos* KO rats.

Therefore, the *c-Fos* KO strain was established from the founder rat KO12. The gene expression of *c-Fos* in homozygous *c-Fos* KO rats was examined by quantitative PCR (qPCR), and it was significantly lower than that in the WT and heterozygous *c-Fos* KO rats (Supplementary Fig. 1).

Appearance feature of *c-Fos* KO rats

At birth, homozygous *c-Fos* KO rats could not be distinguished. However, after ~3 weeks, homozygous *c-Fos* KO rats were found to be smaller than other littermates and had shortened snouts and round heads (Fig. 2a). In contrast, heterozygous *c-Fos* KO rats seemed to be normal. Additionally, all homozygous *c-Fos* KO rats were toothless (Fig. 2b). The body weight of male homozygous *c-Fos* KO rats significantly decreased compared with that of WT rats after 3 weeks of age but that of heterozygous *c-Fos* KO rats did not decrease compared with that of WT rats (Fig. 3a). The body weight of female homozygous *c-Fos* KO rats was also similar (Fig. 3b). To confirm the fertility of homozygous *c-Fos* KO rats, one male and two female homozygous *c-Fos* KO rats were crossed. Offspring were obtained from all of 3 homozygous *c-Fos* KO rats, and an average of 8.2 pups were obtained.

Tooth and bone disorder in homozygous *c-Fos* KO rats

The jawbone of homozygous *c-Fos* KO rats was smaller than that of WT rats (Fig. 4a). H&E staining revealed that adult homozygous *c-Fos* KO rats had a tooth disorder (Fig. 4b). H&E staining of the femur showed that the diaphysis of homozygous *c-Fos* KO rats was short and had many small medullary cavities compared with that of WT rats (Figs. 5a and b). This study focused on the epiphysis of the distal femur (Fig. 6a).

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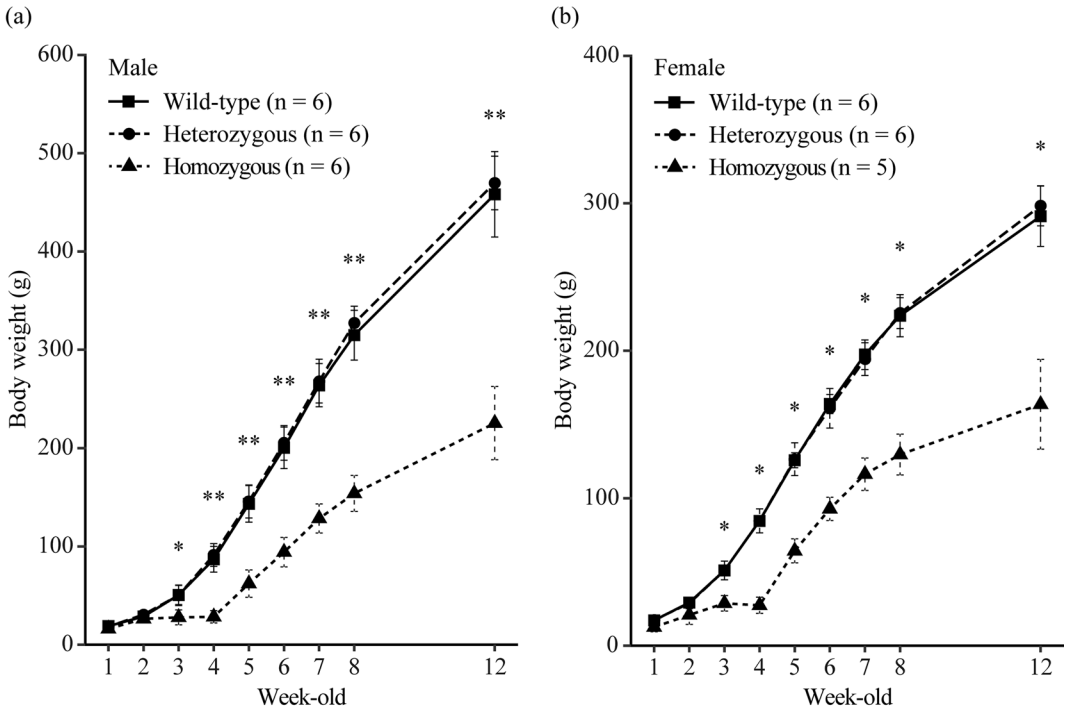

Fig. 3. Body weight of *c-Fos* KO rats. (a and b) Body weight of male (a) and female (b) *c-Fos* KO rats [female homozygous *c-Fos* KO rats (n=5) and others (n=6)]. The rats were weaned and fed powder diets at 4 weeks of age. The error bar shows the standard deviation, and the significant differences were tested using the Steel test. **P*<0.05; ***P*<0.01.

The distal epiphysis and medullary cavity of homozygous *c-Fos* KO rats were significantly smaller than those in WT rats (Figs. 6b and c).

Discussion

The *c-Fos* KO rat strain was established using the CRISPR/Cas system, and the heterozygous *c-Fos* KO rat was normal in the appearance and growth. However, the homozygous *c-Fos* KO rat had a severe growth and bone development phenotype.

When using the CRISPR/Cas system, we needed to be aware off-target effects, of which unintended gene modifications could occur except for the target gene. However, founder rats were bred up to seven generations. We keep breeding *c-Fos* KO rats with Slc:SD rats. Unexpected off-target effects would fade away with each generation. Even if there was an off-target effect, *c-Fos* KO rat phenotypes were unlikely to have been affected by the off-target effect because the same phenotype was observed across all homozygous *c-Fos* KO rats.

The *c-Fos* gene was slightly expressed in homozygous *c-Fos* KO rats (Supplementary Fig. 1) despite of the fact that the deletion region extended to the promoter region and 1st ATG of *c-Fos* gene (Fig. 1b). Although some transcripts might be expressed from downstream of the deletion region, the *c-Fos* transcript in homozygous *c-Fos* KO rat wouldn't be normal. Actually, the severe phenotype had emerged in homozygous *c-Fos* KO rat.

Homozygous *c-Fos* KO rat remarkably resembled *c-Fos* KO mice. Regarding the appearance, homozygous *c-Fos* KO rat had a shortened snout, round head, and lack of incisor, as shown in Fig. 2a. Johnson *et al.* described that *c-Fos*-deficient mice had a foreshortened snout, domed skull, and lack of incisor eruption [10]. The Mendelian ratio of homozygous *c-Fos* KO rat derived from crossbreeding of heterozygous *c-Fos* KO rats was significantly lower than that of the WT, which is similar to the *c-Fos* KO mice study (Supplementary Table 1). In addition, another *c-Fos*-deficient mouse strain that Wang *et al.* established had similar features [9]. The body weight of male homozygous *c-Fos* KO rat was approximately 40% decreased compared with that of WT and heterozygous *c-Fos* KO rat after 3 weeks of age (Fig. 3a). This rate was close to that of the *c-Fos* KO mice that Wang *et al.* generated [9]. The tooth defect of homozygous *c-Fos* KO rat is a serious problem for food intake for survival. Homozygous *c-Fos* KO rat were given a powder diet after weaning; then, these rats could survive beyond 12 weeks of age, and produce offspring.

Besides, homozygous *c-Fos* KO rat showed bone development abnormalities similar to those seen in *c-Fos* KO mice. *c-Fos* is an essential factor that induces osteoclast differentiation from progenitor cells [11, 12]. This

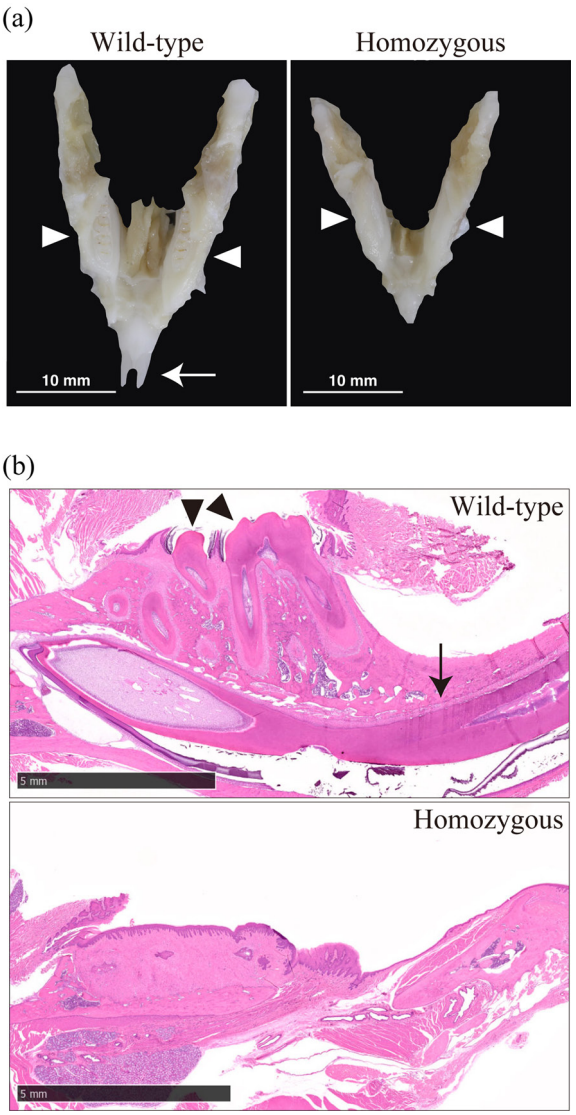

Fig. 4. *c-Fos* KO rats have a molar and incisor disorder. (a) Mandible of *c-Fos* KO rats. White arrows and white arrowheads show the incisor and molar, respectively. (b) H&E staining of the mandible of *c-Fos* KO rats. Black arrows and black arrowheads show the incisor and molar, respectively. The black bar indicates 5 mm.

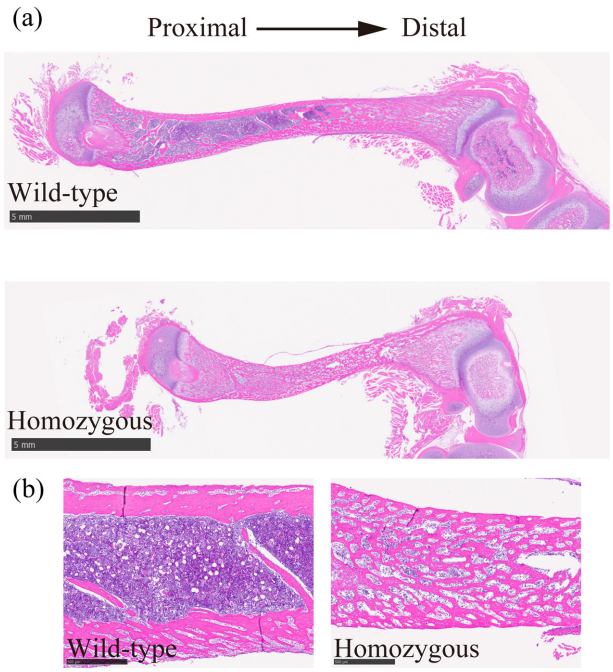

Fig. 5. Histological features of the femur of *c-Fos* KO rats at 4 weeks of age. (a) Overview of the femur stained with H&E. Scale bar, 5 mm. (b) Diaphysis of the femur stained with H&E. Scale bar, 1mm.

suggests that homozygous *c-Fos* KO rat might have bone absorption abnormalities resulting from a deficiency of osteoclasts, similar to *c-Fos* KO mice. There is a positive correlation between body height and weight, and bone growth is essential for an increase in body height. Therefore, bone abnormalities often cause growth retardation. This possibility could explain why the body weight of homozygous *c-Fos* KO rats was lower than that of heterozygous and WT rats. *c-Fos*-deficient mice are used as model mice of osteopetrosis. Thereafter, *c-Fos* KO rat might be model rats of osteopetrosis.

To ensure proper expression of reporter genes, the KI technique is more rigorous than random integration of transgenes. In KI, the reporter gene is inserted into the endogenous target gene, and then target gene expression from the KI allele is often affected or disrupted. In some cases, the disruption of one allele might cause abnormal phenotypes, such as the disruption of methyl CpG binding protein 2 and fibroblast growth factor receptor 3 genes [23, 24]. The phenotype of disruption of target gene should be confirmed in detail to choose which gene modification style, e.g., conventional transgenic (TG), conditional KI, and other methods, is suitable for generating reporter animals. In present study, the *c-Fos* KO phenotypes were not shown in heterozygous *c-Fos* KO rat. Hence, the *c-Fos* locus is a permissible locus for KI.

We succeeded to establish *c-Fos* KO rats and observed their phenotype. The *c-Fos* KO rat phenotypes were not observed in the heterozygous *c-Fos* KO rat. However, KI rats targeted to the *c-Fos* locus must be maintained as heterozygous. Otherwise, when the *c-Fos* gene is null due to gene KI, it would not be suitable for neurophysiology experiments due to severe phenotypes. As a result, it might be inferred that the generation of *c-Fos* reporter KI rats is possible as in mice.

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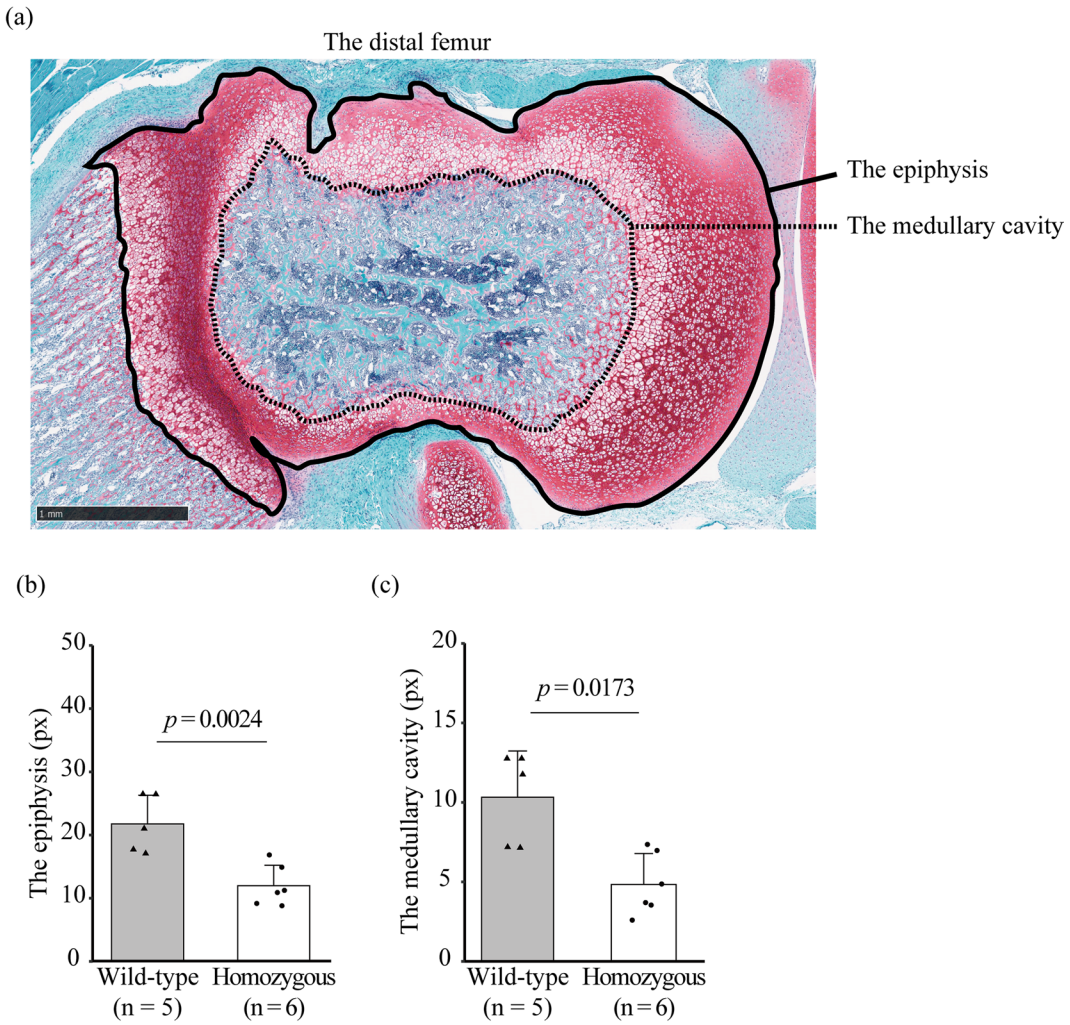

Fig. 6. Disorder of the epiphysis of the distal femur in male *c-Fos* KO rats at 4 weeks of age. (a) Image of the area measured as the epiphysis and medullary cavity in the distal femur. The solid and dashed lines show the edge of the epiphysis and medullary cavity, respectively. Scale bar, 1 mm. (b and c) Area of the epiphysis (b) and medullary cavity (c) of the distal femur [WT (n=5) and homozygous *c-Fos* KO rats (n=6)]. The error bar shows the standard deviation, and the significant differences were tested using Student's *t*-test (b) and Wilcoxon rank-sum test (c).

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