Generation of a dystrophin mutant dog by nuclear transfer using CRISPR/Cas9-mediated somatic cells

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Short report

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Abstract

Dystrophinopathy is caused by mutations in the dystrophin gene, which lead to progressive muscle degeneration, necrosis, and finally death. Recently, golden retrievers have been suggested as a useful animal model for studying human dystrophinopathy, but the model has limitations due to difficulty in maintaining the genetic background using conventional breeding. In this study, we successfully generated a dystrophin mutant dog using the CRISPR/Cas9 system and somatic cell nuclear transfer. The dystrophin mutant dog displayed typical phenotypes, such as elevated serum creatine kinase, dystrophin deficiency, skeletal muscle defects, an abnormal ECG, and avoidance of ambulation, all of which are consistent with human dystrophinopathy. These results indicate that dystrophin mutant dogs can be a reliable and effective animal model for preclinical studies into new therapies for human dystrophinopathy.

Background

Canis familiaris has drawn considerable attention as a model for investigating human diseases. Dogs show over 450 naturally occurring diseases, of which approximately 360 are analogous to human diseases [1]. Based on their size, biological features, and ease of behavioral evaluation and handling, dogs can be good animal models. Since humans and dogs share a common environment, food, and carcinogenic load, it is not surprising that the dog has emerged as a viable model for human disease [2]. Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are the most common X-linked recessive muscular dystrophies caused by mutations in the dystrophin gene leading to a defective dystrophin-glycoprotein complex [3]. DMD and BMD are classified as dystrophinopathies because they are caused by alterations in the dystrophin gene. These mutations lead to progressive muscle degeneration and, finally, to necrosis [3, 4]. This can result in substantial physical and locomotor deficits leading to the need of wheelchair use and early death due to heart failure. Both laboratory-generated and naturally occurring animal models are available to study the pathogenesis and to develop potential new treatments for dystrophinopathy [5]. Dystrophin-deficient mdx mice have been most commonly used for DMD research, but this model has limitations. For example, mdx mice exhibit minimal clinical symptoms and have only a 25% reduction in longevity, unlike DMD patients with a 75% reduction in life span [6]. In addition, there is a weak correlation between the effect of therapeutic interventions in the rodent model and the effect observed in humans [7]. Thus, rodents are not good models of human dystrophinopathy. Recently, canine models have been suggested as a suitable translational bridge between mice and humans [8, 9] because they more closely mimic the human disease compared other existing models of dystrophin deficiency [10].

Canine X-linked muscular dystrophy models have been reported over the last 50 years. Generally, the clinical phenotype of canine dystrophinopathy is more similar to that of human patients in severity and in selective muscle injury compared with mdx mice [9]. Overall, golden retriever muscular dystrophy (GRMD) has been the most extensively examined and characterized for research on human DMD [11]. Mutations in the canine dystrophin gene have been identified in golden retrievers [12], german short-hair pointers

[13], and cavalier king charles spaniels [14]. However, GRMD dogs have a high degree of variability despite an identical causative mutation leading to a great phenotypic range resulting from variation of alternatively spliced dystrophin gene and truncated translational products in the muscles. Additionally, using large golden retrievers has a significant ethical consequences regarding animal welfare, and their maintenance and care are expensive. To address these issues, the GRMD model was bred with a beagle, and a new colony with canine X-linked muscular dystrophy (CXMD) was generated [15]. In addition to the clinical resemblance, the CXMD model also shows histological lesions similar to those seen in affected humans. However, it is difficult to produce individuals with the same genetic background as the CXMD model using conventional breeding methods and maintain individuals for use in preclinical studies. To increase the availability of a canine dystrophinopathy model, a corresponding loss of function using site-directed mutagenesis of the desired gene is needed.

Recently, the clustered regularly interspaced short palindromic repeats (CRISPRs)/CRISPR-associated (Cas) 9 system was developed to edit specific genes with high efficiency [16, 17]. Using this technique, numerous genome edited animals from different species have been generated for biomedical modeling [18, 19], for human disease modeling [20, 21], and for xenotransplantation [22, 23]. In fact, apoE knockout (KO) dogs [24] and myostatin KO dogs [25] were successfully produced by microinjecting CRISPR/Cas9 into zygotes. Although zygote microinjection may be very highly efficient in editing, not all the resulting embryos are true KOs, and the approach can generate mosaic embryos. Somatic cell nuclear transfer (SCNT) is currently the only technique to ensure that all the fetuses are KOs because the experimenter can select the donor cell after establishing donor cells that are edited with the desired genes before the SCNT procedure. In this study, a dystrophin mutant dog was successfully generated using the CRISPR/Cas9 system combined with SCNT technology.

Methods

Ethics statement

In this study, female mixed dogs from 2 to 4 years of age were used as oocyte donors and embryo transfer recipients. The dogs were housed indoors and fed once a day with water ad libitum. All experiments involving animals, methods and protocols were approved by the Committee for Accreditation of Laboratory Animal Care and the Guideline for the Care and Use of Laboratory Animals of Seoul National University (SNU-1700310-14-1). All methods and protocols were carried out in accordance with the relevant guidelines and regulations.

Generation of CRISPR/Cas9

The pET plasmid that encodes His-tagged Cas9 was transformed into BL21(DE3). Expression of Cas9 was induced using 0.5 mM IPTG for 4 h at 25°C. The Cas9 protein was purified using Ni-NTA agarose resin (Qiagen) and dialyzed against 20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM DTT and 10% glycerol. RNAs were in vitro transcribed through run-off reactions by T7 RNA polymerase. The template for sgRNA was generated by annealing and extension of two complementary oligonucleotides. Transcribed sgRNAs

were preincubated with DNase I to remove template DNA, and purified using PCR purification kits (Macrogen). Purified RNA was quantified by spectrometry.

Cell culture and transfections

Canine fetal fibroblasts were derived from the torso of a 27 day fetus post coitum and were cultured in DMEM (Gibco) with 10% FBS. Cas9 and sgRNA were co-transfected into canine fetal fibroblasts using electroporation. The transfected cells were transferred onto 12 well plates and further expansion was performed.

In vivo matured oocytes collection

After vaginal bleeding was first shown, blood was collected daily from the cephalic vein and sera were separated by centrifuging for 10 min. Serum progesterone concentration was monitored by an IMMULITE 1000 (Siemens Healthcare Diagnostics Inc., Flanders, NJ, USA). The day when the progesterone concentration reached 4.0 ng/ml to 10.0 ng/ml was considered the day of ovulation. 72 h after ovulation, in vivo matured oocytes were collected by oviductal flushing using HEPES-buffered tissue culture medium-199 (TCM, Invitrogen) supplemented with 10% bovine serum albumin and 2 mM NaHCO₃.

Somatic cell nuclear transfer and embryo transfer

Cumulus cells were removed from in vivo matured oocytes by gentle pipetting in tissue culture medium-199 supplemented with 0.1% hyaluronidase. Metaphase chromosomes and extruded first polar bodies were removed under ultraviolet light by aspiration in HEPES-buffered TCM drops containing cytochalasin B and Hoechst 33442. Single donor cells were inserted into the perivitelline space of oocytes. Each donor cell-cytoplasm couplet was fused with two pulses of DC 72 V for 15 μ s using an Electro-Cell Fusion apparatus (NEPA GENE Co. Chiba, Japan). Fused embryos were activated in modified synthetic oviductal fluid (mSOF) medium containing 10 μ M calcium ionophore (Sigma-Aldrich Corp). After chemical activation for 4 min, cloned embryos were transferred into 40 μ l of mSOF with 1.9 mM 6-dimethylaminopurine for 2 h. Reconstructed cloned embryos were transferred into the oviducts of synchronized recipients. Under laparotomy with general anesthesia, embryos were placed into the ampullary part of the oviduct using a 3.5 Fr Tom-Cat catheter (Sherwood, St. Louis, MO, USA).

T7 endonuclease I assay and sequencing

Genomic DNA was isolated using a genome isolation kit (Promega, Madison, USA) according to the manufacturer's instructions. PCR amplicons were denatured at 95°C, reannealed at 16°C to form heteroduplex DNA using a thermal cycler and then digested with 5 units of T7 endonuclease 1 (New England Biolabs, Ipswich, MA) for 20 min at 37°C and then analyzed using agarose gel electrophoresis.

Deep sequencing analysis

Genomic DNA was isolated from transfected cells and tail tissue of the cloned puppy. The target region was amplified using Phusion polymerase (New England Biolabs). Equal amounts of the PCR amplicons were subjected to paired-end read sequencing using Illumina MiSeq from Bio Medical Laboratories.

Insertions or deletions located around the RGEN cleavage site (3 bp upstream of the PAM) were considered to be the mutations induced by RGENs.

Creatine kinase

Blood samples for serum CK analysis were obtained from the jugular vein beginning at 10 days after birth. Samples were collected at 1 week intervals until 8 weeks. No attempt was made to limit exercise prior to sampling. Biochemistry analysis were performed on IDEXX Catalyst Dx (IDEXX VetLab Analysers, Westbrook, Maine, USA). The normal reference range is 99–436 U/L.

Anesthesia for magnetic resonance imaging

The dog was positioned in ventrodorsal recumbency and premedicated with 0.05 mg/kg glycopyrrolate (Mobinul, Myungmoon Pharmaceutical Co., Seoul, Korea). Anesthesia was induced with 0.06mg/kg propofol (Provive, Myungmoon Pharmaceutical Co.) intravenously and was maintained with 1.5% isoflurane (Foran solution, Choongwae Pharma Corporation, Seoul, Korea) in 100% oxygen by endotracheal intubation. Heart rate, respiratory rate and end-tidal CO₂ concentration were monitored (MRI Patient Monitor, GE Medical System, Milwaukee, WI, USA).

Non-quantitative (conventional) magnetic resonance imaging

MRI exams were performed with a 1.5-Tesla scanner (Signa HDx, GE Medical Systems, Milwaukee, USA) using an 8-channel receiver coil (HD Knee PA coil, GE Medical Systems). MRI acquisition planes and parameters are summarized in Additional file 1. T1-weighted images and T2-weighted images with fat suppression were acquired for anatomic reference and to assess damage to the hindlimb muscles, respectively. Gadolinium 0.1 mmol/kg (Magnevist, Bayer Korea, Seoul, Korea) was used intravenously for contrast study of the T1-weighted sequence.

Quantitative (T2 mapping) magnetic resonance imaging

Axial T2 maps were obtained by using multiecho sequences. Imaging parameters were follows: FOV, 18 × 18 mm; slice thickness, 5 mm; interslice gap, 0.3 mm; relaxation time, 1000 ms; echo time (ms), 7.4, 14.8, 22.2, 29.6, 37, 44.4, 51.7, 59.1; matrix, 256×192; Nex, 2. Two radiologists (JH Kim and KD Eom) calculated T2 values of each hindlimb muscle at the mid-femur level. The size of ROI was fixed at approximately 5 mm² considering individual muscle size. Mean and standard deviation of T2 values of each of seven muscles were acquired, including rectus femoris, vastus lateralis, vastus medialis, biceps femoris, semitendinosus, semimembranosus and adductor magnus.

Biopsy and histopathological analysis

For biopsies of biceps femoris muscles, the dystrophin mutant dog and the control dog were anesthetized with ketamine and xylazine via intravenous injection, and anesthesia was maintained with isoflurane. The dogs were positioned in left lateral recumbency, and the biopsy region was prepared aseptically. After a 3 cm skin incision, a sample of the right biceps femoris muscle (1 cm x 1 cm x 0.5 cm) was collected

from each dog. Immediately, the biopsies were flash frozen in isopentane precooled in liquid nitrogen. A standard panel of histochemical stains and reactions was performed on 5 µm muscle cryosections. Additional cryosections were used for immunohistochemical staining using monoclonal antibodies against the dystrophin carboxy terminal, rod domain and utrophin (Novocastra, Newcastle-upon-Tyne, UK).

Western blotting

Proteins of the dystrophin mutant dog and the control dog were extracted from each muscle sample. After measuring protein concentration, equal amounts of proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond; Amersham Biosciences). Membranes were blocked for 1 h in Tris-buffered saline Tween (TBST) containing 5% powdered skim milk and incubated overnight with the following primary antibodies: anti-dystrophin NCL-DYS1 (1:500, Novocastra Laboratories, Newcastle, UK), anti-dystrophin NCL-DYS2 (1:100, Novocastra Laboratories), and anti-utrophin NCL-DRP (1:100, Novocastra Laboratories). Horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000, Santa Cruz Biotechnologies, Piscataway, NJ) was used to detect bound antibodies with the Imaging System from FUSION-Solo (6x, Vilber Lourmat, France).

Results And Discussion

First, we designed an sgRNA targeting the six exons of the dog dystrophin gene [26]. The remaining approximately 7% dystrophin gene mutations are caused by single or multi-exon duplications, with exons 2 to 20 being the most commonly affected (see Additional file 2). It is important to note that the GRMD and CXMD models have a frame shift because of a mutation in the splice acceptor of exon 6 that disrupts exon 7, and inducing a mutation in exon 6 could have a therapeutic effect on these canine dystrophinopathy models [27]. The efficiency of sgRNA was validated by co-transfection with a Cas9 vector into canine male fetal fibroblasts. Fibroblasts expressing green fluorescence protein transiently were used as donor cells. Having confirmed that Cas9/sgRNA was highly active in cultured cells, we performed SCNT to generate a dystrophin mutant dog. Oocytes matured in vivo were enucleated, injected with a donor cell, and fused by electrical stimulation. The fused couplets were activated with 10 µM calcium ionophore and 6-Dimethylaminopurine and then a total of 26 reconstructed oocytes were transferred into three naturally synchronized surrogate recipients. One of the recipients was pregnant to term (33.33%) and gave birth to one puppy (3.85%) (Table 1). The tail tissue of the cloned puppy was collected two days after birth to detect genomic mutations in the target dystrophin locus using the T7E1 assay and deep sequencing. PCR products amplified from the genome of this dog were identified via deep sequencing (see Additional file 3). As shown in Fig. 1A, the puppy had a 57 bp deletion in the dystrophin gene (Fig. 1A). The off-target sites in the dog genome were identified using Cas-OFFinder [28]. No off-target indel mutations at candidate sites were detected (see Additional file 4 and 5).

Table 1
Summary of embryo transfer and generation of the dystrophin mutant cloned pup

Recipient	No. <i>in vivo</i> matured oocytes	No. reconstructed oocytes	No. transferred embryos	Pregnancy	No. births
A	16	6	6	+	1
B	13	7	7	-	0
C	20	13	13	-	0
Total	49	26	26	1 (33.3%) §	1 (3.84%) §§
§ The percentage is based on the total number of recipient dogs.					
§§ The percentage is based on the total number of transferred embryos.					

We next evaluated whether the dystrophin mutant dog shared a remarkably similar clinical course to that of dystrophinopathy patients. In both GRMD dogs and human DMD patients, serum creatine kinase (CK) activity is markedly elevated [29]. CK is most important for pre-neuter evaluations in young dogs because increased CK activity may be an early indicator of underlying muscle disease. In the present study, the CK level was recorded in both the dystrophin mutant dog and an age-matched control at two and eight weeks after birth. Until eight weeks of age, the control group had CK levels within the normal range (99–436 U/L), but the CK level (1,019–19,880 U/L) of the dystrophin mutant dog was much higher than the normal range from two weeks to eight weeks (Fig. 1B). At 10 months of age, the CK levels of the dystrophin mutant dog and the control dog were 261 and 31,540 U/L, respectively. However, after 30 min of exercise, the dystrophin mutant dog had a CK level 300-fold higher than that of the control group (Fig. 1C).

We next investigated the clinical manifestations of dystrophinopathy in the dystrophin mutant dog with increased CK using magnetic resonance imaging (MRI). For profiling skeletal muscles, MRI of the five-month-old dystrophin mutant dog was acquired (Fig. 2). Diffuse hyperintense lesions on T2-weighted and T2 fat suppression sequences were found, especially in the rectus femoris and adductor magnus muscles. On T1-weighted images, atrophy of the quadriceps and strong contrast enhancement were found in the rectus femoris muscle. The biceps femoris, semitendinosus, and semimembranosus muscles tended to be relatively uninvolved. T2 values were acquired using T2 mapping of both control and dystrophin mutant dogs (see Additional file 6). The average T2 values of the dystrophin mutant dog were much higher (mean $\pm$ SD, 45.8 $\pm$ 8.2) than those of the control dog (mean $\pm$ SD, 38.7 $\pm$ 1.9). Significant differences between the two dogs were found in anterior-medial hindlimb muscles including the rectus femoris (control dog, 38.1; dystrophin mutant dog, 62.7) and adductor magnus (control dog, 40.3; dystrophin mutant dog, 49.3) muscles. In contrast, the posterior-lateral hindlimb muscles, including the biceps femoris, semitendinosus, and semimembranosus, showed minimal differences between the two dogs. These MRI findings are similar to those of patients with dystrophinopathy, especially young

boys with DMD, regarding the existence of marked inflammatory and edematous lesions with minimal fatty replacement in the thigh muscles [30, 31]. Since the MRI distribution and patterns were very similar to those of early stage DMD boys, it is likely that muscular atrophy and fatty infiltration will occur as the disease progresses.

To establish dystrophin mutant dogs as a valid dystrophinopathy model, we performed electrocardiogram (ECG) analysis in an age-matched control and the dystrophin mutant dog. Patients with dystrophinopathy often show an abnormal ECG. It is generally believed that end-stage patients develop dilated cardiomyopathy and die from heart failure [32]. The ECG patterns of a five-month-old dystrophin mutant dog and the age-matched control were similar. However, the Q wave in the ECG was deeper in the five-month-old dystrophin mutant dog than in the age-matched control (see Additional file 7). Such ECG changes are well established in human dystrophinopathies, but few studies have been reported in dystrophin-deficient dogs [33, 34]. In addition, we observed walking or exercise-related changes in the dystrophin mutant dog after five months of age. The dystrophin mutant dog started to show "bunny hopping" from six months of age. Over time, its limbs became stiff, with a decreased range of joint motion while moving, more pronounced bunny hopping, difficulty in climbing stairs, and avoidance of movement were revealed. In untreated DMD patients, ambulation loss usually occurs during the early teenage years. Unlike in DMD patients, complete loss of ambulation is not a clinical feature in young DMD dogs [8, 10]. In this study, the 10-month-old dystrophin mutant dog was reluctant to exercise and showed limb muscle atrophy, but was still able to walk. Because of the clinical symptoms seen in DMD, it is necessary to observe whether the dystrophin mutant dog will completely lose ambulation in the future.

Besides its resemblance to the human clinical cases, the dystrophin mutant dog also exhibited histological lesions similar to dystrophinopathy patients. The present study examined the biceps femoris of the dystrophin mutant dog and a control dog at six months of age. Muscle histopathology examination revealed mild fiber size variations, muscle fiber necrosis, and regeneration in focal muscle groups (Fig. 3). Immunohistochemical staining of frozen muscle using monoclonal antibodies against the dystrophin carboxy terminal, rod domain, and utrophin, revealed decreased expression of dystrophin 1 and 2 (Fig. 3F and 3G) along with upregulation of utrophin (Fig. 3H) compared to the control dog muscles (Fig. 3A-D). Dystrophin 1 and dystrophin 2 were detected in the muscle tissue of the control (Fig. 3B and 3C) in western blots, but were barely expressed in the dystrophin mutant dog (Fig. 3F and 3G).

Utrophin is markedly upregulated in dystrophin mutant dog when compared with controls in Western blot (Fig. 4). DMD is caused by out-of-frame mutations and absence of dystrophin protein in skeletal muscles because the dystrophin protein that is produced is truncated as a result of the premature stop codon and, therefore, is unstable [34]. BMD is caused by an in-frame mutation resulting in insufficient dystrophin protein, and clinical progression can be predicted by whether the deletion or duplication maintains or disrupts the translational reading frame [35, 36]. However, there are reports about exceptions to the reading-frame rule occurring in 10% of all DMD-causing mutations [26]. These findings are similar to the

pathologic features in human dystrophinopathy. Our results demonstrated that the clinical characteristics of a dystrophin mutant dog are consistent with the diagnosis of dystrophinopathy in humans.

Conclusion

Our study demonstrated, for the first time, that donor cells with CRISPR/Cas9 for a specific gene combined with the SCNT technique can efficiently produce a dystrophin mutant dog. Furthermore, this dystrophin mutant dog showed many features such as CK elevation, dystrophin deficiency, skeletal muscle defects, abnormal ECG, and avoidance of ambulation, which are consistent with human dystrophinopathy. In a recent study, treatment with Cas9 and sgRNA-51 in spontaneous dystrophin KO dogs showed improved muscle histology. Additionally, human DMD iPSCs treated with Cas9 and sgRNA-51 restored dystrophin production [37]. Therefore, our expectation is that canine dystrophinopathy models with an in-frame dystrophin mutation can help in the successful development of new exon-skipping drugs.

Abbreviations

DMD: Duchenne muscular dystrophy; BMD: Becker muscular dystrophy; GRMD: golden retriever muscular dystrophy; CXMD: canine X-linked muscular dystrophy; CRISPRs: clustered regularly interspaced short palindromic repeats; Cas 9: CRISPR-associated 9; SCNT; Somatic cell nuclear transfer; mSOF: modified synthetic oviductal fluid; CK: creatine kinase; MRI: magnetic resonance imaging; ECG: electrocardiogram

Declarations

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Authors' contributions

HJO and EC conceived and designed the study. HJO, EC, JK, MJK, GAK, SHL, KR, and SP was responsible for performed the experiment, HJO, EC, KE and JK analyzed the data. HJO and EC wrote the paper. JC, JK, and BCL edited and reviewed the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed are available from the corresponding author on request.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

A

B

C

Figure 1

Production of a dystrophin mutant cloned dog by Cas9/sgrNA. (A) Sequences of target dystrophin locus detected in a cloned dog. The cloned puppy had a 57 bp deletion and deleted sequences are shown in a red box. (B) Summary of CK values from two to eight weeks of age in the control and dystrophin mutant dog. (C) Change in creatine kinase in normal and dystrophin mutant dog after exercise.

Figure 2

Magnetic resonance imaging of the dystrophin mutant dog. Rectus femoris (RF, arrowheads) and adductor magnus (AD, open arrowheads) muscles show marked hyperintense, contrast enhancing lesions with minimal fatty replacement. On T1-weighted image (A and B), quadriceps muscles (QD) are moderately atrophied. Note diffuse lesions are found in anterior-medial hindlimb muscles with low T2 values (green color) on F. Posterior-lateral hindlimb muscles including biceps femoris (BF), semitendinosus (ST), and semimembranosus (SM) muscles tend to be uninvolved relatively. A, T1-weighted axial plane; B, T1-weighted contrast enhanced, axial plane; C, T2-weighted coronal plane; D, T2-weighted axial plane; E, T2-weighted fat suppression (IDEAL), axial plane; F, color-coded T2-mapping, axial plane

Figure 3

Histopathological analyses of dystrophic muscle in control (A-D) and dystrophin mutant dog (E-H). Muscle pathology showing focal necrosis and regeneration of muscle fibers (HE), Immunohistochemical staining using monoclonal antibody against dystrophin rod domain and utrophin, showing decrease in DYS (dystrophin)1, 2 expression and increased utrophin expression compared to control muscles.

Figure 4

Western blot confirming the absence of DYS (dystrophin)1, 2 and upregulation of utrophin in dystrophin mutant dog (Mt dog) and control dog.

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