Generation of Genome-Edited Dogs by Somatic Cell Nuclear Transfer

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Research Article

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Abstract

Background: Canine cloning technology based on somatic cell nuclear transfer (SCNT) combined with genome editing tools, such as CRISPR/Cas9, can be used to correct pathogenic mutations in purebred dogs or to generate animal models of disease.

Results: In this study, we constructed a CRISPR/Cas9 vector construct targeting the canine DJ-1 gene. Genome-edited canine fibroblasts were established by transfection of the vector following antibiotic selection. We performed canine SCNT using genome-edited fibroblasts and successfully produced two genome-edited dogs. Both genome-edited dogs had indel mutations at the target locus, and expression of the DJ-1 gene was downregulated or completely repressed.

Conclusion: In conclusion, SCNT successfully produced genome-edited dogs using the CRISPR/Cas9 system for the first time.

Background

Canine cloning technology based on somatic cell nuclear transfer (SCNT) has been used for various purposes since the first cloned dog, Snuppy, was born in 2005 [1]. It was used not only for pet cloning [2] but also for the propagation of elite working dogs, including sniffing dogs [3] and rescue dogs [4]. This technology has also been used to preserve rare canine breeds [5, 6] or endangered canid species, such as wolves [7, 8]. In contrast, canine cloning technology can provide a platform for generating genetically engineered dogs. Previous reports have shown that transgenic dogs, cloned from transgenic canine fibroblasts, express transgenes well [9] and stably transfer the integrated transgene to the next generation [10]. These results are particularly interesting because this technology is expected to produce human disease model dogs [11].

The recent development of genome editing tools, including CRISPR/Cas9 technology, has dramatically changed the field of animal genetic engineering. Unlike previous transgenic technologies that tend to integrate exogenous gene sequences into genomes, genome editing tools recognize specific target sequences in the genome, induce double-strand breaks, and edit genomic sequences as needed very efficiently [12]. This new technology significantly changes the approaches and applications of genetically engineered animals. Scientists and companies have tried to use this technology to improve the genetic traits of livestock and generate disease models. It has been used to produce disease-resistant pigs [13] and cows [14] and enhances the productivity of pigs [15] and cashmere goats [16]. It has also been used to improve animal welfare by producing hornless dairy cattle [17].

However, in the canine research field, only two cases have been reported to use genome editing tools for producing genetically engineered dogs [18, 19]. Although these previous reports successfully showed that the genome-editing tool, CRISPR/Cas9, can be used to generate genetically engineered dogs, it has the limitation that both reports did not use SCNT-based canine cloning technology. The advantage of SCNT-based canine genome editing is that it can maintain the breed, genotypic backgrounds, and phenotypes,

except genome-edited target loci of donor animals. It is particularly important to use the technology to recover pathogenic mutations in purebred dogs or generate inbred animal models to study diseases.

In this study, we successfully produced genome-edited dogs using CRISPR/Cas9 and SCNT technologies. We targeted the DJ-1 gene in the canine genome and confirmed an indel mutation at the target locus of the genome-edited dogs produced in this study. We also confirmed that expression of the DJ-1 gene was downregulated or completely repressed in genome-edited dogs.

Methods

Ethics statement

The experimental procedures and methods used in this study were approved by the Animal Welfare and Ethics Office (CNU-01089), Chungnam National University, Daejeon, and performed according to “the Guide for the Care and Use of Laboratory Animals” published by the IACUC of Chungnam National University. Mixed female dogs from 2 to 6 years of age purchased form Honghwa Inc were used in this study as oocyte donors and embryo transfer recipients. The dogs were housed indoors and fed once daily with water ad libitum. All methods and protocols were performed following relevant guidelines and regulations.

Establishment of DJ-1 knockout canine fibroblasts

The target guide RNA (gRNA) for the CRISPR/Cas9 system was designed using CRISPR RGEN Tools (http://www.rgenome.net/). The selected gRNA sequence, 5’-CCTGTAGATGTCATGAGACGAGC-3’ (Figure 1a), was inserted into the PiggyBac-based commercially available CRISPR/Cas9 vector (VectorBuilder, USA). The vector construct also contained an EGFP/neomycin-resistant fusion gene for visualization and antibiotic selection (Figure 1b). The CRISPR/Cas9 vector construct was co-transfected with transposase vector (System Biosciences) into canine fibroblasts to establish the DJ-1 knockout cell line, using TurboFect reagent (Thermo Fisher Scientific) following the manufacturer’s instructions. After 24 h of transfection, the fibroblasts were treated with 1 μg/ml of neomycin for 14 days and then with 200 ng/ml for antibiotic selection of vector-integrated cells. All fibroblasts were cultured in DMEM (Gibco) supplemented with 15% FBS (Gibco) and 1% P/S (Gibco) at 38 °C and 5% CO_2.

Production of DJ-1 knockout dogs by Somatic Cell Nuclear Transfer

For the production of DJ-1 knockout dogs, SCNT followed by embryo transfer was performed as described in our previous report [29]. Briefly, in vivo-matured canine oocytes with the first polar body were used for micromanipulation. Metaphase chromosomes were removed by oocyte aspiration. A single cell from the cultured genome-edited fibroblasts was transferred into the perivitelline space of an enucleated oocyte, and each donor cell-cytoplast couplets was fused with two pulses of direct current (24–26 V for 15 μs) using an electro-cell fusion apparatus (NepaGene). The fused SCNT embryos were chemically activated by incubating with 10 μM calcium ionophore (Sigma) and then 1.9 mM 6-dimethylaminopurine

(Sigma). The activated SCNT embryos were surgically transferred into oviducts of oestrus-synchronized surrogates. Pregnancy was confirmed by ultrasonography 45 days after embryo transfer, and genome-edited dogs were produced by cesarean section. Sequencing analysis was performed to confirm the genome-edited mutation in the cloned dogs using the PCR primer set F: 5’-CTGCTGTGTTTTCATCTC-3’ and R; 5’-AGTATCCAGGAAATTTAC-3’.

Analysis of target gene, DJ-1, expression in genome-edited dogs

The target gene expression was analyzed with qRT-PCR. First, primary fibroblasts from genome-edited dogs were isolated. Primary cell culture was performed under the same conditions as the SCNT donor cell culture described above. Total RNA was extracted from cultured primary cells, and qRT-PCR was performed to analyze the expression of the DJ-1 gene in the genome-edited cells. The primer set sequences used for qRT-PCR were F: 5'-GGACCTTATGACGTAGTGATT-3' and R: 5'-CTTTGCTTCCAAAACCTATTT-3'.

Results And Discussion

In the current study, we successfully produced CRISPR/Cas9-mediated genome-edited dogs using SCNT technology. In particular, the DJ-1 gene was selected as the genome editing target in this study. DJ-1 is a multifunctional protein expressed in almost all cells and tissues [20]. It was originally discovered as an oncogene [21] and is now well known as a molecule related to various neurodegenerative disorders, including Parkinson’s disease, Alzheimer’s disease, and ischemic stroke [22]. Recently, accumulating evidence has indicated that DJ-1 is a novel therapeutic target for immune and inflammatory diseases [23] or ocular diseases [24]. DJ-1 deficient mice were generated to study the role and function of this gene; however, the disease phenotype of DJ-1 deficient mice were not similar to that of humans [25]. In contrast to the knockout mice models, DJ-1 deficient rat models showed a disease phenotype similar to that of human patients [26, 27]. For this reason, it is expected that larger animal models, such as dogs, can provide more accurate data for studying DJ-1 related human diseases.

We transferred 54 SCNT embryos to five recipient dogs. Among them, one recipient was diagnosed as pregnant, and two DJ-1 deficient offspring were produced (Table 1 and Figure 2a). The overall efficiency of CRISPR/Cas9-based genome-edited dog production by SCNT in this study was 3.7%. This is similar to the efficiency of previous reports using the microinjection method to produce genome-edited dogs (5.7% [19] or 3.1% [18]).

Table 1
Production of DJ-1 knock-out dogs by somatic cell nuclear transfer.

No. of transferred SCNT embryos	No. of Recipients	No. of Pregnancy (pregnancies/recipients, %)	No. of Offspring (births/transferred Embryos, %)
54	5	1 (20)	2 (3.7)

We confirmed that both DJ-1 deficient dogs showed EGFP expression due to the selective reporter on the vector construct (Figure 2b). Genome-edited mutations were analyzed using sequencing analysis. Interestingly, DJ-1 KO #1 showed in-frame (-3bp/-12bp) mutations, while DJ-1 KO #2 had out-of-frame (-7bp/-1 and 1 substitution) mutations (Figure 2c). Consequently, qRT-PCR results showed that DJ-1 expression was partially downregulated in DJ-1 KO #1 dogs and completely repressed DJ-1 expression in DJ-1 KO #2 dogs (Figure 2d). These two different genotypes can provide more options for using these animals to study the function of the DJ-1 gene.

The SCNT method has some advantages as a platform for generating genome-edited dogs. Unlike the microinjection method used in previous studies, it is possible to maintain breed and other genetic traits, except for the target gene, if SCNT was performed to produce genome-edited dogs. This is particularly important for correcting pathogenic mutations in purebred dogs. Due to strictly selective breeding programs with a limited number of founder dogs, purebred dogs have a greater risk of suffering from genetic disorders than any other species [28]. We believe that using genome editing tools, SCNT-based production of pathogenic gene-corrected dogs can be an excellent solution to this problem because it does not change the desired phenotype of the specific breed. On the other hand, SCNT is also useful for producing a group of genome-edited disease model dogs with the same genetic background. It will provide more reliable and stable data to researchers who use animal models.

Conclusion

In conclusion, we successfully developed genome-edited dogs using SCNT with CRISPR/Cas9 technology. DJ-1 knockout dogs showed partial or complete repression of target gene expression. This technology can be used for further studies to produce pathogenic gene-corrected dogs or disease-modeling dogs.

Declarations

Ethics approval and consent to participate

The experimental procedures and methods used in this study were approved by the Animal Welfare and Ethics Office (CNU-01089), Chungnam National University, Daejeon, and performed according to “The Guide for the Care and Use of Laboratory Animals” published by IACUC of Chungnam National University.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing Interests

O.J.K. is employee and shareholder of genome editing company, ToolGen. and M.K.K. are employee and shareholder of genome edited animal company, MK Biotech. The current study was financially supported by MK Biotech. MK Biotech had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Authors' Contributions

Conceptualization, D.E.K., J.H.L., K.B.J., K.S.P., T.Y.K. and M.K.K.; methodology, formal analysis, and investigation, D.E.K.; resources, M.K.K.; original draft preparation, O.J.K.; review and editing, D.E.K., O.J.K., M.K.K.; supervision and project administration, T.Y.K; research ethics and software, O.J.K., M.K.K.; funding acquisition, M.K.K. All authors have read and agreed to the published version of the manuscript.

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Figures

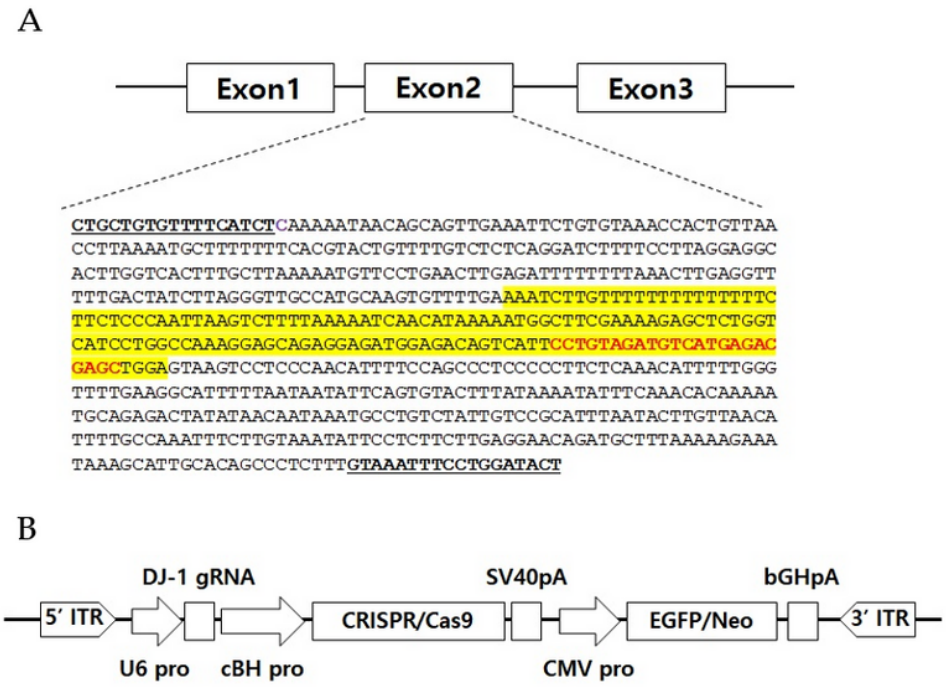

Figure 1

Design and Expression of CRISPR/Cas9 vector used in the study. A The gRNA (red letters) sequence was selected from exon 2 of canine DJ-1 (highlighted in yellow). The bold, underlined letters in the front and end of the sequences were primer sets used for sequencing analysis. B Schemes for CRISPR/Cas9 vector construct used in the study. Expression of the vector in the transfected cells was confirmed by EGFP expression in C bright view and D under UV light.

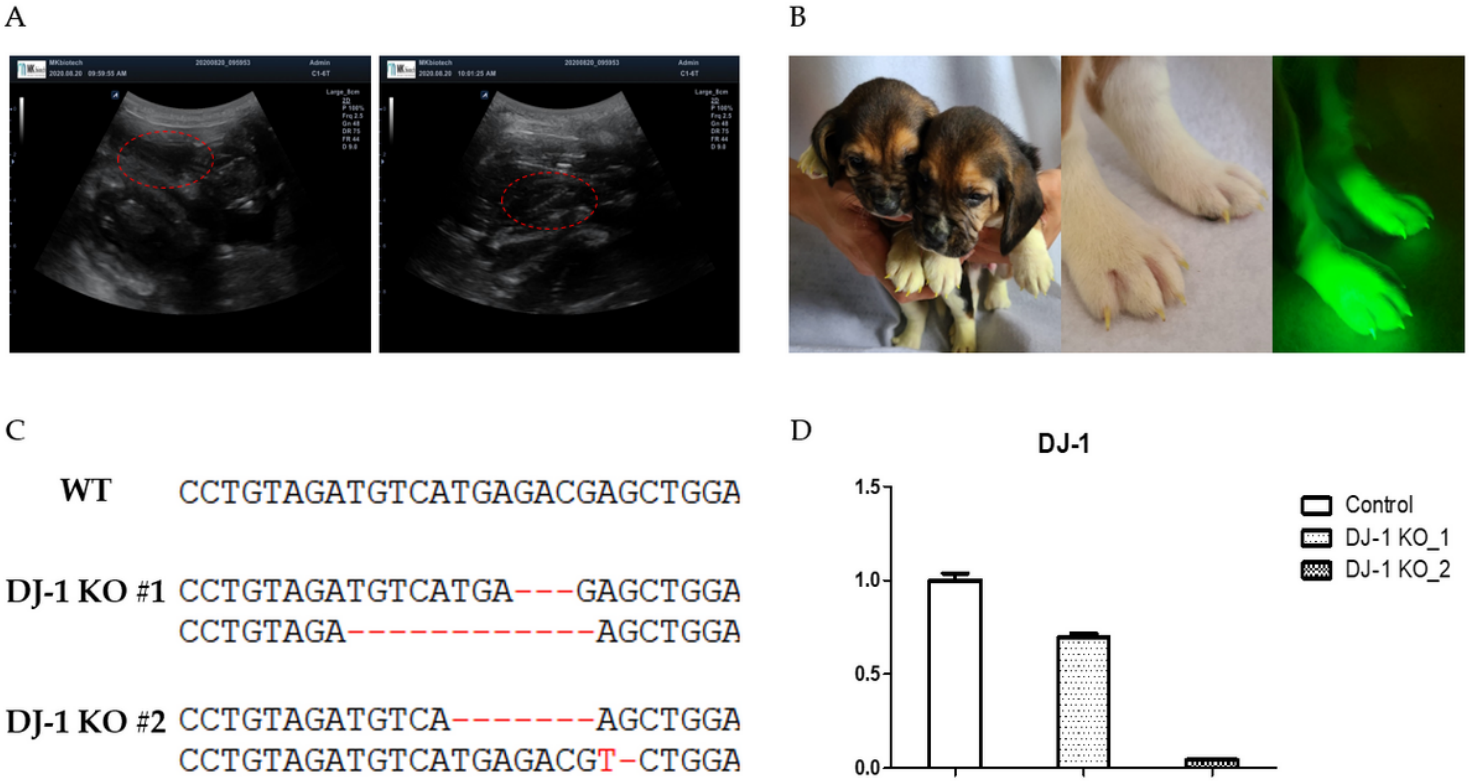

Figure 2

Genetic analysis of genome-edited dogs. A Pregnancy was diagnosed by ultrasound on day 45 after embryo transfer (fetuses circled in red). B DJ-1 knockout dogs at the age of 3 weeks after birth. The right panel shows the EGFP expressed in the dogs. C Sequence analysis of the targeted locus in DJ-1 knockout dogs. D Analysis of DJ-1 gene expression in DJ-1 knockout dogs by qRT-PCR.