Journal of Genetics and Genomics 45 (2018) 47–50

Contents lists available at ScienceDirect

Journal of Genetics and Genomics

Journal homepage: www.journals.elsevier.com/journal-of-genetics-and-genomics/

Letter to the editor

Generation of *ApoE* deficient dogs via combination of embryo injection of CRISPR/Cas9 with somatic cell nuclear transfer

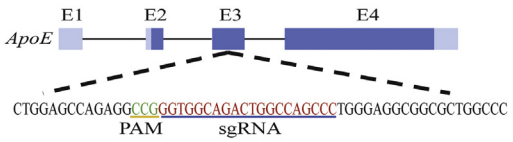
Atherosclerotic cardiovascular disease is the leading cause of death in the world which is resulted from complex interactions among multiple genetic and environmental factors (WHO). Atherosclerosis is a chronic inflammatory disease characterized by accumulation of lipids in the arterial wall (Gofman and Lindgren, 1950). Tremendous clinical and experimental efforts have been made to reveal the pathogenesis of the disease. Nevertheless, the mechanism of atherosclerosis is still unclear. A suitable animal model to study metabolic disorders and subsequent atherosclerosis is a necessity. The traditional method by feeding high fat diet to establish animal models of atherosclerosis disease is time-consuming and laborious, and in many circumstances, the phenotypes are not consistent among the individual models. Apolipoprotein E (ApoE) is a glycoprotein and synthesized mainly in the liver and brain. It is a structural component of all lipoprotein particles and responsible for the transportation and absorption of these particles. *ApoE* knockout mice showed a significant phenotype of atherosclerosis (Piedrahita et al., 1992; Plump et al., 1992; Zhang et al., 1992) and have been widely used in atherosclerosis research (Lusis, 2000). However, the lipoprotein profiles and metabolism in mice are different from humans (Langheinrich et al., 2007), and thus mouse models could not authentically mimic human atherosclerosis.

Dogs (*Canis familiaris*) are ideal models for biomedical research, drug evaluation and studying human disease due to its close similarities to humans in terms of metabolic, physiological, and anatomical characteristics (Tsai et al., 2007). In this study, dog *ApoE* gene was chosen to knock out for generating a cardiovascular disease model. These genetically modified dogs will provide a more effective large animal model for atherosclerosis studies. Genetically modified animals could be generated either by co-injection of *Cas9* mRNA and single-guide RNA (sgRNA) into one-cell stage embryos or combination of gene targeting of somatic cells with somatic cell nuclear transfer (SCNT) (Niemann and Lucas-Hahn, 2012). For dogs, precise genomic editing is difficult by either of the two approaches because of their unique species-specific reproductive characteristics. So far, only one case of gene editing dogs generated by CRISPR/Cas9 system was reported in 2015 (Zou et al., 2015). When *Cas9* mRNA and sgRNA were co-injected into one-cell stage embryos to generate gene editing animals, they mediated DNA double strand breaks (DSBs), repaired by error-prone non-homologous end-joining (NHEJ) in target locus randomly and gave rise to uncertain mutation pattern. Therefore, many of the resulted founder animals were chimeric ones with multiple mutations. To acquire the animals with a single mutation, one or two more rounds of further breeding have to be employed for selection among the offspring, which is time-consuming and costs high, hence, it is not so applicable in the large animals such as dogs with long gestation term and sex maturation time. In the last two decades, SCNT has been the most effective method to produce gene-targeting large animals and overcome the chimera problem. But, the efficiency of SCNT of dog is much lower than those large animals such as pigs, cows and goats. So far, no gene targeting dog via SCNT have been achieved. In order to solve the chimera problem and expedite the process of establishing homozygous gene editing dogs, we combined embryo microinjection of CRISPR/Cas9 with SCNT approaches to produce *ApoE* knockout dogs in this study, attempting to take the advantages and overcome the shortcomings of each approach.

As a first attempt, sgRNA (Fig. 1A) targeting the exon 3 of dog *ApoE* was designed and canine embryonic fibroblasts (CEFs) were chosen for co-transfection of *Cas9* vector and *ApoE* sgRNA to assess *ApoE* gene targeting efficiency. Twenty-three colonies were harvested and lysed to detect site-specific gene modification by PCR. Genomic PCR and Sanger sequencing showed that 19 colonies (82.6%) were mutated at the targeting site with various mutation patterns from 221 bp deletion to 1 bp insertion (Fig. 1B). Of these 19 gene targeting colonies, 4 colonies (17.4%, 4/23) showed monoallelic mutations and 15 colonies (65.2%, 19/23) showed biallelic mutations (Table S1). In view of the high gene editing efficiency of this sgRNA, we then sought to generate *ApoE* knockout dogs. A mixture of *in vitro* transcribed sgRNA and *Cas9* mRNA was microinjected into dog zygotes. Auto-transplantation strategy was employed for embryo transfer to overcome the possible synchronization difficulty of reproduction cycle between the donor embryos and surrogates. Briefly, only one side of the canine oviduct was flushed to collect zygotes. After injected with *Cas9* mRNA and *ApoE* sgRNA, these zygotes were immediately transferred back into the other side (not flushed) of the oviduct of the same donor female. A total of 65 presumptive zygotes (Fig. 1C) were collected and microinjected with *Cas9* mRNA and *ApoE* sgRNA. The injected embryos were transferred into 13 donor-recipient female dogs. Among them, 7 recipients were pregnant to term and gave birth to 13 puppies. Ear punch tissues were collected from all the puppies to initially confirm if any mutation in *ApoE* locus had been created. PCR products amplified from the genome of all the 13 puppies were sequenced. Among these 13 puppies, 2 were confirmed with genetic mutations in *ApoE* locus by DNA sequencing (Figs. 1C, 1D and S1). One (numbered #161207) of the 2 gene-target puppies is male and was named “Apple” with a single mutation pattern of 34 bp deletion and 17 bp insertion at the target locus. The other (numbered #170111) is female and was named “Hulu” with 33 bp

https://doi.org/10.1016/j.jgg.2017.11.003

A



B

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ApoE :          GGAGCCAGAGGCCGGGTGGCAGACTGGCCAGCCCTGGGAGGCGGC   WT
#8, #12, #28, #30 : GGAGCCAGAGGCCGGGTGGCAGACTGGCCAGCCCTGGGAGGCGGC   WT
#4, #18, #20, #23, #24, #27 : GGAGCCAGAGGCCGGGT-GCAGACTGGCCAGCCCTGGGAGGCGGC   Δ 1
#11 :           ----------//----------CCCTGGGAGGCGGC   Δ 216
#13 :           ----------//----------CTGGGAGGCGGC   Δ 51
#21 :           GGAGCCAGAGGCCGGGT----------CCAGCCCTGGGAGGCGGC   Δ 10
#22 :           ----------//----------CTGGGAGGCGGC   Δ 67
#16 :           ----------//----------TGGGAGGCGGC   Δ 44
                GGAGCCAGAGGCCGGGTGGCAGACTGGCCAGCCCTGGGAGGCGGC   WT
#17 :           GGAGCCAGAGGCCGGGT-GCAGACTGGCCAGCCCTGGGAGGCGGC   Δ 1
                GGAGCCAGAGGCCGGG----------CCAGCCCTGGGAGGCGGC   Δ 11
#15, #19 :      GGAGCCAGAGGCCGGGTG-CAGACTGGCCAGCCCTGGGAGGCGGC   Δ 1
                GGAGCCAGAGGCCGGGTGGCAGACTGGCCAGCCCTGGGAGGCGGC   WT
#26 :           GGAGCCAGAGGCCGGGT--CAGACTGGCCAGCCCTGGGAGGCGGC   Δ 2
                GGAGCCAGAGGCCGGGTGGCAGACTGGCCAGCCCTGGGAGGCGGC   +1
                                G
#29 :           GGAGCCAGAGGCCGGGTG-CAGACTGGCCAGCCCTGGGAGGCGGC   Δ 1
                GGAGCCAGAGGCCGGGTGGCAGACTGGCCAGCCCTGGGAGGCGGC   +1
                                G
#31 :           GGAGCCAGAGGCCGGGTG-CAGACTGGCCAGCCCTGGGAGGCGGC   Δ 1
                ----------//----------   Δ 84
#6 :            GGAGCCAGAGGCCGGGTGGCAGACTGGCCAGCCCTGGGAGGCGGC   WT
                GGAGCCAGAGGCCGG----------//----------   Δ 35
#14 :           ----------//----------   Δ 221
                GGAGCCAGAGGCCGGGTGGCAGACTGGCCAGCCCTGGGAGGCGGC   +1
                                G
```

C

Type of transplantation	Flushed embryos	Embryos injected	Embryos transferred	Host mother	Pregnancy	Birth	Mutation	Biallelic mutation
Auto-transplantation	65	65	65	13	7	13	2	2

D

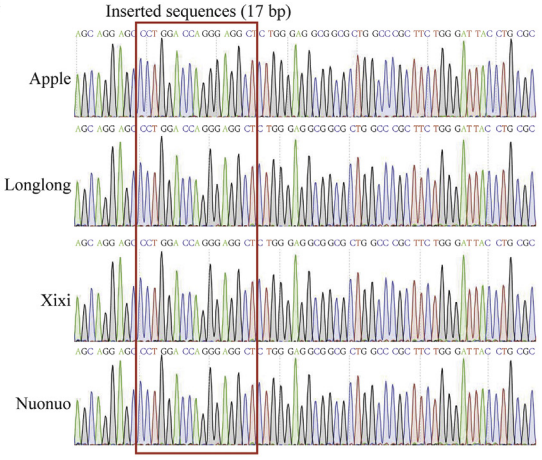
```
ApoE   AGGTCCAGCAGGAGCTGGAGCCAGAGGCCGGGTGGCAGACTGGCCAGCCCTGGGAGGCGGC
Apple  AGGTCCAGCAGGAGC----------CTGGGAGGCGGC   Δ 34,+17 (15/15)
                    ^
                    CCTGGACCAGGGAGGCT
Hulu   AGGTCCAGCAGG----------AGCCCTGGGAGGCGGC   Δ 33(9/15)
       AGG----------AGGCGGC   Δ 51(6/15)
```

E

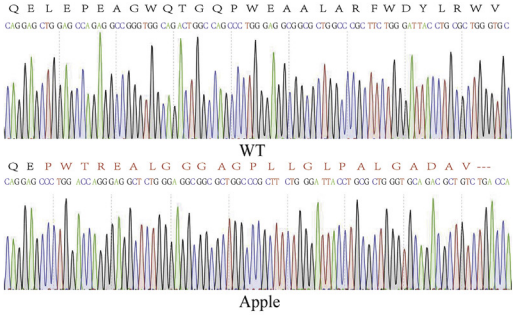
Recipient	Type of transplantation	Number of oocyte donor dogs	Number of oocytes collected	Number of oocytes fused and transferred (%)	Number of puppies born (%)
NTR1217	Allo-transplantation	9	37	21 (56.8)	1 (4.8)
NTR1243	Allo-transplantation	2	15	7 (46.7)	2 (28.6)
NTR1252	Allo-transplantation	3	18	9 (50.0)	0 (0)
NTR1256	Allo-transplantation	2	18	7 (38.9)	0 (0)
Total	–	16	88	44 (50.0)	3 (6.8)

F

G



H



I

Dog ID	Apple	Longlong	Xixi	Nuonuo	161205 (WT)	161206 (WT)
Total cholesterol (mmol/L)	22.92	21.86	13.12	15.82	7.14	7.31
Triglycerides (mmol/L)	2.25	1.85	1.91	1.84	1.57	1.44
HDL (mmol/L)	8.80	3.58	4.24	4.21	5.56	5.51
LDL (mmol/L)	13.10	17.44	8.01	10.77	0.87	1.15

Fig. 1. Generation of *ApoE* deficient cloned dogs. **A**: Schematic of sgRNA target site within exon 3 of dog *ApoE* locus. The sgRNA target site is highlighted in red and underlined in blue. Protospacer-adjacent motif (PAM) sequence is highlighted in green and underlined in orange. **B**: Sequences of modified *ApoE* locus detected in CEF colonies. Target sequences are shown in red; the dash represents mutations; Δ represents deletions; + represents insertions; WT represents wild-type allele. **C**: Summary of results from zygote microinjection of *Cas9* mRNA and *ApoE* sgRNA in beagle dogs. **D**: Sequencing of target site in both mutant puppies. Fifteen TA clones of PCR products were analyzed by DNA sequencing. **E**: Summary of results for generation of gene targeting cloned dogs. **F**: Pictures of 28-day-old cell donor dog Apple (above, left), 28-day-old cloned dog Longlong (above, right), cloned dogs Xixi and Nuonuo (below). **G**: The PCR products from Apple and 3 cloned dogs were examined by Sanger sequencing. The results showed that the 3 cloned dogs had the same gene modification with Apple. **H**: DNA sequencing diagram and amino acid sequences of *ApoE* proximal to target site. Sequencing of nonsense mutations was detected in Apple. – represents the stop codon site. **I**: Total cholesterol, triglycerides, HDL and LDL levels were detected in the plasma derived from Apple, Longlong, Xixi, Nuonuo and two other wild-type dogs at one month old.

deletion in one allele and 51 bp deletion in the other at the target locus (Figs. 1D and S1), both being out-of-frame shift mutation.

An estrus cycle of dogs is from six months to one year and sex maturation age is about one and a half years. If using natural breeding to expand genetically modified dogs achieved by embryo injection, it may take three years to get homozygous gene editing dogs. When applying SCNT approach by using the cells from the founder dogs with *ApoE* mutation as donor nuclei, one can expand homozygous gene editing dogs within six months, which would at least save two and a half years. Therefore, our second attempt was to use ear skin fibroblasts of Apple as donor cells for SCNT to produce *ApoE* gene targeting cloned dogs. Dog metaphase II oocytes were collected by flushing oviducts at 48–72 h after ovulation. For SCNT, the nuclei of dog oocytes were removed by micromanipulation and a single cell from Apple was injected into the perivitelline space of the enucleated oocyte. After fusion and activation, a total of 44 cloned embryos were immediately transferred into 4 surrogates (Fig. 1E). Among these 4 recipients, 2 were pregnant to term and gave birth to 3 puppies (Fig. 1E and F). The cloning efficiency of gene targeting dogs is 6.8% (3/44), similar to that previously reported by using fetal fibroblasts as donor cell (4.0%), but higher than that by using adult fibroblasts (0.2%–1.8%) as donor cell (Hong et al., 2009). Theses puppies were numbered as #170502, #170610 and #170611, and named as "Longlong","Xixi" and "Nuonuo", respectively. To confirm the genetic identity of the cloned dogs, microsatellite analysis was performed with genomic DNA from the blood samples of Apple, Longlong, Xixi, Nuonuo, and the two surrogate female recipients. A total of 15 microsatellite markers were selected and analyzed. Analysis of the 15 microsatellite markers loci confirmed that the cloned dogs were genetically identical to the donor dog (Table S2). Genomic PCR and Sanger sequencing demonstrated that all the 3 cloned puppies had the same mutation pattern with 34 bp deletion and 17 bp insertion at *ApoE* locus (Figs. 1G and S1) of both alleles as that of Apple.

The mutation of 34 bp deletion and 17 bp insertion at the target site resulted in premature termination of *ApoE* translation (Fig. 1H). To assess the potential effects of *ApoE* gene mutation on serum lipids, plasma samples collected from one-month-old knockout puppies and wild-type litter mates were used to conduct serum biochemical analyses. Plasma high density lipoprotein (HDL), low density lipoprotein (LDL), total cholesterol and triglyceride levels were measured. As shown in Fig. 1I, LDL, total cholesterol and triglycerides levels in Apple and the three clones, which were all *ApoE* homozygous knockout dogs, were substantially elevated when compared with those in wild-type litter mates. But the HDL level in the three cloned dogs was similar to that in wild-type litter mates. Total cholesterol levels were increased 3-fold and LDL levels were increased about 10-fold in the 4 *ApoE* knockout dogs when compared with those in wild-type dogs.

To test the specificity of Cas9/sgRNA cleavage in these genetically modified dogs, we identified the 13 most likely off-target sites (OTS1–13) (Table S4) that differed by 2 or 3 nucleotides in the *APOE* target site and amplified these DNA fragments via PCR from ear skin genomic DNA of Apple, Hulu, Longlong and wild-type dogs. The PCR products amplified from OTS1–13 were subjected to T7E1 cleavage assay (Fig. S3) and DNA sequencing analysis. No cleavage bands were found at any potential off-target sites, suggesting that the Cas9/sgRNA did not induce detectable off-target mutation in *APOE* knockout dogs.

In summary, we successfully generated *ApoE* gene knockout dogs by microinjecting CRISPR/Cas9 into dog zygotes, followed by using fibroblasts from the knockout dogs as the donor cells for SCNT. We achieved and expanded dog population of homozygous *ApoE* gene targeting dogs. Similar to that in cell donor dog, the total cholesterol and LDL levels in the cloned puppies were elevated, compared with wild-type dogs. *ApoE* gene targeting dogs with high plasma lipoprotein levels is expected to develop to atherosclerosis with the increase of age, which will provide a powerful large animal model for study of pathogenesis and development of new drugs for treatment and prevention of human atherosclerosis disease. In addition, our results showed that gene targeting dogs could be generated through SCNT approach by using the mutant cells as donor nuclei, which facilitate the production of precise gene editing dogs with different mutation patterns including knockout and knockin and point mutation. Collectively, our study provides a general platform to produce more canine disease models possessing precise gene modification and paves the way to genetic studies and pre-clinical investigation. We believe it will not only greatly facilitate the generation of new disease models for biomedical research but also potentially promote creation of dogs with favorable traits for many more other purposes.

Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jgg.2017.11.003.

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24 October 2017
Available online 2 December 2017

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