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Targeted disruption of Ataxia-telangiectasia mutated gene in miniature pigs by somatic cell nuclear transfer

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ABSTRACT

Ataxia telangiectasia (A-T) is a recessive autosomal disorder associated with pleiotropic phenotypes, including progressive cerebellar degeneration, gonad atrophy, and growth retardation. Even though A-T is known to be caused by the mutations in the Ataxia telangiectasia mutated (ATM) gene, the correlation between abnormal cellular physiology caused by ATM mutations and the multiple symptoms of A-T disease has not been clearly determined. None of the existing ATM mouse models properly reflects the extent to which neurological degeneration occurs in human. In an attempt to provide a large animal model for A-T, we produced gene-targeted pigs with mutations in the ATM gene by somatic cell nuclear transfer. The disrupted allele in the ATM gene of cloned piglets was confirmed via PCR and Southern blot analysis. The ATM gene-targeted pigs generated in the present study may provide an alternative to the current mouse model for the study of mechanisms underlying A-T disorder and for the development of new therapies.

1. Introduction

Ataxia telangiectasia (A-T) is a recessive autosomal disorder in humans with an average frequency of 1:40,000–1:100,000 live births worldwide [1–3]. It is characterized by a wide variety of pleiotropic phenotypes, including progressive cerebellar degeneration, gonadal atrophy, growth retardation, lymphoreticular malignancies, and incomplete sexual maturation. Patients with A-T are extremely sensitive to ionizing radiation, which causes double strand breaks (DSB) in the DNA [4,5]. One of the major clinical symptoms of A-T is cerebellar ataxia, which results from the gradual loss of the Purkinje cells in the cerebellum [6,7]. The disease is progressive, and death generally occurs by the second or third decade of life due to neurologic deterioration or lymphoreticular malignancies, which occur in 10–15% of patients [8]. Ataxia telangiectasia is caused by a deficiency or malfunction of the ataxia telangiectasia mutated (ATM) protein that normally mediates cellular response to DNA damage through multiple transduction pathways. Carriers of the ATM mutation have been estimated to comprise 0.5–1% of the general population [9], and these individuals show none of the severe clinical symptoms seen in A-T patients but do have a predisposition to cancer, particularly breast cancer in women [10].

The ATM gene has 65 exons extending over 160 kb of genomic DNA. It produces a 13-kb transcript that encodes a 350-kDa protein containing highly conserved catalytic domains for phosphatidylinositol-3-kinase, which is presumably involved in mediating cell cycle arrest in response to radiation-induced DNA damage [11,12]. This protein initiates a signaling cascade through the phosphorylation of various downstream genes such as p53, Chk2, Mdm2, BRCA1, H2AX, and Pin2/TRF1, that in turn control cell cycle-check point, DNA double-strand break repair pathways, apoptosis, and telomere metabolism [13–15].

A murine model for A-T has generally been used to characterize the effects of the ATM mutation. Many types of ATM knockout (KO) mice have been generated to study the function of the ATM protein as well as the molecular basis of this pleiotropic and multisystemic disease [16–18]. ATM-deficient mice display a variety of symptoms

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similar to those of human A-T patients, including growth and meiotic defects, immunological abnormalities, radiation hypersensitivity, and predisposition to cancer, which together are the most common pleiotropic roles of ATM. However, the neurological defects in these mice develop more slowly than those clinically observed in humans, and the mice have not died from ataxia, but from other consequences of ATM-deficiency, such as lymphoma.

The neurological abnormalities seen in ATM-mutant mice do not present the same severity as human neurological abnormalities, suggesting that an alternative animal model for human A-T could be beneficial [19]. As such, the pig has already been recognized to be a useful, well-established model for medical studies of human disease. Since the pig has been able to provide insight into the mechanism and treatment of various diseases (such as cancer, diabetes, and atherosclerosis), the ATM gene-targeted pigs could be a valuable model for studying A-T. Moreover, the structure and transcription of the porcine ATM gene has already been characterized [19]. Genomic sequencing of the porcine ATM gene revealed that a similarity between humans and pigs was greater than that between humans and mice, suggesting that, from a molecular standpoint, a porcine model may be more suitable.

The aim of the present study is to produce a novel disease model for ATM that can replace the mouse model that does not fully represent all phenotypes of the disease. We therefore report on the production of cloned miniature pigs via somatic cell nuclear transfer (SCNT) using ATM gene-targeted fetal fibroblasts.

2. Materials and methods

2.1. Animal ethics

All procedures in this study were carried out in accordance with the Code of Practice for the Care and Use of Animals for Scientific Purposes, as approved by the Institutional Animal Care and Use Committee of Dankook University.

2.2. Preparation of porcine fetal fibroblasts

Pig fetuses at 28–39 days of gestation were obtained from Minnesota miniature pigs maintained in specific pathogen-free (SPF) conditions at Seoul National University. The head, dorsal spine of the medial section, and tail of the fetuses were removed. Then, small pieces of the remaining tissues were washed in Dulbecco's PBS (DPBS; Invitrogen) and minced with a surgical blade in a 100-mm petridish. Cells were then dissociated from the tissues in 0.25% Trypsin–EDTA (Invitrogen) for 10 min at 39 °C. After centrifuging the cell suspension three times at 800*g* for 5 min, the pellets were resuspended and seeded on 100-mm culture dishes (Falcon) and cultured for 6–8 days in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone), 1 mM L-glutamine, 100 units/ml penicillin, and 0.5 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air. After removing the unattached clumps of the cells by washing the culture plates with DMEM, the attached cells were cultured until confluent and were then subcultured at intervals of 5–7 days by trypsinization until used for transfection and SCNT.

2.3. Construction of porcine ATM gene targeting vector

A targeting vector was constructed from a genomic DNA fragment containing exons 58 and 59 of the porcine ATM gene (GenBank accession no: AY587061). As shown in Fig. 1, the vector was comprised of a 10,051 bp 5′ long arm of homology corresponding to a region of the ATM gene from exon 58 to exon 59; a 1806 bp mouse phosphoglycerate kinase-1 (PGK) promoter-neomycin resistance gene as a positive selection maker; a 2205 bp 5′ short arm of homology corresponding to a region of ATM gene from exon 59 to intron 59; and a diphtheria toxin A (DTA) gene for negative selection. The long and short arms were amplified with a long arm primer set (forward 5′-ccgcggTTTGGGTTGCTTGTAGTCCTTT-3′ and reverse 5′-cccgggCAGTCGTCTCTAAACCAAACA-3′) and a short arm primer set (forward 5′-gtcgacTGTTTGGTTTGAGAGACGACTG-3′ and reverse 5′-aagcttCCCTTCTTCGAGACCTGACTTA-3′) and were ligated into a PGKneolox2DTA.2 plasmid (Addgene). The targeting vector was linearized with SacII before electroporation-mediated gene transfer into the porcine fetal fibroblasts.

2.4. Generation of gene targeted fibroblasts

A 20 kb linearized targeting vector was introduced into 2×10^7 female fetal fibroblasts using the Gene Pulser II (Bio-Rad) according to the manufacturer's protocol. After culture for 48 h, the genetically modified cells were selected using culture medium containing 500 μg/mL of G418 (Invitrogen) for 10–14 days. After the antibiotic selection, G418-resistant colonies were isolated and transferred onto 0.1% gelatin-coated dishes for clonal culture. The cells were continuously cultured at 39 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.5. Karyotypic analysis

Prior to the SCNT, karyotypic analysis was performed to ensure whether αGT KO fibroblasts maintains normal chromosomes. Briefly, 200 μL of colcemid (Invitrogen) was added to actively dividing fibroblasts before overnight incubation at 39 °C in 5% CO_2. The cells were trypsinized, harvested, and centrifuged for 5 min at 400*g*. After 10 μL of hypotonic solution (0.075 M KCl) was added to the cell pellet, then the cells were resuspended, incubated for 20 min at 39 °C in 5% CO_2, and centrifuged for 5 min at 400*g*. Then, the hypertonic solution was removed, and 500 μL of Canoy's fixative (methanol:acetic acid = 3:1) was added and mixed with the cells. The fixative containing fibroblasts was dropped and spread on clean glass slides, and the slides were then baked at 60 °C for 30 min and were treated with 50% H_2O_2 for 3 min. Finally, karyotyping with Giemsa staining (GTG banding) was performed using ChIPS-Karyo (GenDix), a chromosome image processing system.

2.6. Somatic cell nuclear transfer

The in vitro maturation (IVM) of the oocytes and the SCNT were performed as previously described [20]. Briefly, 42 h after the onset of IVM, the oocytes were enucleated using a glass pipette with a 20-μm internal diameter by aspiration of the first polar body and the second metaphase plate with a small volume of surrounding cytoplasm in HEPES-buffered TCM-199 supplemented with 0.3% bovine serum albumin (BSA) and 5 μg/mL cytochalasin B. After enucleation, the oocytes were stained with 5 μg/mL bisbenzimide (Hoechst 33342) for 5 min and were observed under a Nikon TE-300 inverted microscope equipped with epifluorescence. Oocytes containing DNA materials were excluded from the subsequent experiments. The fibroblasts were trypsinized into single cells and transferred into the perivitelline space of the enucleated oocytes. The resulting couplets were equilibrated for 1 min in 0.3 M mannitol solution containing 0.5 mM HEPES, 0.05 mM $CaCl_2$, and 0.1 mM $MgCl_2$ in a chamber containing two electrodes. A BTX Electro-Cell Manipulator 2001 (Harvard Apparatus) was used to fuse the couplets with a double DC pulse of 1.5 kV/cm for 45 μs. After electrical stimulation, the reconstructed oocytes were cultured in porcine zygote medium-3 (PZM-3) supplemented with

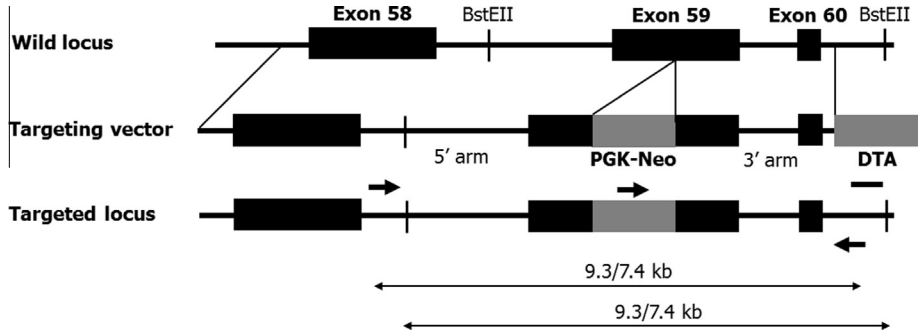

Fig. 1. Diagram of the ATM gene-targeting vector. The arrows represent PCR primers to detect homologous recombination, and the bar represents a probe for Southern blot analysis.

3 mg/mL fatty acid-free BSA and 7.5 μg/mL cytochalasin B for 3 h to suppress extrusion of the second polar body.

On the day after SCNT, the reconstructed embryos were surgically transferred to naturally cycling surrogate sows on the second day of standing estrus. Abdominal ultrasonograpy was performed to test for pregnancy at days 25–30 after the embryo transfer, and thereafter, the pregnant recipients were examined weekly via ultrasound.

2.7. Detection of homologous recombination

As shown in Fig. 1, the genomic DNA obtained from the G418-resistant fibroblasts and tail biopsies of the cloned piglets were analyzed with respect to the disrupted porcine ATM allele. The genomic DNA was extracted from the cells and tissues using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocol. To perform a PCR analysis of the gene targeting events, genomic DNA was amplified using the Maxime PCR Premix Kit (Intron Biotechnology) in 20 μL reaction volume. The amplification of the targeted gene was carried out using forward 5′-TCAAGTCCCTCTTTA CCACCTC-3′ and reverse 5′-CAGCCTGGCTTTTGATAGTCTT-′3 primers. The conditions for PCR were as follows: 30 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, extension at 68 °C for 10 min plus 20 s increment per cycle, and one final cycle of 15 min at 68 °C. Amplification products (7.4 kb fragment of the wild type allele and 9.3 kb fragment for the targeted allele) were analyzed via electrophoresis in 0.4% agarose gel.

Southern blot hybridization was performed using DIG High Prime DNA Labeling and Detection Kit 2 (Roche). Ten micrograms of genomic DNA were digested overnight with a BstEII at 60 °C. As shown in Fig. 1, digestion generates a 7.4-kb fragment in the wild allele and a 9.3-kb fragment in the targeted allele. The samples were separated on a 0.8% agarose gel. Following electrophoresis, the restricted genomic DNA was transferred to a nylon membrane. Then, the membrane was hybridized with a 520 bp DIG-labeled probe in DIG Easy Hyb solution (Roche) at 53 °C for 16 h. After hybridization, the membrane was washed twice in 0.5× SSC with 0.1% SDS at 68 °C and exposed to a camera for 10 min.

3. Results

3.1. Generation of ATM gene-targeted fibroblasts

To disrupt the porcine ATM gene, both male and female fetal fibroblasts were separately transfected with a conventional targeting vector (Fig. 1). After antibiotic selection, 87 male and 138 female G418-resistant colonies were obtained. Among these colonies, one male and three female fibroblast colonies were identified

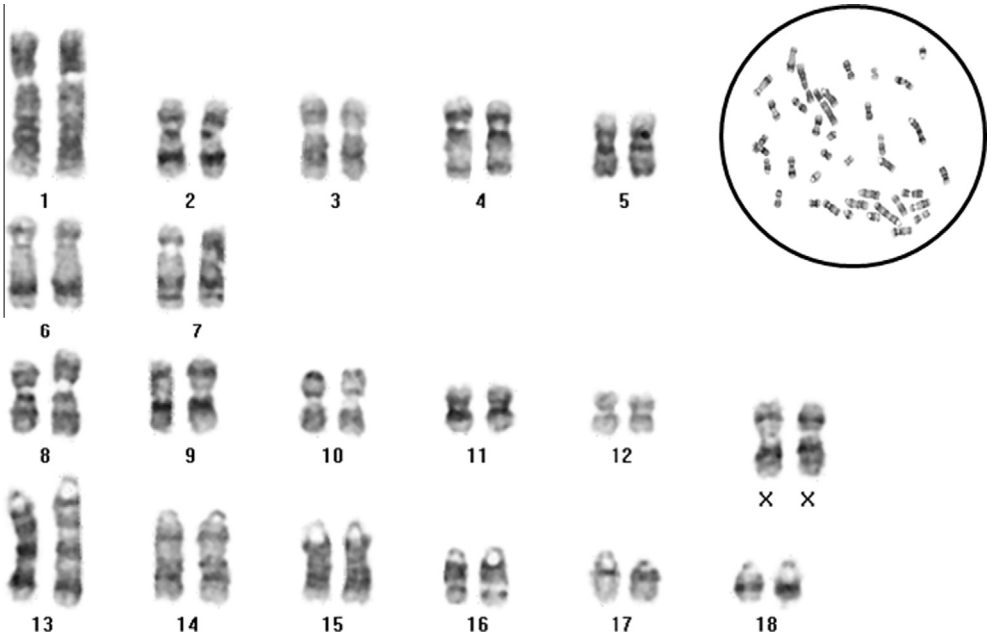

Fig. 2. Karyotype of ATM gene-targeted fetal fibroblasts. Cells contained a normal karyotype of female swine ($2n = 38$, XX).

Fig. 3. ATM gene-targeted miniature pigs produced via somatic cell nuclear transfer.

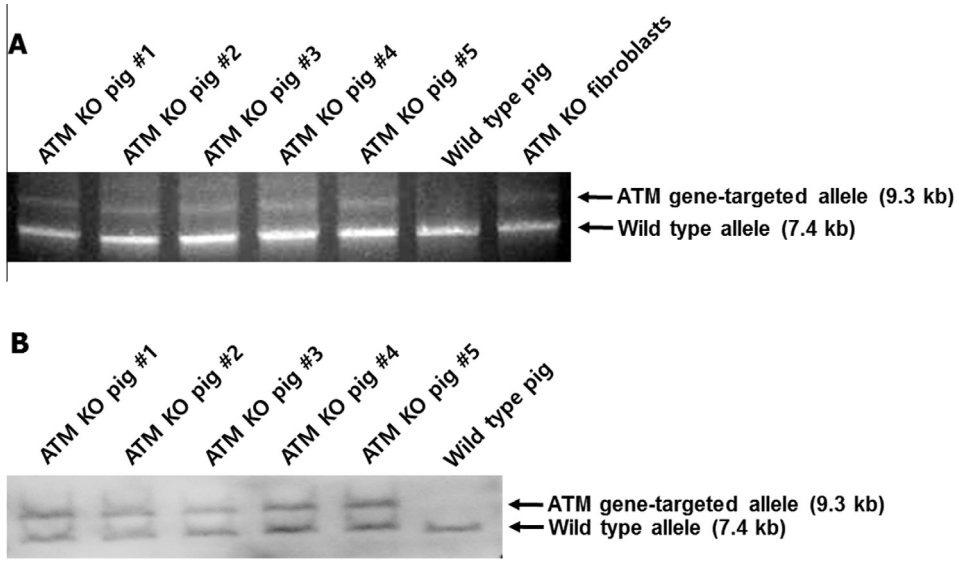

Fig. 4. Analysis of ATM gene-targeted pigs. (A) PCR analysis for ATM gene-targeting. (B) Southern blot for ATM gene-targeting.

as homologous recombinants based on PCR analysis. As shown in Fig. 2, one female colony containing normal karyotype ($2n = 38$, XX) was chosen for subsequent SCNT experiment.

3.2. Production of ATM gene-targeted pigs

A total of 611 reconstructed oocytes were produced via SCNT using ATM gene-targeted fetal fibroblasts. These cloned embryos were surgically transferred to five surrogate gilts. Early pregnancies were observed in three gilts, and two ultimately went to full term. Five cloned piglets were delivered, four from one surrogate and one from the other (Fig. 3). To confirm whether the cloned piglets were derived from the nuclear donor cells, genomic DNA extracted from tail biopsies was screened by PCR and Southern blot. As shown in Fig. 4, all piglets contained one allele of the ATM gene disrupted by PGK-Neo, resulting in an increased size of ATM gene fragment in PCR and in Southern blot.

4. Discussion

In the present study, we described a novel large animal model for preclinical research of A-T disease. We produced heterozygous ATM knockout miniature pigs via SCNT using ATM gene-targeted cells as nuclear donors. The ATM mutations were broadly categorized to be of two types: the missense mutation (ATM^{mis}) and the truncating mutation (ATM^{trunc}). The former includes non-silent mutations that cause a substitution or short in-frame insertion or deletion of amino acids. The ATM protein is expressed in this mutation, but does not have its proper functions. The latter is a truncating and null mutation so that little or no ATM proteins are expressed [21]. Approximately 70% of the ATM mutations identified in A-T patients are truncating mutations, and the remaining 30% are missense mutations [3,21,22]. Clinical phenotypes could be different depending on the types of ATM gene mutations [3]. Truncating mutations could cause lethal effects such as tumorigenesis before the emergence of neuronal degeneration, whereas missense mutations do not lead, generally, to the progressive neurodegenerative phenotype [23]. In addition, A-T patients appear to have an increased risk of cancer, particularly breast cancer [10]. Although the frequency of ATM mutation carriers in western populations are as high as 0.5–1% [24], approximately 5% of breast cancer patients could be ATM mutation carriers [21]. Therefore, the increased risk for breast cancer in ATM carriers suggests that ATM heterozygosity might be one of the conditions predisposing patients to breast cancer [25]. This was one of the reasons for which we produced female clone pigs with ATM heterozygosity (Figs. 3 and 4), providing a pig model susceptible to breast cancer.

Different types of mutations may have different effects cancer risks, and it is still uncertain which type of mutation is more

dominant for cancer outbreaks in ATM carriers. Missense mutations in ATM have a higher risk for cancer than truncating mutations due to dominant negative effects. However, if a stable truncated protein is expressed, it would contain the leucine zipper domain and could potentially act in a dominant-negative manner [21,26]. Hence, we decided to disrupt exon 59 of the porcine ATM gene where kinase activity exists, in turn causing truncating mutations (Fig. 1). Through this targeting strategy, a truncating mutation of ATM would be able to operate in a dominant-negative manner.

In this study, ATM gene-targeted cloned piglets were produced via SCNT. To the best of our knowledge, this is the first report of an A-T disease model in large animals. The heterozygous ATM KO pigs described here would be an essential step for the production of ATM-deficient pigs. A porcine ATM heterozygous model may not only provide tools to study the effects of ATM haploinsufficiency on the development of cancer, but may also, followed by production of homozygous ATM KO pigs through breeding, expand our understanding of the mechanism underlying the development and nature of A-T to ultimately assist in finding effective therapies.

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