

ORIGINAL ARTICLE

Heterozygous troponin T-K210 del mutant iPSCs generated from a patient with familial dilated cardiomyopathy and CRISPR-Cas9 genome editing experiment

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Abstract

Background: The advent of clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR-Cas9) system has markedly accelerated research employing induced pluripotent stem cells (iPSCs) for the analysis of gene function and gene therapy. In this study, we performed exome sequencing to identify genetic mutations in patients with familial dilated cardiomyopathy (DCM). Subsequently, we generated iPSCs from the patient and performed genome editing experiment using the CRISPR-Cas9 system.

Methods and Results: Exome sequencing was conducted on genomic DNA extracted from two siblings with familial DCM. The results revealed the presence of the heterozygous troponin T (TnT)-K210 del mutation (Δ K210/WT) in both individuals. One of the patients' peripheral blood mononuclear cells were reprogrammed into iPSCs through electroporation of episomal plasmids encoding the reprogramming genes. The Δ K210/WT iPSCs were successfully differentiated into cardiomyocytes. Despite the generation of homozygous TnT-K210 del mutant (Δ K210/ Δ K210) iPSCs using the CRISPR-Cas9 system, the Δ K210/WT iPSCs could not be repaired into the WT/WT iPSCs.

Conclusions: The TnT- Δ K210/WT was detected in patients with DCM, and iPSCs were generated. The CRISPR-Cas9 system was employed to successfully generate the Δ K210/ Δ K210 iPSCs, but not the WT/WT iPSCs from the Δ K210/WT iPSCs. Further studies are necessary to generate WT/WT, Δ K210/WT, and Δ K210/ Δ K210 iPSCs.

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Key words: Dilated cardiomyopathy; Induced pluripotent stem cells; Troponin T mutation; CRISPR-Cas9 system.

Introduction

Dilated cardiomyopathy (DCM) is characterized by the presence of ventricular dilatation, systolic dysfunction, and progressive heart failure. The etiology of DCM has long been unclear, however, recent analysis has distinguished between hereditary (familial) and non-hereditary (non-familial) categories. Approximately 20% to 35% of DCM cases are familial, indicating a substantial genetic component. The major genes associated

with this condition are titin gene (*TTN*), lamin A/C gene (*LMNA*), myosin heavy chain 7 gene (*MYH7*), and cardiac troponin T gene (*TNNT2*)¹⁾. Approximately 40 causative genes for familial DCM have been identified, and abnormalities in any of these genes can result in the pathology of DCM²⁾. A large-scale genomic cohort study of DCM conducted in Europe using next-generation sequencing reported that more than 38% of cases had multiple mutations, and 13% had three or more mutations³⁾. While some genetic

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abnormalities have been reported to reduce the calcium sensitivity of myocardial contraction⁴⁾, many aspects of the mechanisms by which these abnormalities cause DCM remain unclear. The establishment of experimental models is therefore required to unravel these mechanisms.

The recent advancements in induced pluripotent stem cells (iPSCs) technology and the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR-Cas9) system have significantly expanded the scope of biomedical research. For example, Shiba *et al.* reported that desmosome deficiency is the primary etiology of clinically diagnosed idiopathic DCM⁵⁾. They generated disease-specific iPSC lines that were differentiated into cardiomyocytes and performed genome editing using the CRISPR-Cas9 system, thereby providing a possible concept for gene replacement therapy for DCM.

The objective of this study is to elucidate the underlying mechanisms of familial DCM. To this end, exome sequencing was initially employed to identify genetic mutations in two siblings with familial DCM. Subsequently, iPSCs were generated from the patient, and genome editing was performed using the CRISPR-Cas9 system.

Methods

Study cohort

Two siblings with familial DCM were recruited for this study. The study was approved by the ethics committee of the Hirosaki University Graduate School of Medicine (Approval No. 2021-156). Written informed consent was obtained from the subjects before the study.

Extraction of genomic DNA and direct sequencing

Genomic DNA was extracted from whole blood using the QIAamp DNA Blood Kit (Qiagen, Chatsworth, CA, USA), and from

cardiac biopsy specimens using the QIAamp DNA FFPE Tissue Kit (Qiagen). Polymerase chain reaction (PCR) amplification was performed using PrimeSTAR[®] HS (Premix) (Takara, Japan). The isolated DNA was purified using the QIAamp DNA Mini Kit (Qiagen). Both strands of each amplicon were sequenced with a fluorescent dye terminator reaction using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems).

Exome sequencing

Exome sequencing and variant annotation were conducted at the Hirosaki University Healthy Aging Innovation Center on two siblings with familial DCM. Genomic DNA from two siblings with familial DCM was subjected to whole exome sequencing using the NextSeq 550 sequencing system (Illumina, San Diego, CA, USA). The exome sequencing library was constructed using the Nextera DNA Library Prep for Enrichment kit (Illumina) and the xGen Exome Panel (Integrated DNA Technology, Coralville, IA, USA). Paired-end sequencing with 76-bp read lengths was conducted using a NextSeq 500/550 High Output kit (Illumina). Sequence reads mapping and annotations were performed using Ingenuity[®] Variant Analysis[™] software (www.ingenuity.com/variants) from Ingenuity Systems with the human reference genome (GRCh37/hg19, dbSNP build 132).

Generation and culture of iPSCs

The protocol for derivation of iPSCs in a feeder-free culture system was performed as previously reported^{6, 7)}.

Peripheral blood mononuclear cells from one of the DCM patients were reprogrammed into iPSCs by the electroporation of episomal plasmids encoding the reprogramming genes OCT3/4, SOX2, KLF4, L-MYC, LIN28, and

mouse p53DD. These iPSCs were cultured in a laminin-coated 6-well dish, using StemFit AK02N (Ajinomoto, Japan) without supplement C on day 1, followed by regular StemFit AK02N medium. After 3 weeks, when the colonies had grown and were visible to the naked eye, iPSCs were picked from some colonies. The picked iPSCs were cultured under feeder-free conditions using StemFit AK02N. The banding pattern of each chromosome was analyzed by trypsin-Giemsa staining (G-band analysis) to assess whether it was normal or abnormal.

Cardiac differentiation from iPSCs

The cell culture and induction protocol for ventricular cardiomyocytes was conducted as previously reported with some modifications^{8,9}.

Once the iPSCs reached 80-90% confluence, embryoid bodies (EBs) were generated by dissociating the iPSCs into single cells using TrypLE™ Select (Gibco) + 0.5 mM EDTA/PBS. The cells were then seeded at 2×10^6 cells per well in 6-well dishes coated with poly (2-hydroxyethyl methacrylate) (HEMA, Sigma-Aldrich) in a basal medium of StemPro-34 (Gibco) supplemented with penicillin/streptomycin (1%, Gibco), L-glutamine (1%, Sigma), ascorbic acid (50 µg/mL, Sigma), monothioglycerol (MTG) (50 µg/mL, Sigma), and transferrin (150 µg/mL, Wako), as well as Rho-associated coiled-coil forming kinase (ROCK) inhibitor Y-27632 (10 µM, Fuji Film) and bone morphogenetic protein 4 (BMP4) (2 ng/ml, R&D Systems). After 24 hours, an equal volume of basal medium was added to achieve final concentrations of BMP4 (10 ng/mL), basic fibroblast growth factor (bFGF, 5 ng/mL, R&D Systems), and activin A (6 ng/mL, R&D Systems). On day 3 of differentiation, the EBs were washed with Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) and transferred to basal medium containing inhibitor of Wnt production-3 (IWP3, 1 µM, Stemgent), vascular

endothelial growth factor (VEGF) (10 ng/mL, Bio-Techne), SB431542 (5.4 µM, Sigma), and dorsomorphin (6 µM, Sigma). On day 7 of differentiation, the EBs were transferred to basal medium supplemented with VEGF (5 ng/mL). This basal medium supplemented with VEGF (5 ng/mL) was changed every 3-4 days. After 3 weeks, the cardiomyocytes were stained with APC lineage markers (CD31, CD49a, CD140b, CD90) and signal regulatory protein alpha (SIRPa) antibodies. SIRPa is the previously identified marker for cardiomyocytes derived from iPSCs, and CD31, CD49a, CD140b, and CD90 are surface markers that are non-specific for cardiomyocytes and are expressed by endothelial cells, fibroblasts, smooth muscle cells, and others. Flow cytometry was used to sort cells that were CD31⁻, CD49a⁻, CD140b⁻, CD90⁻, and SIRPa⁺, which were designated as cardiomyocytes.

Genome editing with CRISPR-Cas9 system

A guide RNA (gRNA) specific for the introduction and repair of the target gene was designed using CRISPRdirect (<https://crispr.dbcls.jp/>) and CRISPOR (<http://crispor.gi.ucsc.edu/>). A single-stranded donor DNA (ssDNA) template containing the correct sequence was also designed.

For CRISPR-Cas9 delivery, cells were electroporated with a CRISPR-Cas9 plasmid containing the designed gRNA and the ssDNA template using the 4D-Nucleofector (Lonza). To isolate single-cell clones, cells were plated at low density using the limiting dilution method. Edited clones were then isolated and expanded. Genomic DNA was extracted, and PCR amplification of the target region was performed. Successful gene repair was confirmed by Sanger sequencing.

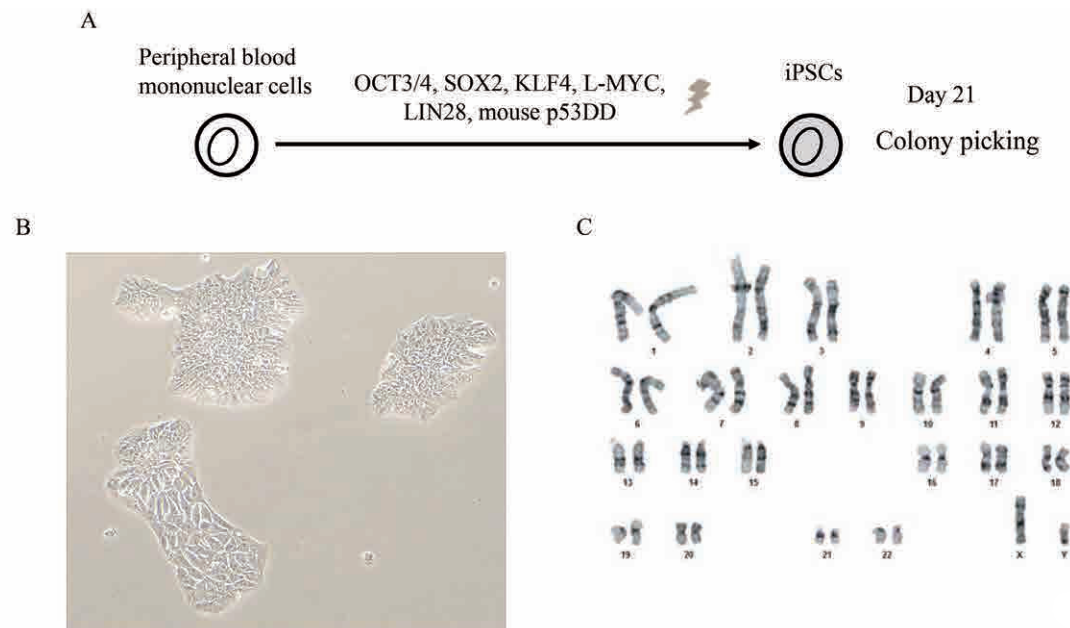


Figure 1 Generation of heterozygous troponin T (TnT)-K210 del mutant (Δ K210/WT) induced pluripotent stem cells (iPSCs). A, Peripheral blood mononuclear cells from a patient with familial dilated cardiomyopathy (DCM) were reprogrammed into iPSCs by electroporation of episomal plasmids encoding the reprogramming genes OCT3/4, SOX2, KLF4, L-MYC, LIN28, and mouse p53DD. After 3 weeks, when the colonies had grown and were visible to the naked eye, iPSCs were picked from some colonies. B, Picked iPSCs were cultured under feeder-free conditions using StemFit AK02N. C, Δ K210/WT iPSCs were confirmed to have normal karyotypes.

Results

Heterozygous troponin T-K210 del mutation in two siblings with familial DCM

In this study, genomic DNA was extracted and analyzed from cardiac biopsy specimens obtained from one sibling and from whole blood obtained from the other. Exome sequencing of genomic DNA extracted from two siblings with familial DCM revealed a deletion of lysine 210 (Δ K210) in one allele of *TNNT2*, which encodes cardiac troponin T (TnT) in both siblings: the heterozygous TnT-K210 del mutation (Δ K210/WT). The presence of this mutation was also confirmed by direct sequencing. No gene mutations were identified in *TTN*, *LMNA*, and *MYH7* that would result in amino acid substitutions or other severe functional impairment.

TnT- Δ K210/WT iPSCs generation

As shown in Figure 1A, iPSCs were generated

from peripheral blood mononuclear cells of a DCM patient with the Δ K210/WT. The Δ K210/WT iPSCs formed colonies. iPSCs were picked from some colonies and their ability to proliferate was confirmed (Figure 1B). Then, the banding pattern of each chromosome in the picked Δ K210/WT iPSCs was analyzed by G-banding analysis and showed normal karyotypes (Figure 1C).

Differentiation of Δ K210/WT iPSCs into cardiomyocytes

As shown in Figure 2A, Δ K210/WT iPSCs were differentiated into cardiomyocytes. Spontaneous beating was observed as early as day 14 after differentiation (Figure 2B). And after 3 weeks, flow cytometry was performed to sort CD31⁺, CD49a⁺, CD140b⁺, CD90⁺, and SIRPα⁺ cells, indicating normally differentiated cardiomyocytes (cardiomyocyte purity > 60%) (Figure 2C). Immunostaining confirmed the expression of TnT

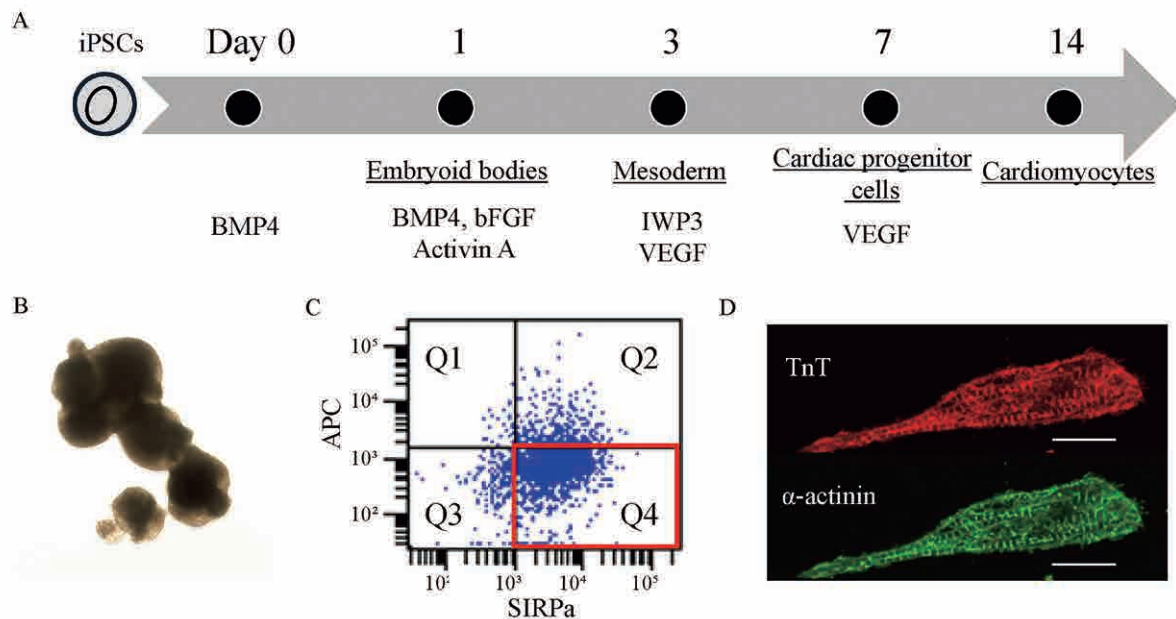


Figure 2 Differentiation of heterozygous troponin T (TnT)-K210 del mutant (Δ K210/WT) induced pluripotent stem cells (iPSCs) into cardiomyocytes. A, Schematic representation of cardiomyocyte differentiation. B, At day 14 post differentiation, spontaneous beating of cardiomyocytes was observed. C, At day 21 post differentiation, cardiomyocytes were stained with lineage markers-APC (CD31, CD49a, CD140b, CD90) and signal regulatory protein alpha (SIRPa) antibodies. Q4 indicates normally differentiated cardiomyocytes that were CD31-, CD49a-, CD140b-, CD90-, and SIRPa+. Cardiomyocyte purity > 60%. D, Immunostaining for sarcomeric TnT and α -actinin at day 21 after differentiation. Cardiomyocytes showed a punctate distribution pattern of sarcomeric TnT and α -actinin. Scale bar: 10 μ m. BMP4, bone morphogenetic protein 4; bFGF, basic fibroblast growth factor; IWP3, inhibitor of Wnt production-3; VEGF, vascular endothelial growth factor.

and α -actinin in these cells (Figure 2D). Δ K210/WT iPSCs-derived cardiomyocytes showed a punctate distribution pattern of sarcomeric TnT and α -actinin, indicating a disorganized myofilament structure.

Genome editing of Δ K210/WT iPSCs

We used these iPSCs to attempt introduction and repair of the TnT-K210 del mutation using the CRISPR-Cas9 system. As shown in Figure 3A, the gRNA recognizes the target DNA sequence and the Cas9 protein cleaves the DNA double strand. This cleavage then initiates repair, allowing the donor DNA sequence to be incorporated at the desired site. The gRNA used for introduction and repair of the TnT-K210 del mutation is shown in Figure 3B. We successfully introduced the TnT-K210 del mutation into Δ K210/WT iPSCs to generate the homozygous

TnT-K210 del mutant (Δ K210/ Δ K210) iPSCs (Figure 4). However, we were unable to repair the TnT-K210 del mutation to generate the WT/WT iPSCs from the Δ K210/WT iPSCs.

Discussion

In this study, we detected the TnT- Δ K210/WT in DCM patients and generated iPSCs, which were differentiated into cardiomyocytes. We also successfully generated the Δ K210/ Δ K210 iPSCs from the Δ K210/WT iPSCs using the CRISPR-Cas9 system. However, we were unable to generate the WT/WT iPSCs from the Δ K210/WT iPSCs. According to CRISPRdirect, the gRNA used to generate the WT/WT iPSCs from the Δ K210/WT iPSCs had no significant specificity issues, and showed relatively high cleavage activity in CRISPOR. However, this

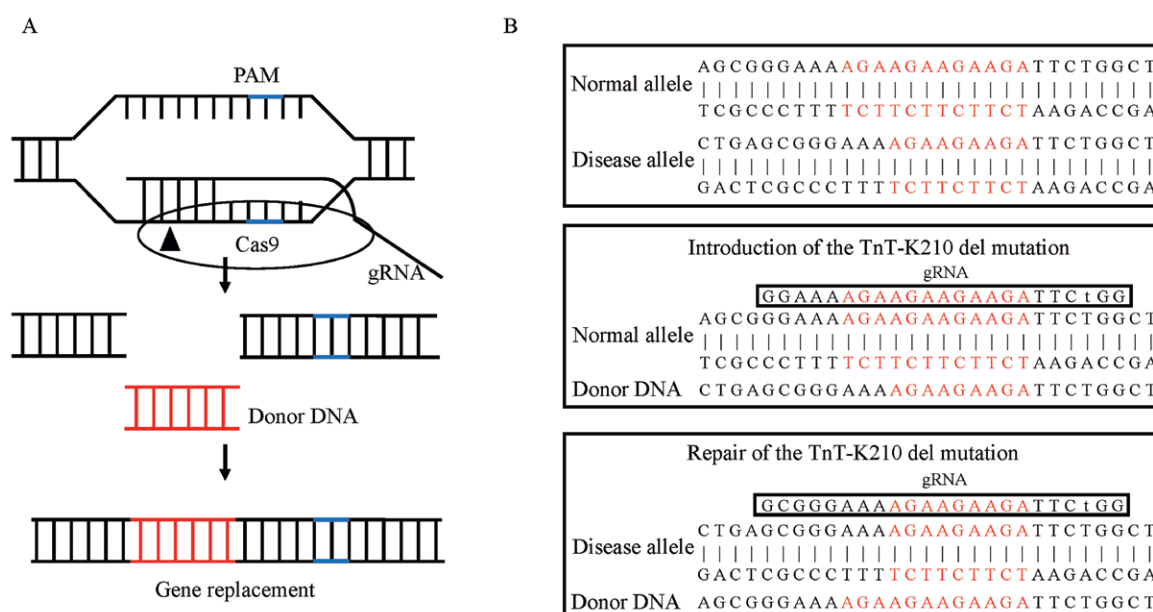


Figure 3 Genome editing of heterozygous troponin T (TnT)-K210 del mutant (Δ K210/WT) induced pluripotent stem cells (iPSCs) using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR-Cas9) system. A, Guide RNA (gRNA) recognizes the target DNA sequence. The Cas9 protein and gRNA complex locate the target site, and the Cas9 protein cleaves the double-stranded DNA. In order for the Cas9 protein to function, it also requires a specific protospacer adjacent motif (PAM) sequence (“NGG”) adjacent to the target site. Donor DNA is used to mend the broken strand, resulting in precise repair. B, gRNA and donor DNA used for introduction and repair the TnT-K210 del mutation.

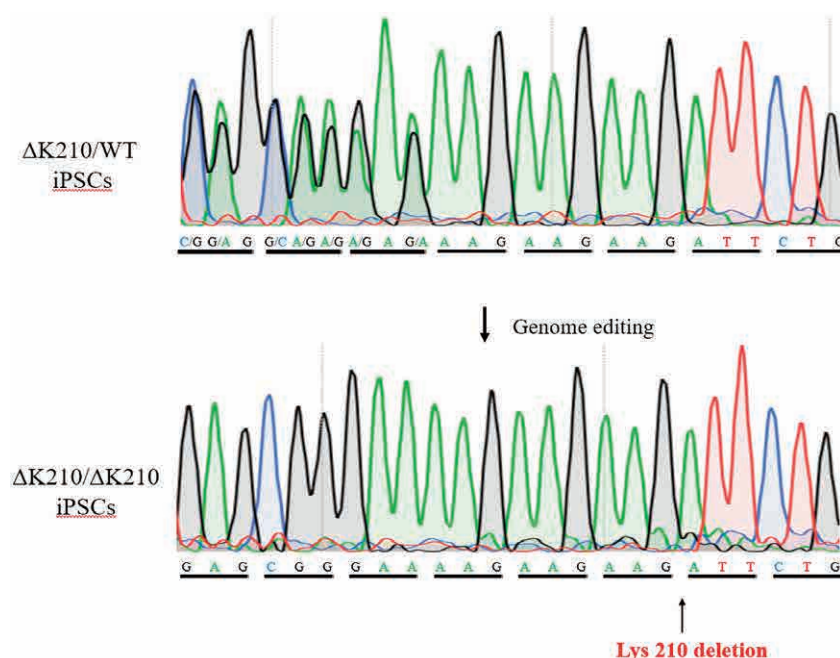


Figure 4 Sequence pattern of the human cardiac troponin T gene (*TNNT2*) detected by Sanger method with a reverse primer. Using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR-Cas9) system, we introduced the troponin T (TnT)-K210 del mutation into heterozygous TnT-K210 del mutant (Δ K210/WT) induced pluripotent stem cells (iPSCs) to generate homozygous TnT-K210 del mutant (Δ K210/ Δ K210) iPSCs. We confirmed an in-frame “AGA” deletion by direct sequencing. Lys, lysine.

gRNA contained one of the motifs described by Graf et al.¹⁰⁾. Specifically, the guide ends with TTC or TTT or contains only T and C in the last four nucleotides, with more than two Ts or at least one TT and one T or C ('TT motif'). In the design of gRNAs for the CRISPR-Cas9 system, the presence of such motifs should be avoided if the objective is to achieve consistent and high gRNA expression. However, only a single protospacer adjacent motif (PAM) sequence was available in the vicinity of this genetic abnormality. Moreover, this gRNA was used in this study due to the absence of any alternative gRNA capable of differentiating between the "AGA" x4 repeat allele and the "AGA" x3 repeat allele. In addition to gRNA, it is essential to consider the concentration of donor DNA, the conditions of electroporation, and the use of iPSCs in the logarithmic growth phase. Despite implementing the requisite modifications to these variables, the generation of WT/WT iPSCs from Δ K210/WT iPSCs was not achieved.

Conversely, we successfully generated Δ K210/ Δ K210 iPSCs from Δ K210/WT iPSCs. The gRNA used in this case had slightly lower specificity according to CRISPRdirect, slightly lower cleavage activity in CRISPOR, and also contained the TT motif. Despite these limitations, this gRNA was able to successfully modify the target genes. While evaluations using CRISPRdirect and CRISPOR are important, experimental verification remains essential.

The TnT-K210 del mutation has been identified in two siblings with familial DCM. This mutation is located in coding exon 12 of the *TNNT2* gene and results in an in-frame AGA deletion between nucleotide positions 629 and 631. This variant was first reported by Kamisago M et al. and was identified in families with familial DCM¹¹⁾. Since then, it has been found repeatedly in unrelated patients and in large, unrelated families with DCM^{4, 12, 13)}. This variant has also been reported to occur *de novo* in

individuals with DCM^{13, 14)}. *In vitro* studies have shown that the decline in cardiac function due to this variant is caused by a decrease in Ca^{2+} sensitivity and ATPase activity^{4, 15)}. It has been reported that mice overexpressing the TnT-K210 del mutation had mild DCM; however, when one allele of *TNNT2* was deleted in these mice to make them heterozygous, the DCM became severe¹⁶⁾.

When the TnT-K210 del mutation was introduced into single membrane-permeabilized cardiomyocytes of left ventricular tissue by exchanging the recombinant troponin complex, troponin I and TnT protein levels were reduced by 51% and 53%, respectively¹⁷⁾. In addition, TnT protein levels were reduced in cardiomyocytes differentiated from human embryonic stem cells carrying the TnT-K210 del mutation¹⁸⁾. The mechanism by which this mutation leads to reduced protein levels remains to be elucidated. However, structural abnormalities in sarcomeres, including a reduction in myofibrils, an increase in disorganized sarcomere configuration, and a punctate cellular distribution of TnT, have been documented in cardiomyocytes that have undergone differentiation from human embryonic stem cells with the TnT-K210 del mutation¹⁸⁾. Although this is a different genetic abnormality, cardiomyocytes differentiated from iPSCs with the TnT-R173W mutation have also been suggested to have difficulty maintaining sarcomeric structure, making them more susceptible to chronotropic stress from norepinephrine¹⁹⁾. This suggests that the sarcomere structure composed of TnT with the mutations may be fragile and that instability of the sarcomere structure may be involved in the reduction of TnT protein levels. This may be an issue to be addressed in future research.

Conclusions

We detected the TnT- Δ K210/WT in DCM

patients and generated iPSCs. Using the CRISPR-Cas9 system, we successfully generated the Δ K210/ Δ K210 iPSCs, but not the WT/WT iPSCs from the Δ K210/WT iPSCs. Further studies are needed to generate WT/WT, Δ K210/WT, and Δ K210/ Δ K210 iPSCs.

Conflicts of Interest

All authors have no conflicts of interest directly related to the content of this article.

Acknowledgments

None.

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