MethodsX 8 (2021) 101404

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Method Article

Production of TRPM4 knockout cell line using rat cardiomyocyte H9c2

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A B S T R A C T

The method presented in this article are related to the research article entitled as "Role of the TRPM4 channel in mitochondrial function, calcium release, and ROS generation in oxidative stress" [1]. TRPM4, a non-selective monovalent cation channel, is not only involved in the generation of the action potential in cardiomyocytes, but also thought to be a key molecule in the development of the ischemia–reperfusion injury of the brain and the heart [2–5]. However, existing pharmacological inhibitors for the TRPM4 channel have problems of non-specificity [6]. This article describes methods used for targeted genomic deletion in the rat cardiomyocyte H9c2 using the CRISPR-Cas9 genome editing system in order to suppress TRPM4 protein expression. Confocal microscopy, flow cytometry, Sanger sequencing, and western blotting are performed to confirm vector transfection and the subsequent knockout of the TRPM4 protein.

- These data provide information on the comprehensive analyses for knocking out the rat TRPM4 channel using CRISPR/Cas9. The analyses include confocal microscopy, flow cytometry, Sanger sequencing, and western blotting.
- This dataset will benefit biological and medical researchers studying the function of TRPM4-expressing cells including neurons, cardiomyocytes, and vascular endothelial cells. It is also useful to study the involvement of the TRPM4 channel in pathological processes such as cardiac arrhythmia and ischemia–reperfusion injury.
- The dataset can be used to guide the experiment of knocking out the TRPM4 gene and its subsequent application to the study of disease process caused by the gene.

DOI of original article: 10.1016/j.bbrc.2021.03.077
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https://doi.org/10.1016/j.mex.2021.101404

ARTICLE INFO
Method name: CRISPR knockout of rat TRPM4 gene
Keywords: TRPM4, Cardiomyocyte, H9c2, CRISPR/Cas9, Confocal microscopy, DNA sequencing, Flow cytometry, Western blotting
Article history: Received 29 March 2021; Accepted 28 May 2021; Available online 29 May 2021

Specifications table

Subject Area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	Cell biology, molecular pathophysiology
Method name:	CRISPR knockout of rat TRPM4 gene
Name and reference of original method:	N.A.
Resource availability:	N.A.

Background

The rat cardiomyocytes cell line H9c2 is derived from embryonic BDIX rat heart tissue [7] and extensively used in cell biology research. It has been revealed that the TRPM4 channel, originally found as an ion channel involved in the development of cardiac arrhythmia, is also associated with the development of cancer such as prostate cancer and large B cell lymphoma [8]. CRISPR/Cas9 provides more convenient methods for genome editing including knock-out than the conventional methods using zinc-finger nucleases and transcription activator-like effector nucleases [9]. The method of knocking out rat TRPM4 channel using CRISPR/Cas9 system can contribute to the development of research in various fields such as cardiovascular diseases and cancer research.

Methods

TRPM4 knockout in the H9c2 rat cardiomyocyte line was achieved using the Guide-it CRISPR-Cas9 System (Clonetech Laboratories, CA, USA) for the cloning and expression of a target single guide RNA (sgRNA). The included vector simultaneously express Cas9, a target specific sgRNA, and tdTomato fluorescent protein.

Designing and cloning of sgRNAs

The target sequence of the rat TRPM4 gene was determined using chopchop server [10], which reduces off-target mutagenesis by determining potential off-target sites with up to three bases mismatches. Of the candidate oligonucleotide sequences, a sequence pair close to the N terminus was chosen: oligo 1: 5′-ccggGGCTGTGAGGGACCACCAGA-3′, oligo 2: 5′-aaacTCTGGTGGTCCCTCACAGCC-3′. These oligonucleotides were ligated to the pGuide-it-tdTomato Vector.

The ligated vector was cloned using competent cells, and a single colony of competent cells was picked, inoculated into LB medium containing ampicillin (final concentration: 100 μg/ml), and incubated with shaking overnight at 37 °C. After purification, the DNA was purified using QIAGEN Plasmid Midi Kit (QIAGEN, Germantown, MD, USA) and then quantified using spectrophotometer (DeNovix DS-11+, Wilmington, DE, USA). Sequencing analysis was conducted on ABI 3130xl (Applied Biosystems, USA) using 500 ng plasmid sample and the Guide-it Sequencing Primer 1 in the Guide-it CRISPR-Cas9 Systems.

Transfection of plasmid vector

H9c2 cells (American Type Cell Culture, Manassas, VA, USA) were cultured on a 6-well plate (10^6 cells/well) in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator (5% CO_2, 20% O_2) at 37 °C. The plasmid vector (2.5 μg) was transfected into H9c2

cells using the Lipofectamine LTX reagent (12 µl) (Thermo Fisher Scientific) with the PLUS reagent (2.5 µl) in culture medium without serum (300 µl in total). Transfected cells were harvested 24 h after transfection via fluorescence-activated cell sorting (FACS Aria III, BD Biosciences, NJ, USA). Single colonies of the transfected H9c2 cells were obtained by limiting dilution using a 96-well plate. After 4 weeks, the colonies were isolated, and the mutation was ascertained by DNA sequencing and western blot analysis.

Optimization of vector transfection protocol

Transfection was confirmed on confocal microscope (Olympus FV1000, Tokyo, Japan) by visualizing tdTomato fluorescence at 2, 4, 6, 18, and 24 h after transfection. After the microscopic observation, cells were detached using trypsin, filtered through a 30 µm CellTrics disposable filter (Sysmex, Kobe, Japan), and then used for flow cytometry analysis and cell sorting (FACS Aria III, BD Biosciences, NJ, USA).

Immunocytochemistry staining of Cas9 protein

For the expression analysis of Cas9 protein, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10 min at room temperature within 24 h after transfection. Cells were then permeabilized and blocked with phosphate buffered saline (PBS) containing 0.3 M glycine, 1% bovine serum albumin, and 0.1% Triton X-100 for 1 h at room temperature.

Next, cells were incubated with anti-CRISPR-Cas9 antibody (1:100 in PBS) (ab191468, Abcam, Tokyo, Japan) overnight at 4 °C. Cells were then incubated with goat anti-mouse Alexa Fluor 488 antibody (1:1,000 in PBS) (A11001, Thermo Fisher Scientific) and 4',6-diamidino-2-phenylindole (1 drop/ml in PBS) (NucBlue Fixed Cell ReadyProbes, Thermo Fisher Scientific) for 1 h at room temperature. Then we performed three-color confocal imaging to visualize the cell nucleus, Cas9 protein, and tdTomato protein.

Sequencing of genomic DNA

Genomic DNA was obtained using PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific). The extracted DNA was amplified by PCR using the following primers: forward primer, 5'-GTGGGTGTGGCTGTGAGGGACCAC-3'; reverse primer, 5'-GTCTCTGTTCCGGACCACTC-3'. PCR reaction mix (50 µl total) was prepared as follows: 25 µl OneTaq Quick-Load 2X Master Mix with Standard Buffer (Cat no: M0486S, New England Biolabs, Tokyo, Japan), 0.5 µl forward primer (20 µM), 0.5 µl reverse primer (20 µM), ~900 ng extracted DNA, and PCR grade water. The PCR reactions were run using the following program: initial denaturation: 94 °C for 30 s; cycling protocol (40 cycles): denaturation 94 °C for 30 s, annealing 59.7 °C for 60 s, elongation 68 °C for 60 s; final extension: 68 °C for 5 min. Amplified PCR products were separated from the primers via electrophoresis on a 2.5% agarose gel and extracted from the gel using the Monarch DNA Gel Extraction Kit (New England Biolabs, Ipswich, MA, USA). DNA sequencing was performed using ABI 3130xl (Applied Biosystems, USA) and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). 20 µl reaction per sample was prepared by adding the purified PCR product (30 ng) and the reverse primer above (3.2 pmol) to the reaction mix (8 µl) provided in the kit. Genetyx software (Genetyx corporation, Tokyo, Japan) was used for the analysis of the sequencing result.

Detection of TRPM4 protein by western blot

Cells were harvested, washed in cold PBS, and lysed on the ice for 5 min with Pierce RIPA Buffer (Thermo Fisher Scientific). Protein concentration was measured using the Protein Assay Rapid Kit (Wako, Japan). Equal amounts of protein (60 µg) from each sample were separated by 4–12% SDS-PAGE (Bolt bis-tris Plus Gel, Thermo Fisher Scientific) and transferred onto an Invitrolon PVDF membrane (Thermo Fisher Scientific). The membrane was incubated with shaking in Blocking One buffer (Nacalai Tesque, Japan) for 1 h at room temperature to block nonspecific binding.

The membrane was cut horizontally at ~60 kDa to separate the higher molecular weight part that contains TRPM4 (134 kDa) from the lower molecular weight part that contains β-actin (43 kDa). Then the membranes were incubated overnight at 4 °C with primary antibodies: mouse anti-β-Actin antibody (1:1,000; Cell signaling technology, Danvers, MA, USA; Cat. no: 3700S) for the lower molecular weight part and rabbit anti-TRPM4 antibody (1:400; Alomone Labs, Jerusalem, Israel; Cat. no: ACC-044) for the higher molecular weight part. The membranes were washed in PBS buffer containing 0.1% Tween-20 (PBS-T) and incubated with HRP-conjugated secondary antibody (Bio-Rad #161-0380; Hercules, CA, USA) at room temperature for 1 h. The secondary antibody dilution factor was 1:1,000 for anti-β-Actin primary antibody and 1:10,000 for anti-TRPM4 primary antibody. The membranes were washed again with PBS-T, and proteins were visualized using an ECL Prime Detection System (GE Healthcare, Chicago, IL, USA) with a Fujifilm LAS-3000 Imager.

The intensity of band luminescence was quantified using ImageJ [11]. Briefly, a rectangle that surrounds the target band was selected, and then the mean intensity of the pixels that had intensity above background was calculated. The mean intensity of the TRPM4 band was divided by that of the β-actin for normalization.

Statistical analysis

Data were presented as the mean ± standard error of the mean and were analyzed using Prism software (version 8.0, Graphpad Software, USA). Comparisons between two groups were conducted using Student's t-test. Results were considered statistically significant for $p < 0.05$.

Results

Expression of the fluorescent marker and Cas9 proteins

As shown in Fig. 1, tdTomato fluorescence was detected on a certain percentage of H9c2 cells transfected with the plasmid expression vector. While the fluorescence was observed throughout the cytoplasm, it was observed especially intensely in the nucleus. Labeling with anti-Cas9 antibody was particularly intense in the nucleus, suggesting the nuclear localization of the Cas9 protein. The flow cytometry analysis indicated that the expression of the tdTomato protein peaked at 6 h and was maintained at relatively higher level until 24 h after the transfection of the plasmid vector (Fig. 2).

DNA sequencing

As shown in Fig. 3, the DNA sequence that encodes TRPM4 gene in non-transfected (WT) cells was identical to that of the rat TRPM4 gene located at chromosome 1, exon 5 (accession: NC_005100.4), except for the thymine at location 101,316,731 which was intentionally replaced by adenine for subsequent knockout analysis using restriction enzyme (data not shown). In contrast, the sequence obtained from the TRPM4-knockout ($TRPM4^{KO}$) cells lacked a guanine at location 101,316,729. This site is precisely four residues after the protospacer adjacent motif (PAM) sequence (shown as "CCG" in Fig. 3, whose reverse complementary sequence is CGG) for Cas9 nuclease, suggesting that this single-residue deletion resulted from cleavage by this enzyme. Of the seven samples obtained in the limited dilution after FACS sorting, deletion of the expected residue was found in two samples (Fig. 3, right).

Assessment of the expression of TRPM4 protein using western blot

Expression of the TRPM4 protein in WT and $TRPM4^{KO}$ cells was assessed using western blot (Fig. 4). TRPM4 expression was significantly lower in $TRPM4^{KO}$ than in WT cells (0.03 ± 0.01 vs. 1.00 ± 0.04, respectively; $^{****}p < 0.0001$).

Limitation

Although the sgRNA was designed to minimize off-target effects, gene mutations due to off-target cleavage should always be considered [12].

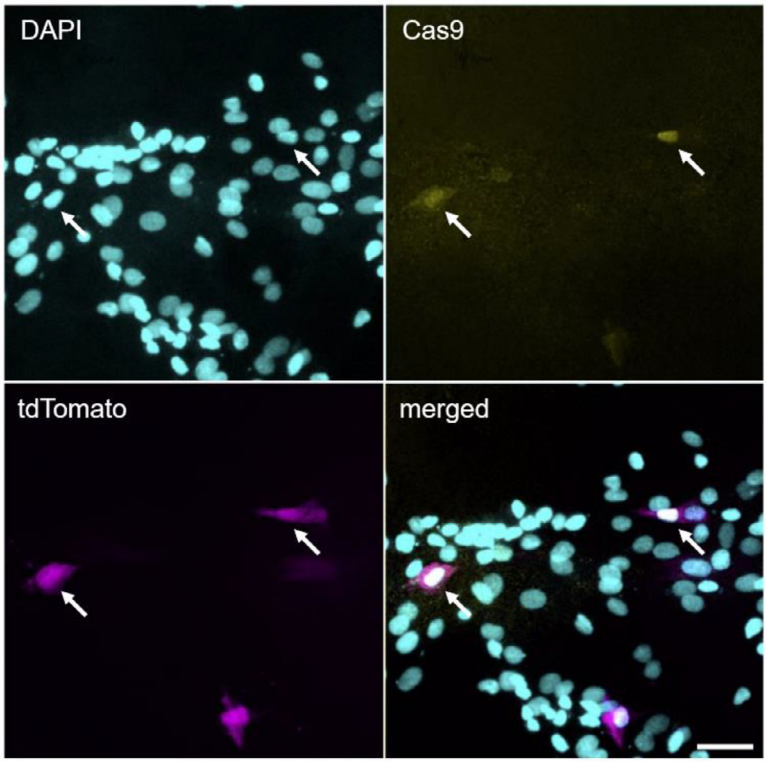

Fig. 1. Simultaneous detection of vector transfection and Cas9 expression. Rat H9c2 cardiomyocytes were labeled with anti-Cas9 antibodies (yellow, top right) and stained with DAPI (cyan, top left). Magenta fluorescence (bottom left) indicates tdTomato fluorescence derived from the transfected plasmid vector. Arrows indicate the cells that express tdTomato and Cas9 proteins simultaneously. Scale: 20 μm.

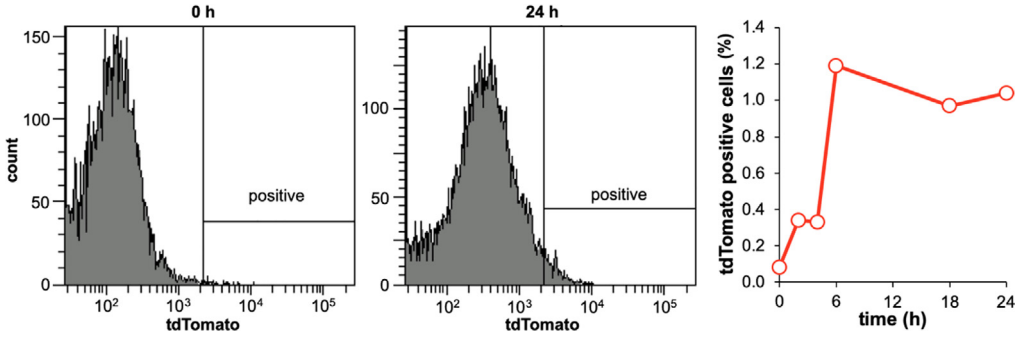

Fig. 2. Time course of the ratio of vector expressing cells. Flow cytometry analysis of tdTomato expression on rat H9c2 cardiomyocytes. The vertical axis of the histogram indicates the number of cells. Samples were obtained 0, 2, 4, 6, 18, and 24 h after transfection of the plasmid vector encoding tdTomato and Cas9 proteins. Right: The ratio of tdTomato positive cells over time after transfection of the expression vector.

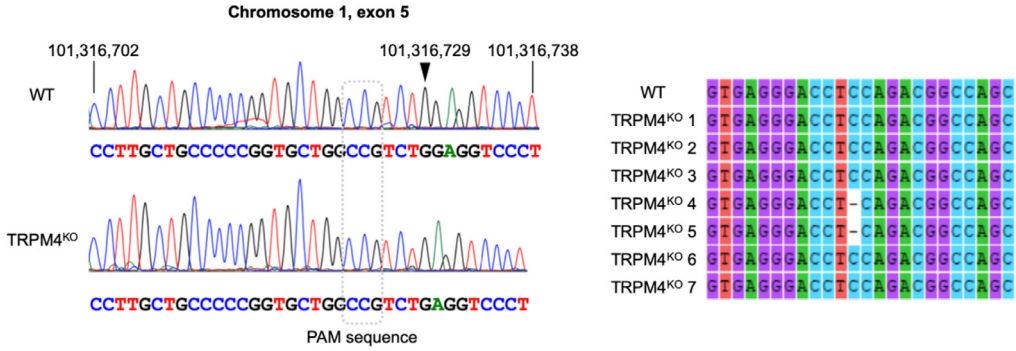

Fig. 3. Comparison of DNA sequence between the WT and TRPM4^{KO} cells. Left: Sequencing chromatographs of genomic DNA samples obtained from WT and TRPM4^{KO} cells. The numbers indicate locations in the rat chromosomal DNA (accession: NC_005100.4). The guanine at site 101,316,729 (arrowhead) is missing in the DNA of TRPM4^{KO} cells. Right: Sequencing results of 8 samples (reverse complemented). The cytosine residue between thymine and cytosine is missing in TRPM4^{KO} 4 and TRPM4^{KO} 5 samples.

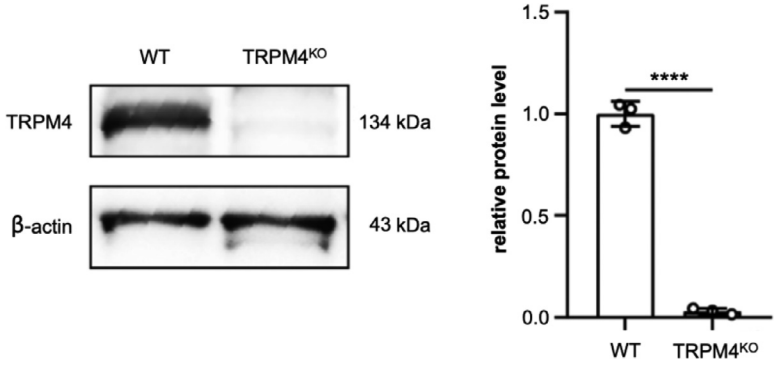

Fig. 4. Detection of TRPM4 protein using western blotting. TRPM4 protein expression was significantly lower in TRPM4^{KO} than in WT cells. β-actin was used as loading control. The TRPM4 expression level was normalized by dividing the band intensity of TRPM4 by that of β-actin. Chart represents average of three independent experiments. Error bars represent SEM. $^{****}p < 0.0001$.

Conclusion

The TRPM4 channels in H9c2 rat cardiomyocytes can be successfully knocked out by transfecting a plasmid vector that simultaneously expresses the sgRNA, Cas9 protein, and the fluorescent marker tdTomato. This method may also be used to knock out the TRPM4 channel in other rat-derived cells.

Acknowledgements

This research was funded by JSPS KAKENHI, Fund for the Promotion of Joint International Research (Fostering Joint International Research), 17KK0168 and JSPS KAKENHI Grant-In-Aid for Scientific Research (B), 20H04518. We thank the Central Research Laboratory, Okayama University Medical School for their technical support for the flow cytometry and the DNA sequencing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] C. Wang, J. Chen, M. Wang, K. Naruse, K. Takahashi, Role of the TRPM4 channel in mitochondrial function, calcium release, and ROS generation in oxidative stress, Biochem. Biophys. Res. Commun. (2020) In Press. Elsevier production reference: YBBRC45815. https://authors.elsevier.com/tracking/article/details.do?aid=45815&jid=YBBRC&surname=Takahashi, doi:10.1016/j.bbrc.2021.03.077.

[2] J.M. Simard, K.T. Kahle, V. Gerzanich, Molecular mechanisms of microvascular failure in central nervous system injury–synergistic roles of NKCC1 and SUR1/TRPM4, J Neurosurg 113 (3) (2010) 622–629 PMID: 20035575; PMCID: PMC3032917, doi:10.3171/2009.11.JNS081052.

[3] J. Wang, K. Takahashi, H. Piao, P. Qu, K. Naruse, 9-Phenanthrol, a TRPM4 inhibitor, protects isolated rat hearts from ischemia-reperfusion injury, PLoS One 8 (7) (2013) e70587 PMID: 23936231; PMCID: PMC3723883, doi:10.1371/journal.pone.0070587.

[4] H. Piao, K. Takahashi, Y. Yamaguchi, C. Wang, K. Liu, K. Naruse, Transient receptor potential melastatin-4 is involved in hypoxia-reoxygenation injury in the cardiomyocytes, PLoS One 10 (4) (2015) e0121703 PMID: 25836769; PMCID: PMC4383534, doi:10.1371/journal.pone.0121703.

[5] C. Wang, K. Naruse, K. Takahashi, Role of the TRPM4 Channel in Cardiovascular Physiology and Pathophysiology., Cells 7 (6) (2018) 62 PMID: 29914130; PMCID: PMC6025450, doi:10.3390/cells7060062.

[6] R. Guinamard, T. Hof, C.A. Del Negro, The TRPM4 channel inhibitor 9-phenanthrol, Br. J. Pharmacol. 171 (7) (2014) 1600–1613 PMID: 24433510; PMCID: PMC3966741, doi:10.1111/bph.12582.

[7] P. Witek, A. Korga, F. Burdan, M. Ostrowska, B. Nosowska, M. Iwan, J. Dudka, The effect of a number of H9C2 rat cardiomyocytes passage on repeatability of cytotoxicity study results, Cytotechnology 68 (6) (2016) 2407–2415 Epub 2016 Mar 5. PMID: 26946144; PMCID: PMC5101311, doi:10.1007/s10616-016-9957-2.

[8] Y. Gao, P. Liao, TRPM4 channel and cancer, Cancer Lett 454 (2019) 66–69 Epub 2019 Apr 10. PMID: 30980865, doi:10.1016/j.canlet.2019.04.012.

[9] H. Wang, M. La Russa, L.S. Qi, CRISPR/Cas9 in genome editing and beyond, Ann. Rev. Biochem. 85 (2016) 227–264 Epub 2016 Apr 25. PMID: 27145843, doi:10.1146/annurev-biochem-060815-014607.

[10] K. Labun, T.G. Montague, J.A. Gagnon, S.B. Thyme, E. Valen, CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering, Nucleic Acids Res. 44 (W1) (2016) W272–W276 Epub 2016 May 16. PMID: 27185894; PMCID: PMC4987937, doi:10.1093/nar/gkw398.

[11] C.T. Rueden, J. Schindelin, M.C. Hiner, B.E. DeZonia, A.E. Walter, E.T. Arena, K.W. Eliceiri, ImageJ2: ImageJ for the next generation of scientific image data, BMC Bioinformatics 18 (1) (2017) 529 PMID: 29187165; PMCID: PMC5708080, doi:10.1186/s12859-017-1934-z.

[12] X.H. Zhang, L.Y. Tee, X.G. Wang, Q.S. Huang, S.H. Yang, Off-target Effects in CRISPR/Cas9-mediated genome engineering, Mol. Ther. Nucleic Acids 4 (11) (2015) e264 PMID: 26575098; PMCID: PMC4877446, doi:10.1038/mtna.2015.37.