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# CRISPR/Cas 9 system for the treatment of dilated cardiomyopathy: A hypothesis related to function of a MAP kinase

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ABSTRACT

Dilated cardiomyopathy (DCM) is a disease with high incidence and mortality rates. Its therapies have one primary goal, which is to minimize symptoms and it has only one effective approach to healing, the heart transplantation. As it is widely associated with genetic causes, the use of gene therapies, such as the CRISPR/Cas9 system, is a promising alternative to treat DCM. For this purpose, it is necessary to analyze possible target genes for this approach and what would be the implications of their use. Here, we hypothesized that cardiac troponin I type 3 interacting kinase (TNI3K), involved with superoxide production in DCM patients, besides other factors, could be a good target for the use of gene editing.

## Introduction

Dilated cardiomyopathy (DCM) is characterized by progressive heart failure, with mortality rates ranging from 16% in 10 years to 80% in five years [1]. It is the most frequent cardiomyopathy (around 90% of all) and its incidence is estimated in 5 to 8 cases per 100,000 people. The preeminent morphological modification in DCM is the dilation of both ventricles, which leads to systolic dysfunction, and in some cases, of atria as well [2]. The main symptoms of DCM are left heart failure with progressive dyspnea, frequently progressing to global heart failure with ascites, hydrothorax and jugular swelling [3]. According to Lipshultz et al. (2013), the primary goal of DCM therapies is to increase survival, mitigate disease progression, minimize risk factors, and alleviate symptoms [1]. Conventional therapies, in addition to stimulating a healthy lifestyle, include pharmacological treatments and surgical interventions; heart transplant has been the only effective treatment for the disease [4]. Furthermore, about 40% of DCM cases are associated with a genetic cause [5,6], with the most frequent mutations being located in genes encoding cytoskeletal proteins (leading to sarcomere disruption), cellular metabolism proteins, and intracellular calcium maintenance proteins [7,8]. In this context, genomic edition systems becomes a promising alternative in the treatment of DCM.

To date, the clustered regularly interspaced short palindromic repeats (CRISPR) system and its associated protein Cas9 has been largely applied in experimental medicine [9–11]. Our group has also highlighted potential applications of this system for cardiovascular diseases [12]. The CRISPR/Cas9 system is a simple and low cost method when compared to existing genomic editing methods, such as plasmid vectors and restriction enzymes. It is based on the adaptation of a process present in the immunity of bacteria that uses a small RNA guide to direct an endonuclease (the Cas9) to a specific location on the DNA [13]. The methodology can repair dysfunctional genes, correcting expression levels, or promoting loss or gain of function [14]. In this way, CRISPR genes are first transcribed into a single-stranded RNA, to be processed into a small CRISPR RNA (crRNA), which directs the nucleolytic activity of Cas9 enzyme to degrade specific nucleic acids [13]. Originated from *Streptococcus pyogenes*, Cas9 is widely used and has been already applied in the genomic edition for several species and cell types [15].

In this scenario, we are proposing that genomic editing based on the CRISPR/Cas9 system could be used for DCM targets. Our hypothesis is that modifications induced by the system can be directed to the disease related genes, causing loss of function in those whose expression is altered in patients with DCM. Such modifications could prevent that ventricular dilation occurs, which would clinically benefit patients suffering from a disease of difficult treatment.

## Hypothesis

When analyzing the possible genes involved in the DCM, some are highlighted for being overexpressed in patients with the disease, being necessary to perform knock-down of the same. Therefore, it is possible

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**Table 1**
Genes whose expression is disturbed and has known effect on susceptibility or cause of dilated cardiomyopathy.

| Gene target | Function | Justification for editing | References |
|---|---|---|---|
| *ACTC1* | Encodes the sarcomeric actin protein, fundamental for the normal functioning of cardiomyocytes. | The single nucleotide polymorphism rs113178069 in exon 1 generates a missense mutation that occurs in a binding domain for alpha-actinin, which anchors the thin filaments | [17] |
| *Calr* | Encodes a calcium-buffering chaperone of the endoplasmic reticulum. It is highly expressed in the embryonic heart and is essential for cardiac development. | Up-regulated expression of calreticulin induces DCM. | [18] |
| *Csq* | Encodes a calcium-binding protein, responsible for calcium storage in the sarcoplasmic reticulum. | The gene is a target for point mutations, whose overexpression lead to DCM | [19,20] |
| *TNNT2* | The troponin T (TnT) proteins are central players in the calcium regulation of actin thin filament function and is essential for the contraction of striated muscles. The isoforms are generate by alternative splicing. *Tnnt2* act on cardiac muscle. | The *Tnnt2* gene polymorphism loci rs3729547 is related to idiopathic DCM. | [21,22] |
| *TNNI3K* | Encodes the cardiac troponin I interacting kinase, the protein responsible for the regulation of troponin I type 3. | When overexpressed or even in its normal expression, it is associated with increased susceptibility to DCM. | [19] |

*ACT1*, Actin, alpha, cardiac muscle 1; *Calr*, calreticulin; *Csq*, calsequestrin; *TNNT2*, cardiac troponin T type-2; *TNNI3K*, troponin I type 3 interacting kinase.

to permanently disrupt gene function by generating a mutation in the sequence, which can be achieved by insertion or deletion generated by Non-homologous end joining (NHEJ) or even by deleting entire DNA fragments due to double cutting of the DNA in sites flanking the target sequence to be excised. The target of this mutation may be an exon or essential domains for protein formation [16]. Some of the possible targets, whose modification would be viable to correct, are listed on Table 1.

According to Wheeler et al. (2009), selective inhibition of some of these genes could slow down the progression of the DCM [19]. In case of cardiac troponin I type 3 interacting kinase (TNNI3K), our main study object, its function corroborates with injury through increased mitochondrial superoxide production and impaired mitochondrial function, which is largely dependent on p38 mitogen-activated protein kinase (MAPK) activation [23]. Thus, Vagnozzi et al. (2013) also showed that inhibition of TNNI3K could reduce oxidative stress, as well as the injurious remodeling in the heart. Thus, when it comes to the targets pointed in the Table 1, our hypothesis is that the gene *tnni3k* would be the most important and suitable for genomic editing experiments. Besides, *tnni3k* gene encodes a protein that belongs to the MAP kinase kinase kinase (MAPKKK) family of protein kinases. The protein contains ankyrin repeat, protein kinase and serine-rich domains, being important in cardiac physiology [24]. Nonetheless, DCM is a disease whose genetic base is caused by modifications that lead to great structural changes in cardiac tissue, and may occur very early during the embryonic stage. Thus, validation experiments end up becoming difficult. In this context, the use of stem cells as the mesenchymal stromal cells (MSC), which are from the mesoderm and able to generate muscle tissue in adulthood, or induced pluripotent stem cells (iPSC), could be of great value to the expression analysis of genomic editing targets [25]. Additionally, for this cell type, both viral vectors [12] and non-viral (plasmids) can be used, as demonstrated by Chicaybam et al. (2017) [26].

## Hypothesis evaluation

To the *TNNI3K* gene, for example, guide RNAs whose targets are exons 4 and 5 will be used, since they have a greater amount of base pairs unique to that gene, generating a deletion of a portion of the gene. Forward and reverse primers will be made for each exon, and, after annealing, they will be cloned into the CRISPR vector pSpCas9(BB)-2A-GFP (PX458). The plasmids will be used to gene modify the target mammalian cells and the deletion can be detected by conducting the Polymerase Chain Reaction (PCR) with primers flanking the deleted region, followed or not by amplicon sequencing. According to the result, it is possible to evaluate whether there was successful editing and whether the clones are homozygous or heterozygous for the deletion [16].

## Implications of the hypothesis

The biggest advantage of using the CRISPR/Cas9 system for editing genes causing DCM and the validation that shows the processes initiated by action or overexpression of protein products can be controlled is the high range of applicability at the clinic. First, because this can be performed in addition to cell therapy, providing “carrier cells” for modified genes and capable to attach to target tissues (in this case, the heart muscle) correcting local processes [27]. Second, because multiplex strategies may be used, where multiple genes could be modified. Ousterout et al. (2015) conducted experiments using the system to modify multiple targets (several exons) on a gene that causes Duchenne Muscular Dystrophy and the modified myoblasts were implanted in mice, showing that the dystrophin was expressed and muscle fibers were formed after 4 weeks [28]. Although, the implications of using CRISPR to provide selective advantage on a tissue or to remove deleterious genes and how this may affect the outcome and efficiency of the editing must be taken into account. Considering a syncytial tissue, as the embryo, for example, it is possible that even after editing, if the cellular product has been shared in the syncytium, the function gain is not seen [29].

The ethical issues involved in using this method are also of importance. Considering the fact that it is a recently developed method, it must be considered if it a safe method to be used. In the case of DCM, for example, the CRISPR/Cas 9 system could be used *in vitro* in the transformation of stem cells or cell lines or in vivo by intramuscular injections of plasmids encoding the CRISPR/Cas 9 system or ribonucleoproteins, such as the protein Cas 9 complexed with the gRNA. In addition, it is also necessary to deliberate whether such a method could be used to treat the disease after the appearance of symptoms, or only prior to the development of clinical aspects, as a form of prevention. The assessment of this aspect must be consistent with the actual screening commonly performed in newborns, which means it should be assessed whether the genetic predisposition is usually detected before or only after the onset of symptoms. It is important to emphasize that DCM, as well as most cardiovascular diseases, is multifactorial, and it is not clear how significant the genetic factor is and whether the CRISPR system would actually be effective in treating the disease or alleviating symptoms. Therefore, many studies are needed to assess how genomic editing can stop or soften the symptoms caused by the disease. Nonetheless, this powerful tools are emerging as a light at the end of the tunnel for this disease.

## Conclusions and perspectives

We believe that the CRISPR/Cas 9-based gene editing is a promising approach to study the function of genes related to cardiovascular diseases. It could be attempted for DCM with the objective of inactivating erroneously expressed genes that are associated to the disease. Thus, our group is currently pursuing to inactivate the function of the *TNNI3K* gene through this method and we will test this intervention in MSC. We hope that the results found may provide benefits to the treatment of DCM.

## Founding

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## Declaration of Competing Interest

None.

## References

[1] Lipshultz SE, Cochran TR, Briston DA, Brown SR, Sambatakos PJ, et al. Pediatric cardiomyopathies: causes, epidemiology, clinical course, preventive strategies and therapies. Future Cardiol 2013;9:817–48.
[2] Burke MA, Cook SA, Seidman JG, Seidman CE. Clinical and mechanistic insights into the genetics of cardiomyopathy. J Am Coll Cardiol 2016;68:2871–86.
[3] Maron BJ, Towbin JA, Thiene G, Antzelevitch C, Corrado D, Arnett D, et al. Contemporary definitions and classification of the cardiomyopathies: an American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention. Circulation 2006;113:1807–16.
[4] Pietra BA, Kantor PF, Bartlett HL, Chin C, Canter CE, Larsen RL, et al. Early predictors of survival to and after heart transplantation in children with dilated cardiomyopathy. Circulation 2012;126:1079–86.
[5] Vatta M, Mohapatra B, Jimenez S, Sanchez X, Faulkner G, Perles Z, et al. Mutations in cypher/ZASP in patients with dilated cardiomyophaty and left ventricular non-compactation. J Am Coll Cardiol 2003;42:2014–27.
[6] Tayal U, Prasad S, Cook SA. Genetics and genomics of dilated cardiomyopathy and systolic heart failure. Genome Med 2017;9:20.
[7] Shaw T, Elliott P, McKenna WJ. Dilated cardyomyophaty: a genetically heterogeneous disease. Lancet 2002;360:654–5.
[8] Towbin JA. Pediatric myocardial disease. Pediatr Clin North Am 1999;46:289–312.
[9] Giau VV, Lee H, Shim KH, Bagyinszky E, An SSA. Genome-editing applications of CRISPR–Cas9 to promote in vitro studies of Alzheimer's disease. Clin Interv Aging 2018;13:221–33.
[10] Miano JM, Zhu QM, Lowenstein CJ. A CRISPR path to engineering new genetic mouse models for cardiovascular research. Arterioscler Thromb Vasc Biol 2016;36(6):1058–75.
[11] Zhen S, Takahashi Y, Narita S, Yang Y-C, Li X. Targeted delivery of CRISPR/Cas9 to prostate cancer by modified gRNA using a flexible aptamer-cationic liposome. Oncotarget 2017;8:9375–87.
[12] Arend MC, Pereira JO, Markoski MM. The CRISPR/Cas9 system and the possibility of genomic edition for cardiology. Arq Bra Cardiol 2017;108:81–3.
[13] Richter C, Chang JT, Fineran PC. Function and regulation of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) systems. Viruses 2012;4:2291–311.
[14] Van Agtmaal EL, André LM, Willemse M, Cumming SA, Van Kessel IDG, Van Den Broek WJAA, et al. CRISPR/Cas9-induced (CTG·CAG)n repeat instability in the myotonic dystrophy type 1 locus: implications for therapeutic genome editing. Mol Ther 2017;25:24–43.
[15] Hsu PD, Lander ES, Zhang F. Development and application of CRISPR-Cas9 for Genome Engineering. Cell 2014;157:1262–78.
[16] Addgene. CRISPR 101: A desktop resource. 1st edition. Jan 2016.
[17] Olson TM, Michels VV, Thibodeau SN, Tai YS, Keating MT. Actin mutations in dilated cardiomyopathy, a heritable form of heart failure. Science 1998;280:750–2.
[18] Lee D, Oka T, Hunter B, Robinson A, Papp S, Nakamura K, et al. Calreticulin induces dilated cardiomyopathy. PLoS ONE 2013;8:e56387.
[19] Wheeler FC, Tang H, Marks OA, Hadnott TN, Chu PL, Mao L, et al. Tnni3k modifies disease progression in murine models of cardiomyopathy. PLoS Genet 2009;5:e1000647.
[20] Hwang JJ, Allen PD, Tseng GC, Lam CW, Fananapazir L, Dzau VJ, et al. Microarray gene expression profiles in dilated and hypertrophic cardiomyopathic end-stage heart failure. Physiol Genomics 2002;10:31–44.
[21] Li Y-D, Ji Y-T, Zhou X-H, Li H-L, Zhang H-T, Xing Q, et al. TNNT2 Gene polymorphisms are associated with susceptibility to idiopathic dilated cardiomyopathy in Kazak and Han Chinese. Med Sci Monit 2015;21:3343–7.
[22] Wei B, Jin J-P. TNNT1, TNNT2, and TNNT3: isoform genes, regulation, and structure-function relationships. Gene 2016;582:1–13.
[23] Vagnozzi RJ, Gatto GJ, Kallander LS, Hoffman NE, Mallilankaraman K, Ballard VLT, et al. Inhibition of the cardiomyocyte-specific kinase TNNI3K limits oxidative stress, injury, and adverse remodeling in the ischemic heart. Sci Transl Med 2013;5:207ra141.
[24] Zhao Y, Meng XM, Wei YJ, et al. Cloning and characterization of a novel cardiac-specific kinase that interacts specifically with cardiac troponin I. J Mol Med 2003;81:297–304.
[25] Yang J, Al-Aama M, Stojkovic M, Keavney B, Trafford A, Lako M, et al. Concise review: cardiac disease modeling using induced pluripotent stem cells. Stem Cells 2015;33:2643–51.
[26] Chicaybam L, Barcelos C, Peixoto B, Carneiro M, Limia CG, Redondo P, et al. An efficient electroporation protocol for the genetic modification of mammalian cells. Front Bioeng Biotechnol 2017;4:99.
[27] Motta BM, Pramstaller PP, Hicks AA, Rossini A. The impact of CRISPR/Cas9 technology on cardiac research: from disease modelling to therapeutic approaches. Stem Cells Int 2017;2017:8960236.
[28] Ousterout DG, Kabadi AM, Thakore PI, Majoros WH, Reddy TE, Gersbach CA. Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause duchenne muscular dystrophy. Nature Comm 2015;6:6244.
[29] Liang P, Xu Y, Zhang X, et al. CRISPR/Cas9-mediated geneediting in human tripronuclear zygotes. Protein Cell 2015;6:363–72.