Brief Report

The Genetic Mutation of ANO5 in Rabbits Recapitulates Human Cardiomyopathy

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Abstract: The limb girdle muscular dystrophy type 2L (LGMD2L) is caused by mutations of the ANO5 gene in humans which encodes a 913 amino-acid integral membrane protein. Although cardiomyopathy has been reported in patients with an ANO5 mutation, the ANO5 mutant mice did not recapitulate this phenotype in previous studies. This study demonstrated that the ANO5−/− rabbits recapitulated the typical signs of cardiomyopathy with decreased ejection fraction (EF) and fraction shortening (FS) with increased interstitial fibrosis. This ANO5−/− rabbit model would promote basic research to comprehend the pathogenesis and mechanism of ANO5-related cardiomyopathy.

Keywords: CRISPR/Cas9; anoctamin 5; cardiomyopathy; rabbit

1. Introduction

The ANO5 gene, which encodes a 913 amino acid integral membrane protein, is highly expressed in skeletal muscle, cardiac muscle, and bone tissues [1–4]. It has been reported that the recessive mutations of ANO5 (ANO5−/−) are associated with limb-girdle muscular dystrophy 2L (LGMD2L), the characteristics of which resemble that of dysferlinopathies; gnathodiaphyseal dysplasia, bone fragility, cortical thickening, and sclerosis of tubular bone diaphysis; and also cardiomyopathy in previous studies [5–7].

Ten anoctamin family members have been identified so far with important functions in various physiological process [8–10]. Due to the structural similarity between the ANO5 gene and other anoctamin proteins, it was proposed that ANO5 may have function of Ca^{2+}-activated Cl− channels (CaCC). Recently, some anoctamins, including ANO1, ANO2, ANO8, and ANO9, have been linked to the regulation of CaCC activities; however, other anoctamins, such as ANO3 and ANO7, which are short on CaCC activities, have localization in cellular activities [6,11,12]. This suggests that the anoctamin family could possess different functional properties among different family members and also that the molecular and cellular mechanisms of the ANO5 gene still remains unclear.

Previous studies have reported that ANO5 knockout (KO) mice have different pathological manifestations. The ANO5 gene trapped mouse model shows signs of muscular dystrophy [13] without any obvious cardiomyopathy [14], reminiscent of the phenotype of LGMD2L patients. Currently, there are no ideal animal models to study the cardiomyopathy phenotype reported in ANO5-mutated patients, and also the cellular functional studies of the ANO5 function in skeletal muscle and myocardium still need to be further confirmed. Moreover, rabbits may be more popular animal models compared to mice in simulating some human diseases and have higher similarity with human beings in some respects, such as genetics, physiology, and anatomy, compared to mice [15].
Therefore, the phenotype of cardiomyopathy was studied using ANO5−/− rabbits generated in our previous study [16]. Our data demonstrate that the typical phenotype of cardiomyopathy was identified in this ANO5−/− rabbit model for the first time.

2. Materials and Methods

2.1. Animal and Ethics Statement

The ANO5 gene editing rabbits were generated by the CRISPR/Cas9 system in our laboratory [16] and kept at the Jilin University Laboratory Animal Centre.

2.2. Genotyping of Rabbits

Genotyping of ANO5 KO rabbits was performed according to the manufacturer’s instructions as described previously [17,18]. Briefly, the exon of the ANO5 gene was amplified by PCR and Sanger sequenced for rabbits’ mutations. The obtained sequences were compared with the corresponding reference sequence and analyzed using Snapgene (NC_013669.1). The same set of PCR primer was used as in our previous study [16].

2.3. Histology Analysis

The tissue sample of cardiac muscle was collected from ANO5+/−, ANO5−/−, and wild-type (WT) rabbits (15 months of age). Histology analysis was performed as per a previous study [16]. The 5 µm sections for HE, Masson’s trichrome, and Van Gieson staining were performed as described previously [19,20]. The pictures of stained sections were captured by a Nikon TS100 microscope.

2.4. Echocardiography

Echocardiography was carried out as described previously [21,22]. The cardiac dimensions left ventricular end-diastolic diameter (LVDd), the percentage of fractional shortening (FS), and left ventricular ejection fraction (EF) were determined.

2.5. Statistical Analysis

The data were statistically analyzed by GraphPad prism 8.0.2 (t-test), and p < 0.05 was used as statistically significant, * p < 0.05.

3. Results

3.1. The Breeding and Genotyping of ANO5 Gene-Edited Rabbits

The ANO5 KO rabbits were generated in our previous study [16]. To breed ANO5 gene-edited rabbits, the heterozygous male was mated with heterozygous female ANO5 gene-edited rabbit (Figure S1A). The genotyping PCR result showed that 4 ANO5+/− and 6 ANO5−/− pups were generated in this study (Figure S1B).

3.2. Pathological Changes of Cardiac in the ANO5−/− Rabbits

To determine whether the disruption of ANO5 in rabbits induces the typical phenotype of cardiomyopathy, the histological and functional cardiac changes were evaluated and compared between gene-edited ANO5 (ANO5+/− and ANO5−/−) and WT rabbits. The results showed that the increased interstitial fibrosis was determined in ANO5−/− rabbits, but there was no significant difference in ANO5+/− rabbits compared to WT control at the age of 15 months (Figure 1A); 6 month old ANO5−/− rabbits did not show significant differences compared to WT control (Figure S2). In addition, the echocardiography result showed the significantly increased diastolic diameter of the left ventricle (LVDD), while EF and FS decreased in the ANO5−/− rabbits, compared to the WT controls (Figure 1B–E).
These data suggest that the typical phenotype of cardiomyopathy was identified in the ANO5−/− rabbit models.

4. Discussion

The high expression of the ANOS5 gene in human skeletal muscle, heart muscle, and bone [1–3]. Together with cardiomyopathy was reported in ANOS5-deficient patients [7,23]. In our study, the ANO5−/− rabbits exhibited cardiomyopathy at 15 months as demonstrated by histological and functional cardiac analysis, while there is no obvious cardiomyopathy with normal thickness of the interventricular septum and cardiac functions in the ANO5−/− mouse [13,14]. The 15 month old ANO5−/− rabbits showed cardiac changes close to ANO5-deficiency patients (age 20–50) [24] and mice (age 6 months or later) [13] with the only symptoms muscular dystrophy typically appearing. Interestingly, the function of cardiac appeared abnormal by reduced left ventricular EF and FS. However, in some patients with cardiac arrhythmia [24], while the echocardiogram displayed LV dilatation and LV dysfunction as seen in other patients, but dilated cardiomyopathy may be a complication in muscles of ANO5-deficient patients [7].

There are some differences between mice and rabbits, which may trigger the performance of some aspects of the genetic disorder. The type of natural mutations is crucial for different consequences in animal models of different species. The rabbits, with a small indel in exon 12 were used to perform the
determination of cardiac pathology. The CRISPR-induced indels within the exon 12 or 13 of the ANO5 gene lead to the development of pathological alterations in various muscles and cardiac changes of the rabbit, resembling human patients with ANO5 mutations [25,26]. In contrast, mice with exon 1 or exon 2 deletion did not develop muscle and cardiac phenotypes [14] which may illustrate the different consequences of cardiac pathology in ANO5 mutant models.

To our knowledge, this novel animal model which recapitulates human cardiomyopathy will be beneficial for studying the potential impact of the disruption of ANO5 on cardiac changes and would also promote understanding of the pathogenesis mechanism of ANO5-related cardiomyopathy in the future.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/14/4976/s1. Figure S1: The generation of F1 carrying ANO5 mutant rabbits; Figure S2: The histological of WT and ANO5−/− at the age of 6 months.

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Conflicts of Interest: The authors declare no conflict of interest.

References
1. Tsutsumi, S.; Kamata, N.; Vokes, T.J.; Maruoka, Y.; Nakakuki, K.; Enomoto, S.; Omura, K.; Amagasa, T.; Nagayama, M.; Saito-Ohara, F.; et al. The novel gene encoding a putative transmembrane protein is mutated in gnathodiaphyseal dysplasia (GDD). *Am. J. Hum. Genet.* 2004, 74, 1255–1261. [CrossRef] [PubMed]
2. Mizuta, K.; Tsutsumi, S.; Inoue, H.; Sakamoto, Y.; Miyawaki, K.; Noji, S.; Kamata, N.; Itakura, M. Molecular characterization of GDD1/TMEM16E, the gene product responsible for autosomal dominant gnathodiaphyseal dysplasia. *Biochem. Biophys. Res. Commun.* 2007, 357, 126–132. [CrossRef] [PubMed]
3. Stohr, H.; Berger, C.; Frohlich, S.; Weber, B.H. A novel gene encoding a putative transmembrane protein with two extracellular CUB domains and a low-density lipoprotein class A module: Isolation of alternatively spliced isoforms in retina and brain. *Gene* 2002, 286, 223–231. [CrossRef]
4. Di Zanni, E.; Gradogna, A.; Schole-Starke, J.; Boccaccio, A. Gain of function of TMEM16E/ANO5 scrambling activity caused by a mutation associated with gnathodiaphyseal dysplasia. *Cel. Mol. Sci. CMLS* 2018, 75, 1657–1670. [CrossRef] [PubMed]
5. Bolduc, V.; Marlow, G.; Boycott, K.M.; Saleki, K.; Inoue, H.; Kroon, J.; Itakura, M.; Robitaille, Y.; Parent, L.; Baas, F.; et al. Recessive mutations in the putative calcium-activated chloride channel Anoctamin 5 cause proximal LGMD2L and distal MMD3 muscular dystrophies. *Am. J. Hum. Genet.* 2010, 86, 213–221. [CrossRef]
6. Duran, C.; Qu, Z.; Osunkoya, A.O.; Cui, Y.; Hartzell, H.C. ANOs 3-7 in the anoctamin/Tmem16 C/-channel family are intracellular proteins. *Am. J. Physiol. Cell Physiol.* 2012, 302, C482–C493. [CrossRef] [PubMed]
7. Wahbi, K.; Behin, A.; Becane, H.M.; Leturcq, F.; Cossee, M.; Laforet, P.; Stojkovic, T.; Carlier, P.; Toussaint, M.; Gaxotte, V.; et al. Dilated cardiomyopathy in patients with mutations in anoctamin 5. *Int. J. Cardiol.* 2013, 168, 76–79. [CrossRef]
8. Manoury, B.; Tamulevicute, A.; Tammaro, P. TMEM16A/anoctamin 1 protein mediates calcium-activated chloride currents in pulmonary arterial smooth muscle cells. *J. Physiol.* 2010, 588, 2305–2314. [CrossRef]
9. Jia, L.; Liu, W.; Guan, L.; Lu, M.; Wang, K. Inhibition of Calcium-Activated Chloride Channel ANO1/TMEM16A Suppresses Tumor Growth and Invasion in Human Lung Cancer. *PloS ONE* 2015, 10, e0136584. [CrossRef]
10. Wong, X.M.; Younger, S.; Peters, C.J.; Jan, Y.N.; Jan, L.Y. Subduced, a TMEM16 family Ca(2)(+)-activated Cl(-)channel in Drosophila melanogaster with an unexpected role in host defense. *eLife* 2013, 2, e00862. [CrossRef]
11. Schreiber, R.; Uliyakina, I.; Kongsuphol, P.; Warth, R.; Mirza, M.; Martins, J.R.; Kunzelmann, K. Expression and function of epithelial anoctamins. *J. Biol. Chem.* 2010, 285, 7838–7845. [CrossRef] [PubMed]
12. Kim, J.H.; Kim, K.; Kim, I.; Seong, S.; Kim, S.W.; Kim, N. Role of anoctamin 5, a gene associated with gnathodiaphyseal dysplasia, in osteoblast and osteoclast differentiation. *Bone* 2019, 120, 432–438. [CrossRef] [PubMed]
13. Griffin, D.A.; Johnson, R.W.; Whitlock, J.M.; Pozsgai, E.R.; Heller, K.N.; Grose, W.E.; Arnold, W.D.; Sahenk, Z.; Hartzell, H.C.; Rodino-Klapac, L.R. Defective membrane fusion and repair in Anoctamin5-deficient muscular dystrophy. *Hum. Mol. Genet.* 2016, 25, 1900–1911. [CrossRef]
14. Xu, J.; El Refaey, M.; Xu, L.; Zhao, L.; Gao, Y.; Floyd, K.; Karaze, T.; Janssen, P.M.; Han, R. Genetic disruption of Ano5 in mice does not recapitulate human ANO5-deficient muscular dystrophy. *Skelet. Muscle* 2015, 5, 43. [CrossRef] [PubMed]
15. Yan, Q.; Zhang, Q.; Yang, H.; Zou, Q.; Tang, C.; Fan, N.; Lai, L. Generation of multi-gene knockout rabbits using the Cas9/gRNA system. *Cell Regen.* 2014, 3, 12. [CrossRef]
16. Sui, T.; Xu, L.; Lau, Y.S.; Liu, D.; Liu, T.; Gao, Y.; Lai, L.; Han, R.; Li, Z. Development of muscular dystrophy in a CRISPR-engineered mutant rabbit model with frame-disrupting ANO5 mutations. *Cell Death Dis.* 2018, 9, 609. [CrossRef]
17. Sui, T.; Lau, Y.S.; Liu, D.; Liu, T.; Xu, L.; Gao, Y.; Lai, L.; Li, Z.; Han, R. A novel rabbit model of Duchenne muscular dystrophy generated by CRISPR/Cas9. *Dis. Models Mech.* 2018, 11. [CrossRef]
18. Simurda, T.; Zolkova, J.; Snahnicanova, Z.; Loderer, D.; Skornova, I.; Sokol, J.; Hudecek, J.; Stasko, J.; Lasabova, Z.; Kubisz, P. Identification of Two Novel Fibrinogen Bbeta Chain Mutations in Two Slovak Families with Quantitative Fibrinogen Disorders. *Int. J. Mol. Sci.* 2017, 19. [CrossRef]
19. Sui, T.; Yuan, L.; Liu, H.; Chen, M.; Deng, J.; Wang, Y.; Li, Z.; Lai, L. CRISPR/Cas9-mediated mutation of PHEX in rabbit recapitulates human X-linked hypophosphatemia (XLH). *Hum. Mol. Genet.* 2016, 25, 2661–2671. [CrossRef] [PubMed]
20. Bergmann, B.; Molne, J.; Gjertsson, I. The Bone-Inflammation-Cartilage (BIC) Stain: A Novel Staining Method Combining Safranin O and Van Gieson’s Stains. *J. Histochem. Cytochem.* 2015, 63, 737–740. [CrossRef] [PubMed]
21. Sui, T.; Liu, D.; Liu, T.; Xu, L.; Gao, Y.; Lai, L.; Li, Z.; Han, R. LMNA-mutated Rabbits: A Model of Premature Aging Syndrome with Muscular Dystrophy and Dilated Cardiomyopathy. *Aging Dis.* 2019, 10, 102–115. [CrossRef] [PubMed]
22. Kane, A.M.; DeFrancesco, T.C.; Boyle, M.C.; Malarkey, D.E.; Ritchey, J.W.; Atkins, C.E.; Cullen, J.M.; Kornegay, J.N.; Keene, B.W. Cardiac structure and function in female carriers of a canine model of Duchenne muscular dystrophy. *Res. Vet. Sci.* 2013, 94, 610–617. [CrossRef] [PubMed]
23. Witting, N.; Duno, M.; Petri, H.; Krag, T.; Bundgaard, H.; Kober, L.; Vissing, J. Anoctamin 5 muscular dystrophy in Denmark: Prevalence, genotypes, phenotypes, cardiac findings, and muscle protein expression. *J. Neurol.* 2013, 260, 2084–2093. [CrossRef]
24. Liewluck, T.; Winder, T.L.; Dimberg, E.L.; Crum, B.A.; Heppelmann, C.J.; Wang, Y.; Bergen, H.R., 3rd; Milone, M. ANO5-muscular dystrophy: Clinical, pathological and molecular findings. *Eur. J. Neurol.* 2013, 20, 1383–1389. [CrossRef] [PubMed]
25. Hicks, D.; Sarkozy, A.; Muela, N.; Koehe, K.; Huebner, A.; Hudson, G.; Chinnery, P.F.; Barresi, R.; Eagle, M.; Polvokoski, T.; et al. A founder mutation in Anoctamin 5 is a major cause of limb-girdle muscular dystrophy. *Brain J. Neurol.* 2011, 134, 171–182. [CrossRef] [PubMed]
26. Savarese, M.; Di Fruscio, G.; Tasca, G.; Ruggiero, L.; Janssens, S.; De Bleecker, J.; Delpach, M.; Musumeci, O.; Toscano, A.; Angelini, C.; et al. Next generation sequencing on patients with LGMD and nonspecific myopathies: Findings associated with ANO5 mutations. *Neuromuscul. Disord. NMD* 2015, 25, 533–541. [CrossRef]