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Insights into restrictive cardiomyopathy from clinical and animal studies

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Abstract

Cardiomyopathies are diseases that primarily affect the myocardium, leading to serious cardiac dysfunction and heart failure. Out of the three major categories of cardiomyopathies (hypertrophic, dilated and restrictive), restrictive cardiomyopathy (RCM) is less common and also the least studied. However, the prognosis for RCM is poor as some patients dying in their childhood. The molecular mechanisms behind the disease development and progression are not very clear and the treatment of RCM is very difficult and often ineffective. In this article, we reviewed the recent progress in RCM research from the clinical studies and the translational studies done on diseased transgenic animal models. This will help for a better understanding of the mechanisms underlying the etiology and development of RCM and for the design of better treatments for the disease.

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1 Introduction

Restrictive cardiomyopathy (RCM) is a disease of the cardiac muscle that is characterized by restricted ventricular filling due to a high ventricular stiffness. Along with other cardiomyopathies which include dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM), RCM is responsible for severe cardiac dysfunction.[1] While RCM cases are less common than both HCM and DCM and represent about 2%−5% of all pediatric cardiomyopathies, they have the worst prognosis and are associated with cases of sudden death.[2−6] The incidence of mortality is very high in RCM pediatric patients with 50% of deaths occurring within 2 years after diagnosis.[7] So far, no effective treatment is available for RCM [8].

Most RCM cases are described as idiopathic RCM, i.e., etiology is unknown.[9,10] Recently, with the advancement of molecular biology, genetic factors, particularly mutations in sarcomeric protein genes, have been associated with the disease. Several case reports have described familiar RCM in childhood and most reported inherited RCM cases are described as autosomal dominate patterns.[11−15] Such finding allows the screening of individuals and families and permits the identification of people or groups most susceptible to the development of the disease. In general, in the case of DCM, heart failure is characterized by a systolic dysfunction (i.e., reduced ejection fraction), whereas HCM and RCM are associated with diastolic dysfunction (i.e., impaired relaxation).[16] Nevertheless, different mutations in the same sarcomeric gene can lead to different phenotypes such as HCM, DCM or RCM suggesting that many complex processes are involved in the manifestation of these diseases.[17] In addition, a significant heterogeneity of RCM manifestation has been clinically observed in RCM patients. It is difficult to find the clues for these puzzles because of the technical difficulties of obtaining cardiac samples from the patients to determine the gene mutations (single gene or multi genes) responsible for the disease and the incorporation rate of the mutant myofibril proteins in the heart. Recently, transgenic animal models have been generated to mimic RCM by expressing the mutant myofibril proteins observed in human patients.[18,19] The development of animal models of these single-gene disorders will undoubtedly improve our understanding of the underlying mechanisms of cardiomyopathies and provide us with clues for the prevention and treatment of these diseases. In this review paper, we will discuss the clinical manifestation of RCM and the implication of transgenic mouse models to RCM studies.

2 Clinical manifestations of RCM

2.1 Characteristics of RCM

Unlike HCM and DCM, which can be recognized based

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on cardiac morphological changes, RCM is mainly defined by its cardiac hemodynamic characteristics. The restricted heart usually presents normal systolic function with the EF usually above 50%. The main impairment occurs in the diastolic function of the heart though the systolic function may deteriorate at the later stage of the disease.[6] The RCM heart is less compliant (more stiffness) which means that increase of pressure in the ventricles leads to only minor increase of volume. The RCM phenotype resembles a lot that of constrictive pericarditis (CP) which is a surgically treatable cardiac disease resulting from a thickened and calcified pericardium. The RCM heart usually undergoes bi-atrial enlargement due to a chronic elevation of the atrial pressure in the restricted heart.[12,20] Such atrial dilatation can be worsened when there is mitral and tricuspid regurgitation.[21] Enlargement of ventricular cavities and increase in the septal and ventricular wall thickness, which are characteristics of DCM and HCM respectively, barely occur in RCM.[7] The American Heart Association defines RCM as restrictive filling with non-hypertrophied, non-dilated ventricles, normal or reduced diastolic volumes, normal or near-normal systolic function.[22]

2.2 Diagnosis

Clinical presentations of RCM are greatly heterogeneous. It is almost impossible to suspect RCM just according to patients' complaints. Patients referred to cardiologists might have various symptoms, or might not be aware of any cardiac anomaly until subjected to cardiac examination based on their family history or upon the detection of visible abnormalities following a well-being checkup. For youngsters and adults, dyspnea is the major complaint (71%), followed by edema, palpitation, fatigue, orthopnea and chest pain. As for the pediatric population, the most common initial presentation of the disease is pulmonarily related (47%), and these patients were not referred to a cardiologist until when cardiomegaly was noted on chest X-ray. An abnormal physical examination (such as murmurs, a gallop rhythm, loud P2, edema, ascites, and hepatomegaly) is the second reason for referring a child to a cardiologist. Syncope, palpitation, fatigue, chest pain are less than 9% among this population. These conclusions are drawn based on two studies on large series of idiopathic RCM patients: the study by Ammash *et al*.[10] that included 94 patients (age 10-90 years old) and the study by Denfield *et al*.[23] who reviewed 79 pediatric patients.

In spite of the various referring reasons, generally, clinical diagnosis of RCM is largely based on echocardiography supported by electrocardiogram (ECG), cardiac catheterization (evaluate hemodynamics), and endomyocardial biopsy. Sometimes, coronary angiography is performed to evaluate coronary artery structure and myocardial perfusion. The typical demonstrations of RCM in echocardiography are (1) marked enlargement of atria; (2) normal or reduced left ventricular end diastolic dimension (LVEDD) and/or volume (LVEDV); (3) normal ventricular wall thickness; (4) restrictive left ventricular filling pattern evidenced by increased ratio of early diastolic filling to atrial filling (E/A ratio); and (5) normal or near normal systolic function indicated by left ventricular fractional shortening (FS) or EF. However, some cases may present decreased or reversed E/A ratio and/or increased isovolumetric relaxation time (IVRT), indicating impaired relaxation. Among these observations, prolonged IVRT is the earliest manifestation of impaired relaxation ahead of the development of typical restrictive physiology.[24,25] Even though the systolic function is initially preserved in RCM, this parameter can deteriorate with disease progression. Therefore, RCM patients may already present abnormal systolic function upon clinical presentation and may be not classified as such due to the disparity between their symptoms and the typical manifestation of RCM. Thus, those patients were usually excluded from the retrospective studies of RCM.[10,24,26–28] Since most of the available clinical information of RCM is based on retrospective studies, the exclusion may set a bias to the study on disease progression.

Some RCM hearts may present mild or moderate hypertrophic ventricles with restrictive physiology. Actually there is uncertainty whether to classify them into RCM or HCM. For retrospective studies of RCM, some groups excluded mild or moderate hypertrophied hearts while others did not. In some of the prospective studies and case reports, initially diagnosed RCM patients could develop hypertrophy at the late stage of the disease while in others, some patients presenting mild or asymmetrically hypertrophied ventricles might have received a RCM diagnosis.[29] Additionally, members in cardiomyopathy families carrying the same disease-causing genetic mutation could all present with restrictive physiology but with presence of hypertrophy in some patients and its absence in others.[15,27,29] Furthermore, while hypertrophied myocytes, fibrosis and myofiber disarray are characteristics of HCM histopathology and usually used as diagnostic criteria, those histological changes can also be observed in typical RCM patients without identifiable hypertrophy via echocardiography. According to a study on one of the largest RCM population (endomyocardial biopsy performed on 33 patients), interstitial fibrosis was observed in 81% of idiopathic RCM patients while 86% of them had myocytes hypertrophy.[10]

Actually, varied degree of interstitial fibrosis, with or without myocytes hypertrophy or disarray are common in both old and young patients and in all clinical studies we reviewed. However, questions still exist as regard to whether RCM is a part of the HCM spectrum and whether some HCM with typical RCM physiology should be considered as a specific group.

ECG recording is good for screening because it is abnormal in 99% of RCM patients. However, its pattern and degree vary greatly. The most common abnormality reported is tall, biphasic P wave, indicating left and/or right atrium enlargement. ST-T wave abnormalities are prevalent in RCM but controversial for their specificity. In a study on 94 patients, 75 of them presented with abnormal ST-T waves which were argued as nonspecific.[10] In another study on 12 patients, resting ST segments obliquely elevated with late T wave was speculated to be characteristics of RCM. ST segment depression, which is associated with ischemia, could be induced in RCM patients by exercising or might manifest in the late stage of disease. However, these electrocardiograms were not regarded as signs of ischemia in that study because of the non-supportive data from myocardial perfusion imaging.[30] On the other hand, a different study on 18 patients with ischemia evidenced by histological examination did associate those ST segment changes with ischemia.[6] This raise further questions about the role of prevalent ST-T wave changes in RCM.

2.3 Cause of death

RCM has been associated with a high rate of mortality especially in the young. The death of RCM patients happens either spontaneously (sudden death) or results from chronic cardiac illness and its associated complications. Congestive heart failure often develops in the latter case. However, the underlying causes of sudden death or progressive heart failure in RCM patients are not very clear and need further exploration.

Ischemia is suspected to be a cause of sudden death in RCM patients. Rivenes *et al*.[6] suggested a correlation between ischemia and sudden cardiac death (SCD) in RCM, based on the clinical profile of five SCD patients. All those five patients presented with syncope or chest pain and ischemic ECG demonstrating a ST segment depression or T-wave inversion, but no sign or symptom of heart failure even at the time of their cardiac arrest. Four hearts available for autopsy demonstrated evidences of acute and/or chronic ischemia in the subendocardium and papillary muscle. Another clinical study performed by Palka *et al*.[31] also reported the presence of ischemic signs and the absence of chronic heart failure (CHF) in RCM patients who died suddenly. Several other studies on RCM patients with SCD reported syncope, chest pain and/or ST segment depression, but no histological data support for ischemia.[29,30] Hence, the question whether ischemia is the cause of SCD in RCM is still open due to lack of histological evidence or due to the great variations in histological findings. Furthermore, few reports can be found about a more direct evaluation of coronary microcirculation function in RCM. However, it is not without reasons to expect an occurrence of ischemia in the restricted heart since the elevated left ventricular pressure and wall stress that occur in the restricted heart can cause an increased extravascular compressive force resulting in a reduced subendomyocardial perfusion. Furthermore, reduced capillary density due to interstitial fibrosis and/or myofiber disarray which are often found in RCM patients can also provoke an increase in coronary microvascular resistance which may induce ischemia.[32–35] Therefore, it is worthwhile to further investigate the presence of ischemia in RCM and its role in disease progression.

Arrhythmia is one of the most intensively studied mechanisms in heart disease cases associated with SCD.[36] It has been known to be responsible for many deaths in HCM. A recent study by Baudenbacher *et al*.[37] promotes a concept that increased myofilament Ca^{2+} sensitivity could be an independent risk factor for lethal ventricular arrhythmia (also see review paper by Huke and Knollmann[38]). Since most RCM mutations are associated with an increase in myofilament Ca^{2+} sensitivity, the affected patients may be expected to be susceptible to arrhythmia.[39] However, although abnormal ECG patterns were observed in 99% of RCM patients, only about 15%–30% of them presented arrhythmia. Atrial fibrillation and atrial flutter are the most common electrocardiographic abnormalities encountered while heart block, bradycardia and the usual SCD-related ventricular arrhythmias are less common.[6,10,23,26,40] Two SCD cases with terminal rhythms are available for review: one presented torsade de pointes while the other displayed bradycardia.[6] Those two cases, together with four non-SCD cases in the same study, reported ventricular arrhythmias but also displayed acute/chronic ischemia. It is not clear whether arrhythmia plays a primary role in SCD of RCM patients since the ischemic myocardium is also proved to be a matter for lethal ventricular arrhythmia.[36]

Heart failure related death is the most common outcome of RCM. RCM associated with sarcomere protein mutations seems to cause much severer heart failure compared to the RCM resulting from other factors.[40–44] However, due to the rare incidence of RCM and the lack of early screening

indicators for the disease, there are no completely tracked, large-scale clinical cases that are available for an intensive survey on the clinical course of RCM. Several childhood RCM and early diagnosed RCM adult patient cases provide us with valuable clues to observe the complete disease progression. Based on the review of these cases, the common clinical course of RCM may be described as the initial asymptotic RCM refilling pattern with mild atrial enlargement, followed by dyspnea, intolerance to exercise, syncope or other related symptoms.[15,24-27] Over time (months, years, or even decades), those symptoms aggravate and are associated with worsening diastolic dysfunction and atrial enlargement, leading to eventual lethal heart failure, if no SCD occurs. However, some questions are still there: is the systolic function preserved throughout the course of the disease? If yes, then how can diastolic dysfunction alone generate congestive heart failure? If no, what impairs the systolic function in the RCM heart?

It is interesting to note that the preserved systolic function sometimes becomes impaired at the end stage of RCM.[10,24,26–28] However, few published reports provide sufficient information on the prevalence of end-stage systolic dysfunction in RCM patients or on the role of impaired systolic function in the development of acute/chronic heart failure in RCM patients. Ammash *et al*.[10] observed systolic dysfunction in 16% of 94 RCM patients. Since these analyses were not limited to the end-stage of RCM, the percentage could be larger. Weller *et al*.[26] observed low cardiac output syndrome (low CO) in 18 RCM children: four of them presented with severe low CO and suffered severe right heart failure/syncope; the other 14 had initially mild signs or symptoms but latter developed low CO over 2.8 ± 2.3 years. Although all the 18 patients had preserved ventricular systolic function at diagnosis, six of them later presented a deteriorated ventricular systolic function and 8 others eventually required inotropic support. This suggests that systolic dysfunction may partly contribute to the low CO in advanced RCM or that low CO may be a cause behind the development of systolic dysfunction at the late stage of the disease.

So far, clinical studies are limited by the small number of patients, controversial diagnostic criteria, heterogeneous disease presentations, and lack of screening and/or early diagnostic indicators. The blooming genetic studies of RCM shed light on identifying etiology and provide the basis for animal model development, which will provide us with a better understanding of the mechanisms underlying the disease initiation and progression.

3 Molecular etiology of RCM

3.1 Myofibril mutation and RCM

Depending on the factors behind the development of the disease, RCM has been classified as primary or secondary. Primary RCM includes RCM due to idiopathic causes or to genetically inherited or sporadically acquired mutations. Primary RCM is solely confined to the myocardium. Secondary RCM develops from extrinsic factors and include infiltrative disorders (amyloidosis and sarcoidosis), storage diseases (haemochromatosis, glycogen storage disease and Fabry disease), and inflammatory diseases like in the cases of Loeffler cardiomyopathy, endomyocardial fibrosis, and eosinophilic endomyocardial disease.[43] Restrictive physiology may develop as well in late stage of HCM, DCM, and other cardiac diseases linked to hypertension and ischemia.[41,42,44] However, most of the cases of RCM were classified as idiopathic due to their unknown etiology.[44–46]

Familial RCM has been linked with accumulation and disorganization of desmin which is an intermediate filament protein that connects myofibrils and anchors them to the sarcomere.[13,47] RCM cases associated with desmin accumulation are often accompanied with atrioventricular conduction abnormalities and varying degrees of skeletal myopathies, and so far, only four mutations identified on the *DES* gene have been linked with the disease.[47,48]

Recently, idiopathic RCM has been associated with mutations in sarcomeric contractile proteins.[15] The sarcomere contains different proteins involved in muscle contraction. Two major contractile components are actin which constitutes the backbone of the thin filament, and myosin which makes up the thick filament. The interaction of myosin and actin causing the sliding of the thin filaments along the thick filaments results in muscle contraction and force development. This contraction is crucially regulated in a Ca^{2+} dependent manner by the troponin complex and tropomyosin (Tm) that are located on the thin filaments. The myosin light chain proteins (MLC1 and MLC2) and the myosin binding protein C (MyBPC) are also involved in the regulation of cardiac muscle contraction. The integrity of these proteins is crucial for proper muscle contraction and relaxation.

Indeed, most of these sarcomeric proteins, when mutated at specific sites, are known to induce some type of cardiomyopathy. In fact, HCM, DCM and RCM have all been linked with mutation of myofibril proteins.[49–51] Usually, different mutations on a designed protein lead to distinct cardiac remodeling and pathologies associated with DCM, HCM, or RCM, although there are a few cases when the same mutation may be associated with more than one

type of cardiomyopathies or not clearly defined phenotypic features.[52] Several RCM-causing sarcomeric protein mutations have been identified so far. Mutation screening of different individuals and families presenting the RCM phenotype showed a missense mutation in α-cardiac actin gene, two different RCM causing missense mutations in β-myosin heavy chain, at least seven reported missense mutations and two deletions leading to different frame-shift mutations in cardiac troponin I, and also a missense mutation and a deletion in the cardiac troponin T gene.[15,24,25,40,53,54] All the mutations and alterations reported affect the functionally important and conserved regions of these sarcomeric proteins. Mutation screening to detect genetic alterations in myofibril proteins is becoming a way to discover the etiology of RCM and to identify individuals who are most susceptible to suffer from this myocardial disease.

3.2 Cardiac troponin I mutations and RCM

Most of the RCM causing mutations that have been reported involved cardiac troponin I (cTnI). cTnI is a 24 kDa protein that is expressed only in the heart and replaces the fetal isoform of that protein, slow skeletal troponin I (ssTnI), in the heart during the early stage of development.[55–57] The sequence of cTnI is highly conserved between species.[58] cTnI is a subunit of the contraction regulatory complex troponin which also includes the calcium binding protein troponin C (TnC) and the tropomyosin (Tm) binding protein cardiac troponin T (cTnT). cTnI is the main inhibitor of muscle contraction and its deficiency gravely impairs cardiac relaxation, leading to severe diastolic dysfunction and death.[57,59,60] At low cytosolic Ca^{2+} concentration, cTnI inhibits cross-bridge cycling by binding to actin and through its weak interaction with TnC causing the troponin complex to remain in a conformation that blocks myosin-actin cross-bridge formation. During systole, Ca^{2+} binds to TnC, which causes cTnI to leave its actin binding site to interact more strongly with TnC, inducing a conformational change in the troponin complex, leading to the displacement of Tm which frees up sites within the actin filaments for the ATP free myosin head to attach. Subsequent removal of calcium ions from TnC, will allow cTnI to reclaim its actin binding site and the initiation of cardiac muscle relaxation. Based on the important role of cTnI in inhibiting contraction, it is logical that alteration of its sequence will have major impact on the cardiac contraction and relaxation patterns and also on the whole heart function.

Mutations in cTnI have been associated with DCM, HCM and RCM.[61] Mogensen *et al*.[15] were the first to link cTnI mutations with RCM. They reported six cTnI missense mutations (L144Q, R145W, A171T, K178E, D190G, and R192H) that they observed in restricted hearts. These and other troponin mutations linked to RCM as well as the clinical cases associated with them were reviewed intensively by Parvatiyar *et al*.[62] All the cTnI RCM causing mutations reported occurred in the C-terminal region of cTnI, which contains an inhibitory region and two acto-tropomyosin binding sites. Out of these mutations, the missense mutation D190G was found in 12 patients of the same family and showed a clinical phenotype of mixed appearance of RCM and HCM. In addition, the R145W mutation of cTnI which is responsible for RCM in that study was also observed in patients presenting the HCM phenotype.[63] Such mutation is also located at the same residue as HCM causing mutations R145G and R145Q.[64] Thus, the codon associated with these mutations was suspected to be susceptible to high mutation rate leading to the development of either RCM or HCM phenotype.[63] The K178E and R192H mutations reported by Mogensen *et al*.[15] happened *de novo* and were the most lethal missense mutations of cTnI in that study. In general, *de novo* mutations in the sarcomere protein genes are associated with a younger onset and more severe disease manifestation.[65] The K178E mutation of cTnI has been linked to two additional RCM cases while the R192H mutation has been also observed in a HCM patient presenting restrictive physiology.[29,40] Another RCM mutation of cTnI that has been also associated with HCM, is the R204H, which have been previously encountered in an Australian family linked with HCM, but was observed in two different patients presenting the RCM disease with no sign of hypertrophy.[52,66,67] The clinical heterogeneity associated with these missense mutations of cTnI call for the consideration of other factors (genetic or environmental) that may influence disease manifestation and progression.

Though less common, cases of deletion in the *cTnI* gene, *TNNI3*, leading to frame-shift mutations in the cTnI protein, have been reported in RCM patients. The first case reported presented a deletion in two nucleotides in exon 7 of *TNNI3* which resulted in a frame shift mutation and the introduction of a premature termination codon at amino acid position 209 (E177fsX209).[40] Another case of deletion of one single nucleotide was also detected in exon 7 of *TNNI3* which caused a frame shift in codon 168, and led to the formation of a premature stop codon at position 176 (D168fsX176). Such truncated form of cTnI is missing the second actin and troponin C-binding region of that protein.[68] Different pathological conditions have been linked with the truncation of the C terminus of cTnI, among them include myocardial stunning which is characterized by a reduction of

myocardial contractile capabilities subsequent to ischemic/reperfusion injuries.[69] The C-terminal truncation of cTnI is also known to increase myofilament Ca^{2+} sensitivity and cross-bridge cycling kinetics in skinned rat cardiac muscle.[70] These highlight the importance of the integrity of cTnI for proper myocardial function and the cardiac damage that may result from alteration of this protein's sequence.

4 Study RCM in diseased animal models

4.1 RCM in transgenic animals

Clinical studies showed great heterogeneity among the genetic RCM patients even when they carry the same mutation.[29,52,66,67] The biggest challenge is that no data are available so far to confirm the overall expression and the incorporation rate of the mutant sarcomeric proteins in the heart. Furthermore, other environmental and genetic factors may also contribute to the heterogeneity of the disease manifestation. Recently, transgenic animal models have been generated to mimic RCM by expressing the mutant myofibril proteins observed in human patients.[18,19] The development of animal models of these single-gene disorders will fill the gap in RCM studies between the *in vitro* assays using reconstituted myofibrils and the clinical studies with the patients carrying the mutant genes. Studies on RCM using transgenic mouse models will undoubtedly improve our understanding of the underlying mechanisms of the diseases and provide us with clues for the prevention and treatment of cardiomyopathies linked to diastolic dysfunction.

Indeed, RCM transgenic mice offer many advantages that are not possible with clinical patients. They allow confirming whether the sarcomeric protein mutations discovered in the clinical RCM patients are really responsible for the restrictive physiology of the diseased heart. They can also help reduce the variability between subjects and the animals can be put in controlled environments with their diet and activities monitored intensively to eliminate extrinsic factors that may compromise analyses. More importantly, transgenic mice give us a tool to investigate the dose-dependency of the disease manifestation caused by various expression levels or incorporation rate of the mutant proteins in the heart. Besides, since mice have a relatively short lifespan compared to humans, the whole progression of the disease can be monitored and the cardio-pathophysiology at the end stage of the disease can be studied during a given time. Moreover, the cardiac organ of the animals can be isolated at various stages of the disease for histological and electro microscopic analyses, for cellular assays to determine molecular, morphological, functional and metabolic changes in mutant myofibers and cardiomyocytes, and for *ex vivo* experiments such as Langendorff working heart perfusion assays to obtain a comprehensive understanding of the characteristics of the disease and the factors behind disease progression and deterioration.

Our laboratory is among the first to use transgenic mice to study the mechanisms and pathophysiology of RCM.[18] The mouse models contained the R192H mutant of cTnI (R193H in the mouse genome) which is associated with severe phenotypes and the worst prognosis.[15] These transgenic animals showed similar characteristics as clinical RCM patients carrying the same mutation. Another mouse model developed by Wen *et al*.[19] contains the RCM causing R145W cTnI mutation which is located at the same residue as HCM causing R145G and R145Q mutations.[71] We will discuss in the following sections the information obtained from transgenic mouse model analyses on the molecular mechanisms of the disease and the cause of death in RCM.

4.2 Myofibril calcium sensitivity increases in RCM TG mice

RCM causing sarcomeric protein mutations alter myofilament sensitivity to Ca^{2+}. RCM myofilaments isolated from transgenic animals are more sensitive to Ca^{2+} and show more force at lower Ca^{2+} concentration.[19] This is consistent with experiments done *in vitro* on assembled human and mice RCM myofibers.[39,72,73] The myofilament hypersensitivity to Ca^{2+} is a common feature that RCM shares with HCM. This opposed them to DCM which is linked to systolic dysfunction and reduced myofibril Ca^{2+} sensitivity.[74–78] The increase in Ca^{2+} sensitivity was also linked with greater affinity of TnC to Ca^{2+} in assembled RCM myofibers as reported by Kobayashi *et al*.[79] However, no significant difference was observed in the Ca^{2+} affinity between RCM and HCM myofibers in that study.[79] Increase of myofilament Ca^{2+} sensitivity observed in RCM myofibers may affect the cross-bridge mechanic and favor myofibril shortening at the expense of relaxation which may cause the prolongation of their relengthening time during diastole. The slower rate of relaxation due to the myofilament hypersensitivity to Ca^{2+} may account for the diastolic dysfunction encountered in RCM heart.

Even though both HCM and RCM-linked mutations of myofibril proteins induced a left shift on the force-pCa curve, RCM causing mutations confer greater Ca^{2+} sensitivity on the myofilament than the HCM-related mutations.[19,39] Comparison of the Ca^{2+} sensitivity and the ATPase force of development of the R145G HCM mutant to

the R145W RCM causing mutation occurring at the same residue of the cTnI protein demonstrated that myofibers isolated from RCM transgenic R145W mice had a larger increase in the Ca^{2+} sensitivity of force development and ATPase activity compared to the HCM transgenic myofibers.[19,80] Another difference between RCM and HCM observed in this study is an increase in both maximal Ca^{2+}-activated force and maximal Ca^{2+}-activated ATPase activity in the RCM mutant which was opposed with a decrease in the maximal force in HCM mutants. This phenomenon allows concluding that increased Ca^{2+} sensitivity is a major characteristic of RCM myofibers and may play a role in determining the phenotypic features of DCM, HCM and RCM. A further proof that Ca^{2+} hypersensitivity may be a key factor behind the diastolic dysfunction due to myofibril protein mutation is that the impaired relaxation can be corrected in R193H RCM transgenic animals after the introduction of a Ca^{2+} desensitizing protein in the heart.[81]

Most RCM mutations of cTnI are located in the C-terminal half of the molecule, which contains two or more actin-Tm interacting sites.[79] The C-terminus half of cTnI is also known to be critical for a full inhibitory activity and Ca^{2+} sensitivity of force development.[82] It remains to be determined, however, how the mutations alter the myofilament sensitivity and what conformational changes happened in the cTnI molecule that can affect the interaction between cTnI and actin, cTnI and TnC, and disturb the troponin structure. Determining the mechanisms behind the increase of Ca^{2+} sensitivity observed in RCM myofibers may help us in the design of therapeutic drugs or molecules aimed at desensitizing myofilaments and at restoring their relaxing abilities.

4.3 Initiation of diastolic dysfunction in RCM TG mice

Impaired relaxation is the earliest pathologic manifestation in the transgenic RCM hearts and can be observed using echocardiographic measurements, isolated working heart and cell based assays.[18,83] The R193H mutation of cTnI causes a serious cardiac disorder in the diastolic function of the RCM heart but not in the systolic function. The isovolumetric relaxation time (IVRT) defined as the interval between aortic valve closure and mitral valve opening is significantly longer in RCM mice compared to wild type. The prolonged IVRT is the earliest sign of diastolic dysfunction observed and is noticeable as early as one month in the transgenic RCM mice.[83] As for the isovolumetric contraction time (IVCT) which represents the interval between the mitral valve closure and aortic valve opening, no significant difference is observed throughout most stage of the disease. The impaired relaxation is responsible for the decrease in diastolic filling and the RCM phenotype observed in the diseased animals. Besides, analysis of the isolated working heart function shows a reduced cardiac compliance characterized by a significantly lower increase in the LVEDV in response to increased preload compared to wild type.[83] The decrease in compliance which leads to an increase in the left atrial pressure throughout the cardiac cycle may be the cause for the bi-atrial enlargement observed in transgenic RCM hearts.

The impaired relaxation is also significantly noticeable at the cellular level. RCM cardiomyocytes have defective relaxing properties which are characterized by shorter end diastolic sarcomere lengths and slower sarcomere relaxation rates.[81] This is consistent with the results obtained by Davis *et al.*[84] in which wild type cardiomyocytes are transduced with RCM causing mutated cTnI genes. The shortened resting sarcomere lengths observed in both transgenic and transduced cardiomyocytes suggest that the mutant proteins cause an increase level of basal actin-myosin interaction under resting diastolic condition. Sarcomere contractility analyses reveal no difference in sarcomere contraction time between WT and RCM mice. The fraction shortening of transgenic and wild type mice is similar, indicating no systolic dysfunction.[81] Also, comparison of the sarcomere relaxation rate between cardiomyocytes transduced with HCM and RCM causing mutant genes by Davis *et al.*[85] shows that RCM cardiomyocytes have a slower relaxation rate compared to HCM. The greater relaxation impairment observed in RCM in comparison to HCM may be the reason behind the greater rate of morbidity and mortality found in the RCM patients.

Concomitant with the cardiomyocyte contractions, RCM cardiomyocytes have a delay of decay kinetics of cytosolic free Ca^{2+}. Therefore, cytosolic Ca^{2+} dissipates at a slower rate during diastole in the restricted cardiomyocytes. The delay in Ca^{2+} decay was not due to defects in proteins responsible for Ca^{2+} uptake by the sarcoplasmic reticulum (SR) such as phospholamban, phosphorylated phospholamban, or sarcoplasmic reticulum calcium ATPase (SERCA), which infers that the main factor behind the prolonged calcium transient was the increased myofilament Ca^{2+} sensitivity caused by the RCM mutation.[81] Thus, the hypersensitivity of the RCM myofibers causes the myofilament Ca^{2+} to remain longer on TnC which will cause a delay in cardiac muscle relaxation and Ca^{2+} decay. Reversing the Ca^{2+} hypersensitivity observed in RCM myofibers may facilitate the dissociation rate of Ca^{2+} from TnC and restore the relaxation rate and diastolic function in RCM hearts.

4.4 Progression of the disease in RCM TG mice

Du *et al*.[83] observed the cardiac function of RCM transgenic mice over a 12-month period. Their analyses demonstrate a chronic development of the disease from initial relaxation impairment to fatal heart failure. Overt diastolic dysfunction is prevalent in the majority of the course of RCM, while the systolic function is preserved until at advanced stage of the disease. At one month of age, the increased IVRT is the earliest parameter change in the RCM animals compared to wild type while the LVEDD and LVEDV evaluated with M-mode echocardiography do not present significant differences to wild type of the same age. Over time diastolic dysfunction worsens with further prolongation of the IVRT and increase in deceleration time. LVEDD and LVEDV are reduced in the animals while the EF and left ventricle end systolic dimensions (LV) remain comparable to that of the wild type, indicating no change in the contraction pattern. Mitral pulse Doppler echocardiography indices, previously similar to wild type, are switched to a reversed E to A velocity peak ratio in RCM transgenic mice, indicating a further deterioration of the cardiac relaxation. The reduced E to A ratio indicates that the impaired relaxation compromised the early ventricular filling and that atrial contraction is mainly responsible for the filling of the ventricle in the RCM mutants. Over a time of 10 months, the worsening diastolic dysfunction is accompanied with noticeable impaired systolic function in 12-month-old diseased animals with evidence of decreased EF and stroke volume revealed by both isolated working heart and echocardiography.[83] Upon that time, those RCM mice began to present with signs of congestive heart failure (i.e., lung congestion, hepatomegaly and ascites) and some of them died of heart failure. This disease progression observation is consistent with some of the clinical studies, suggesting that impaired systolic function may be a sign of late stage RCM and responsible for the end stage progression leading to lethal heart failure. Since ischemia is also confirmed with histological analyses of the diseased mice, which show fibrosis and myocardial disarray at the advanced stage of the disease, we propose that the ischemia observed in RCM hearts is a bridge between the progression of diastolic dysfunction and the development of systolic dysfunction at the late stage of the disease.

5 Treatments for RCM

5.1 Current treatments

5.1.1 Cardiac transplantation

So far, there is no designed therapeutic alternative for RCM. Pediatric patients are at great risk of developing diastolic heart failure with a faster deterioration rate compared to RCM adult patients. Since they average a post-diagnostic two year survival rate, the need for efficient treatments that correct, stop or delay disease progression is an emergency.[44] So far, no documentation is provided about the effects that the drugs used in clinical settings have on life expectancy. Cardiac transplantation is the sole procedure that is revealed to be effective. In fact, patients have a greater survival rate post-heart transplantation compared to other RCM patients.[86,87] Since they carry a very high risk of sudden death, it is recommended that children with RCM obtain preference in cardiac transplant waiting list.[6] To prevent the need for heart-lung transplantation due to rapid increased of pulmonary vascular resistance in RCM patients, early consideration for cardiac transplantation is also suggested.[87] Nevertheless, the invasive nature of such procedure and the difficulty of finding cardiac organs for transplantation as evidenced by the report of the death of many RCM patients awaiting cardiac transplants, call for the development of efficient medications or non-invasive devices that improves the function of the restricted heart.

5.1.2 Clinical medications

Other therapeutic options used for the treatment of diastolic heart failure are not efficient as far as RCM is concerned though they may be used with the purpose of reducing the symptoms secondary to the disease. Diuretics are commonly given to RCM patients to reduce preload and treat pulmonary and systemic venous congestion. However, excessive preload reduction may decrease ventricular filling pressure and lower the cardiac output.[8,43] Thus, it is suggested to limit diuretics use to patients presenting symptomatic pulmonary venous congestion and/or right heart failure.[88] Acute administration of angiotensin-converting-enzyme (ACE) inhibitors such as captopril, was not able to increase the stroke volume nor cardiac output but decreased aortic pressure in RCM patients.[89] Afterload reduction using vasodilators even though they may be considered when systolic function is impaired, are not known to prevent deterioration of the disease and are likely to induce hypotension. Therefore, they are considered detrimental for the treatment of RCM.[26] However, these studies are still limited due to the low number of patients tested. Anti-dysrhythmic therapy or implantable cardioverter defibrillators (AICD) may be an option for people presenting atria fibrillation or ischemia.[88] Since people with RCM are susceptible to thromboembolic complications, anti-coagulating agents such as warfarin can be

administered.[8,43]

Since the RCM heart is unable to relax properly, bradycardiac agents can be tried with the purpose of delaying contraction in order to increase the diastolic filling time. They may be considered at the early stage of the disease but with caution since excessive bradycardia can negatively affect the cardiac output. Bradycardiac drugs such as calcium antagonists and β-blockers are usually administered to RCM patients. However, in a study performed by Gewillig *et al*.[12], calcium channel blockers were unable to induce any echocardiography changes when administered to two RCM patients. β-blockers are usually better than calcium antagonists at reducing heart rate and are known to reduce mortality rate in post-infarct and heart failure patients.[90] Thus, they were suggested as a therapeutic option for RCM patients showing sign of ischemia.[6] Another feature of β-blockers that allows considering them for RCM treatment is their effectiveness in controlling heart rate and ameliorating cardiac hemodynamics in patients with atrial fibrillation.[8] However, the lack of documentations about the impact of β-blockers on restricted heart function and lifespan prolongation makes it difficult to really determine the beneficial effect of such drug in RCM patients. Nevertheless, administration of the heart rate slowing agent Ivabradine was shown to improve hemodynamic patterns in a RCM patient.[91] Ivabradine slows down the heart rate by inhibiting the "funny" currents, I_f, located in the sinoatrial node. One advantage that this drug offers over other bradycardiac agents is its selectivity for the pacemaker which minimizes the chance of affecting other parameters that may disturb cardiac function. Nevertheless, the long term effect of this drug and its influence on stroke volume and cardiac output in RCM patients still need to be determined.

Due to the limited amount of RCM patients, the benefits of these different therapies are not well established. RCM patients still deteriorate and die prematurely besides these medications. Their overall effects on life expectancy need to be assessed as well. The development of RCM transgenic animals offer the opportunity to observe the acute and long term effects of these drugs and may help in the suggestion of therapeutic options for RCM.

5.2 Proposed therapeutic strategies

5.2.1 Lusotropic agents

Drugs that aim at correcting the relaxation impairment encountered in RCM may be a future way to treat that disease. So far, there is no drug with strictly lusotropic properties available. More pharmacological emphasis has been on drugs that induce cardiotonic effects and sensitize myofilaments to calcium in order to treat systolic heart failure.[92–94] There are no drugs that improve the diastolic function of the heart without affecting as well the systolic function. β-agonists are known to improve relaxation through phosphorylation of cTnI which desensitizes myofilament to Ca^{2+} and also by phosphorylating phospholamban which stimulates the uptake of Ca^{2+} by the SR.[95–97] However, β agonists also induce contraction by facilitating Ca^{2+} entry through the L type Ca^{2+} channels and Ca^{2+} release from the SR via the ryanodine receptors. Heart rate acceleration due to β adrenergic stimulation may be detrimental to the restricted heart. In fact, β stimulation was determined to be unable to increase the cardiac output in RCM mice and RCM cardiomyocytes seem not to be able to cope with pacing.[83,84] Also, inotropic agents are not recommended for the treatment of RCM since they also reduce the time for the restricted heart to relax. Moreover, they may have a proarrhythmic influence and increase the risk of sudden cardiac death by increasing intracellular calcium concentration. Thus, finding drugs that specifically target molecules involved in muscle relaxation is therefore a necessity and should be a focus in cardiovascular research aimed at treating RCM and diastolic dysfunction.

A calcium binding protein, parvalbumin (PARV), was proposed as a gene therapy option for diastolic heart failure.[98–101] Such protein is normally not expressed in the human heart but in fast-twitch skeletal muscles and the brain.[98,100] It has a high affinity for Ca^{2+} and accelerates relaxation in fast-twitch skeletal muscles by facilitating the shuttling of Ca^{2+} from TnC to the SR.[102,103] By binding to free Ca^{2+}, it also speeds up the decline of intracellular Ca^{2+} and may help prevent damages resulting from defective intracellular Ca^{2+} handling.[101,103] *PARV* gene transfer in slow-contracting rat soleus muscle was shown to increase the speed of relaxation in a dose dependent manner without impairing contraction.[104] *PARV* gene delivery in the myocardium also achieved to accelerate cardiac relaxation *in vitro* as well as *in vivo*.[100,105–108]

5.2.2 Calcium desensitizers

Restoring the impaired relaxation in RCM hearts by targeting the myofilament Ca^{2+} sensitivity is a promising alternative for the treatment of the diastolic dysfunction. Importantly, Ca^{2+} desensitizers, by targeting specifically molecules involved in muscle contraction, do not alter cytosolic Ca^{2+} homeostasis. They also have the potential ability to prevent arrhythmia in diseased animals characterized by myofilament hypersensitivity to calcium.[37] The use of Ca^{2+} desensitizing compounds for the treatment of diastolic dysfunction is practically a novel idea in the field of cardiovascular research. So far, the number of Ca^{2+}

desensitizers available for research, medical trials or therapeutic use is very limited. Myosin ATPase inhibitors such as blebbistatin and 2,3-butanedione monoxime have been used as desensitizing compounds *in vitro* and as excitation-contraction uncouplers for electrophysiological and mechanical studies both *in vitro* and *ex vivo* due to their ability to inhibit actomyosin cross bridge formations.[37,84,109–112] However, these compounds characterized by their strong negative inotropic effects are unsuitable for use in intact animals due to their cardiac toxicities that can result in systolic heart failure and cardiac arrest.

(-)-Epigallocatechin-3-gallate (EGCg), a polyphenol present in green tea, has been shown to have myofilament Ca^{2+} desensitizing abilities through its interaction with troponin C.[113–115] This compound was previously shown to inhibit cardiac hypertrophy induced by reactive oxygen species and/or pressure overload.[116–118] Its therapeutic effect as a Ca^{2+} desensitizer was also analyzed by Tadano *et al.*[115] on a mouse model possessing a HCM causing cTnT mutation and presenting the increased myofilament Ca^{2+} sensitivity associated with the disease. EGCg achieved to reverse the increased myofilament Ca^{2+} sensitivity of the HCM myofibrils in a concentration dependent manner. It also restored the Ca^{2+} transient parameters without affecting the diseased cardiomyocytes resting Ca^{2+} levels.[115] The use of EGCg as a therapeutic alternative for cardiac dysfunction is reasonable since it is attributed many cardiovascular benefits which include anti-oxidative, anti-hypertensive, anti-inflammatory and vaso-relaxing properties.[119–123] Its cardioprotective effects against ischemia/reperfusion injury has been demonstrated as well.[124–126] The use of transgenic animals will allow determining its relevance for the treatment of RCM and the overall effects of Ca^{2+} desensitization on diastolic dysfunction.

Moreover, it has been shown that restrictive cleavage of the N-terminal extension of cTnI decreases Ca^{2+} sensitivity of myofibril actomyosin ATPase and enhances diastolic function in transgenic animals.[127–129] This proteolytic modification of cTnI is known to occur as a functional adaptation in simulated microgravity and also under physiological and pathological stress conditions.[127,130,131] The proteolysed protein loses about 30 amino acid residues at its N-terminus.[127,128] This 30 amino acid extension is conserved within species and absent in both slow skeletal (ssTnI) and fast skeletal (fsTnI) troponin I.[58] Removal of such section eliminates the two serine residues Ser 23/24 which, when phosphorylated by PKA, are known to decrease myofilament Ca^{2+} sensitivity by diminishing the affinity of cTnI to TnC.[95,96] The N-terminal truncated cTnI (cTnI-ND) desensitizes myofilament to Ca^{2+} in similar ways as wild type cTnI when phosphorylated at its Ser 23/24 residues following β-adrenergic stimulation.[128] cTnI-ND also shows the ability to improve the cardiac function of a mouse model characterized by impaired myocardial β-adrenergic signaling.[131] The cTnI-ND protein has a successful incorporation rate in the myofilament and is able to rescue cTnI null mutants.[132] Also, the systolic function of transgenic animals is not impaired by the presence of cTnI-ND while aged mice display better overall cardiac function.[128,129] Transgenic mice with cTnI-ND present improved ventricular filling, increased myocardial relaxation rate and reduced left ventricle end diastolic pressure (LVEDP).[128] The positive effects of cTnI-ND on diastolic function make it a suitable alternative for the treatment of RCM.

The desensitizing effects of cTnI-ND were also analyzed in RCM mice. Such analyses allow determining whether alteration of myofilament Ca^{2+} sensitivity is able to restore diastolic function in RCM hearts. Li *et al.*[81] developed double transgenic mice (Double-TG) expressing both cTnI-ND and the RCM causing transgenic protein ($cTnI^{193His}$) in a wild type null background. Such double transgenic mice expressed the same amount of $cTnI^{193His}$ as the RCM mice with cTnI-ND completely replacing the wild type cTnI protein. cTnI-ND was able to rescue the mice and to desensitize the myofiber to Ca^{2+} even in the presence of the cTnI RCM causing mutant. Force-pCa experiments revealed that cTnI-ND was able to reduce the hyper-sensitivity to normal levels. This effect was reflected at the cellular level as the sarcomeric relaxation rate as well as the Ca^{2+} decay became similar to that of wild type cardio-myocytes. Echocardiographic measurements confirmed the correction of the diastolic dysfunction with the Double-TG hearts showing no relaxation impairment as the LVEDD, the IVRT and DT were restored to wild type values. Such findings confirm that calcium desensitization is a promising alternative to repair diastolic dysfunction in RCM hearts and emphasize the need to further explore this therapeutic option.

6 Conclusion

RCM is a severe cardiac disease linked to diastolic dysfunction. However, the lack of information about the factors behind the clinical heterogeneity of RCM as well as the unavailability of efficient treatments that delay disease progression and prevent early death in the affected patients make it an emergency to further explore the mechanisms behind the pathophysiology of the disease and the cause of death. The discovery of sarcomeric protein mutations responsible for the development of this myocardial disorder

helps identify the etiology of RCM and allows for the screening of potential RCM patients which may facilitate early diagnostic, counseling and proper monitoring and management of these patients. Based on the clinical reports and experimental data obtained from studies using transgenic animals, we summarize the mechanisms underlying the development of genetic RCM and propose the strategies for the treatment of the disease (Figure 1). In fact, RCM transgenic animals provide a translational link between biochemical studies and clinical research. They will also be very useful for the trial of potential drugs or devices designed to correct the diastolic dysfunction associated with RCM. The lack of effective treatments and the unavailability of drugs that selectively correct the diastolic dysfunction of the restricted heart, make the development of new pharmacological agents an urgent necessity. The design of compounds with strictly lusotropic properties or attempts to modify the myofilament Ca^{2+} sensitivity seem to be promising therapeutic options for treating diastolic dysfunction. Alteration of myofilament Ca^{2+} sensitivity for therapeutic purposes is not such uncommon since Ca^{2+} sensitizers are being developed in cardiovascular research to restore systolic function. However, the importance of Ca^{2+} desensitizers for the treatment of diastolic dysfunction has been undervalued so far. The design of Ca^{2+} desensitizers that selectively target myofibril contractile proteins may be a good way to restore diastolic function without affecting the systolic function as research in double transgenic animals shows.[81] Due to the high percentage of heart failure cases associated with diastolic dysfunction, the exploration of such alternatives is worthwhile and may lead to great progress in cardiovascular research.

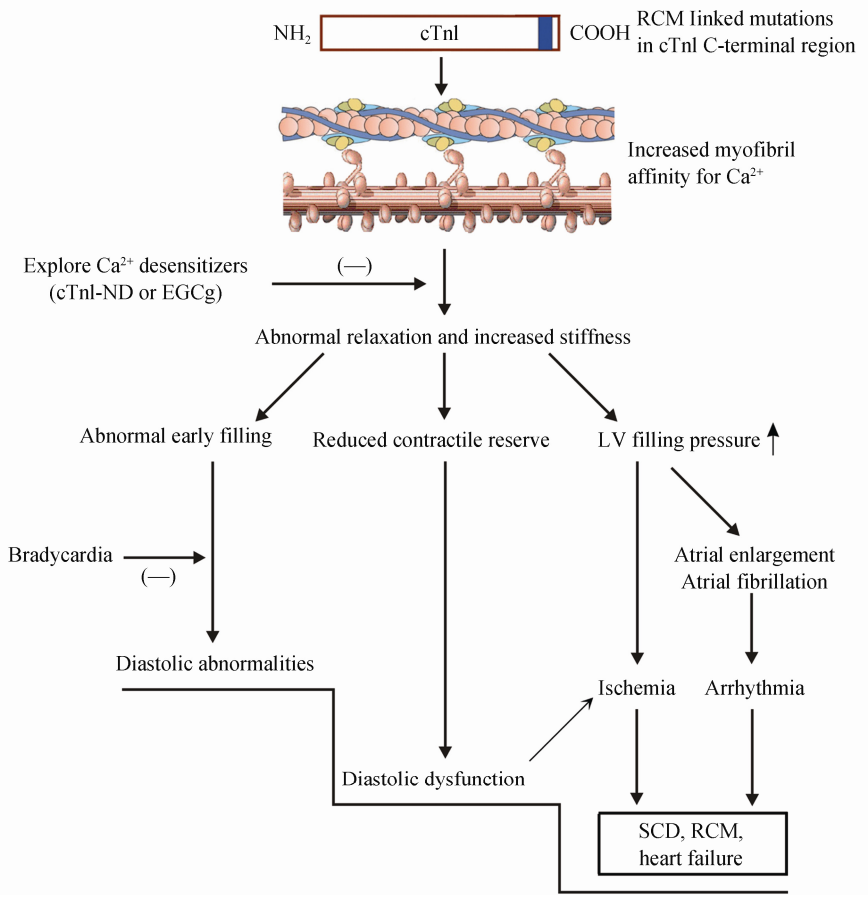

Figure 1. Schematic representation of the development of genetic restrictive cardiomyopathy caused by cardiac myofibril protein troponin mutations and the proposed therapeutic strategies for the disease. cTnI: cardiac troponin I; cTnI-ND: N-terminal deleted cTnI; EGCg: (-)-Epigallocatechin-3-gallate; LV: left ventricle; SCD: sudden cardiac death; RCM: restrictive cardiomyopathy.

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