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## Dystrophin disruption in enterovirus-induced myocarditis and dilated cardiomyopathy: from bench to bedside

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**Abstract** Genetic defects of the dystrophin-glycoprotein complex (DGC) cause hereditary dilated cardiomyopathy. Enteroviruses can also cause cardiomyopathy and we have previously described a mechanism involved in enterovirus-induced dilated cardiomyopathy: The enteroviral protease 2A directly cleaves dystrophin in the hinge 3 region, leading to functional dystrophin impairment. During infection of mice with coxsackievirus B3, the DGC in the heart is disrupted and the sarcolemmal integrity is lost in virus-infected cardiomyocytes. Additionally, dystrophin deficiency markedly increases enterovirus-induced cardiomyopathy in vivo, suggesting a pathogenetic role of the dystrophin cleavage in enterovirus-induced cardiomyopathy. Here, we extend these experimental findings to a patient with dilated cardiomyopathy due to a coxsackievirus B2 myocarditis. Endomyocardial biopsy specimens showed an inflammatory infiltrate and myocytolysis. Immunostaining for the enteroviral capsid antigen VP1 revealed virus-infected cardiomyocytes. Focal areas of cardiomyocytes displayed a loss of the sarcolemmal staining pattern for dystrophin and  $\beta$ -sarcoglycan identical to previous findings in virus-infected mouse hearts. In vitro, coxsackievirus B2 protease 2A cleaved human dystrophin. These findings demonstrate that in human coxsackievirus B myocarditis a focal disruption of the DGC can principally occur and may contribute to the pathogenesis of human enterovirus-induced dilated cardiomyopathy.

**Keywords** Myocarditis · Dilated cardiomyopathy · Dystrophin-glycoprotein complex · Enterovirus · Coxsackievirus B2

### Introduction

Dilated cardiomyopathy, a major cause of heart failure, is a multifactorial disease of genetic and acquired etiologies [9]. Hereditary dilated cardiomyopathy can result from defects of the extra-sarcomeric myocyte cytoskeleton, in particular the dystrophin-glycoprotein complex (DGC) [11]. Dilated cardiomyopathy is a common finding in patients with X-linked dilated cardiomyopathy as well as Duchenne and Becker muscular dystrophy. These diseases are caused by dystrophin mutations, leading to a loss of the typical sarcolemmal localization of both dystrophin and the sarcoglycans [7, 16]. Mutations in various sarcoglycans cause human limb-girdle muscular dystrophy and dilated cardiomyopathy [6, 10]. The DGC collectively connects the internal actin-based cytoskeleton to the extracellular matrix, and is thought to be important for the transmission of mechanical force from the sarcomere to the extracellular space [18].

Acute infection of the heart with enteroviruses such as coxsackievirus B is an acquired etiology of human myocarditis and dilated cardiomyopathy [15]. As much as 30% of human acquired dilated cardiomyopathy is associated with an enteroviral infection of the heart, in particular coxsackievirus B [1]. In mice, transgenic expression of coxsackievirus B3 proteins in the heart induces dilated cardiomyopathy [20]. In analogy to many other virus-mediated illnesses [17], both direct viral effects as well as the host's inflammatory response contribute the pathogenesis of viral heart disease. On one hand, coxsackievirus B3 has direct cytopathic effect(s) on cardiomyocytes [19]. On the other hand, the host immune response, although necessary to eliminate virally infected cells, may induce inappropriate damage by killing uninfected cardiomyocytes [12].

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The coxsackievirus genome encodes two proteases, protease 2A and protease 3C, both of which are essential for the viral life cycle [2, 20]. Substrate recognition by the enteroviral protease 2A depends on a specific amino acid pattern. This amino acid motif was used to establish a neural network algorithm for the *in silico* prediction of putative novel cellular protease 2A-substrates [8].

Based on this computer algorithm, we experimentally demonstrated that the enteroviral protease 2A cleaves dystrophin directly in the hinge 3 region at the computer-predicted site and disrupts the DGC in infected mouse hearts, thus linking the pathogenesis of an acquired cardiomyopathy to that of hereditary cardiomyopathies [2, 3, 13]. Additionally, dystrophin deficiency markedly increases enterovirus-induced cardiomyopathy *in vivo*, suggesting a pathogenetic role of the dystrophin cleavage in enterovirus-induced cardiomyopathy [21].

Here, we extend these experimental findings from cell culture and murine models to a human patient with coxsackievirus-induced dilated cardiomyopathy. Endomyocardial biopsy specimens showed that a focal dystrophin and  $\beta$ -sarcoglycan disruption can principally occur in this human disease.

## Patient and methods

### Patient

Right ventricular endomyocardial biopsies were performed on a previously healthy 33-year-old man who presented with acute-onset dilated cardiomyopathy preceded by fevers to 103°F (39.4°C). There was no coronary artery disease on coronary angiogram, and the ejection fraction of the dilated left ventricular was 27%.

All antibody tests, including the coxsackievirus B2 neutralizing antibody test, were performed at the University of California at San Diego Clinical Laboratories as part of the patient's medical evaluation (<http://www.health.ucsd.edu/labref/labref.html>).

### (Immunohisto)histology

Freshly frozen, unfixed 5- $\mu$ m cryosections were stained with hematoxylin and eosin (HE) or immunostained with an enterovirus group-specific VP1, followed by the Envision/HRP amplification system (both DAKO Systems, Carpinteria, CA) [14]. Primary anti-dystrophin, anti- $\beta$ -sarcoglycan (both Vector Laboratories, Burlingame, CA) or anti-myomesin (gift from H.M. Eppenberger) antibodies were visualized using biotinylated secondary antibodies followed by FITC- or rhodamine-conjugated streptavidin (Vector Laboratories) [2]. Sections were imaged using a Nikon Eclipse E800 microscope (Melville, NY).

### Generation of coxsackievirus B2 protease 2A

The VP1-2A region (nucleotides 3218–3751) was amplified from coxsackievirus B2 (Ohio strain) with reverse transcription-PCR from virus-infected HeLa cells (sense primer: 5'-CCGGAATCCGTTTGGCACAGTATCTTAAAGC-3'; anti-sense primer: 5'-GCTCTAGATTGCTCCATGGCGTCATCTTC-3') and cloned into the *EcoRI/XbaI* sites of pcDNA3.1 mycHis A (Invitrogen, Carlsbad, CA). For enhanced translation *in vitro*, the internal ribosomal entry site (nucleotides 16–530) from pCITE4b (Novagen, Madison, WI) was cloned in front of the VP1 coding

sequence. Site-directed mutagenesis of the active-site cysteine was performed with the Quick Change Mutagenesis Kit (Stratagene, La Jolla, CA). Nucleotide sequencing checked all clones. Recombinant protease was translated *in vitro* using [<sup>35</sup>S]methionine and the rabbit reticulocyte lysate system (TnT T7, Promega, Madison, WI).

### Cleavage of human dystrophin

A human dystrophin miniprotein containing the mapped cleavage site in the hinge 3 region was expressed *in vitro* using [<sup>35</sup>S]methionine (TnT T7, Promega) and dialyzed against protease 2A cleavage buffer [3]. Following incubation with the *in vitro* translated, non-radioactive protease 2A at 30°C, cleavage was detected by SDS gel electrophoresis and autoradiography as described [3].

## Results

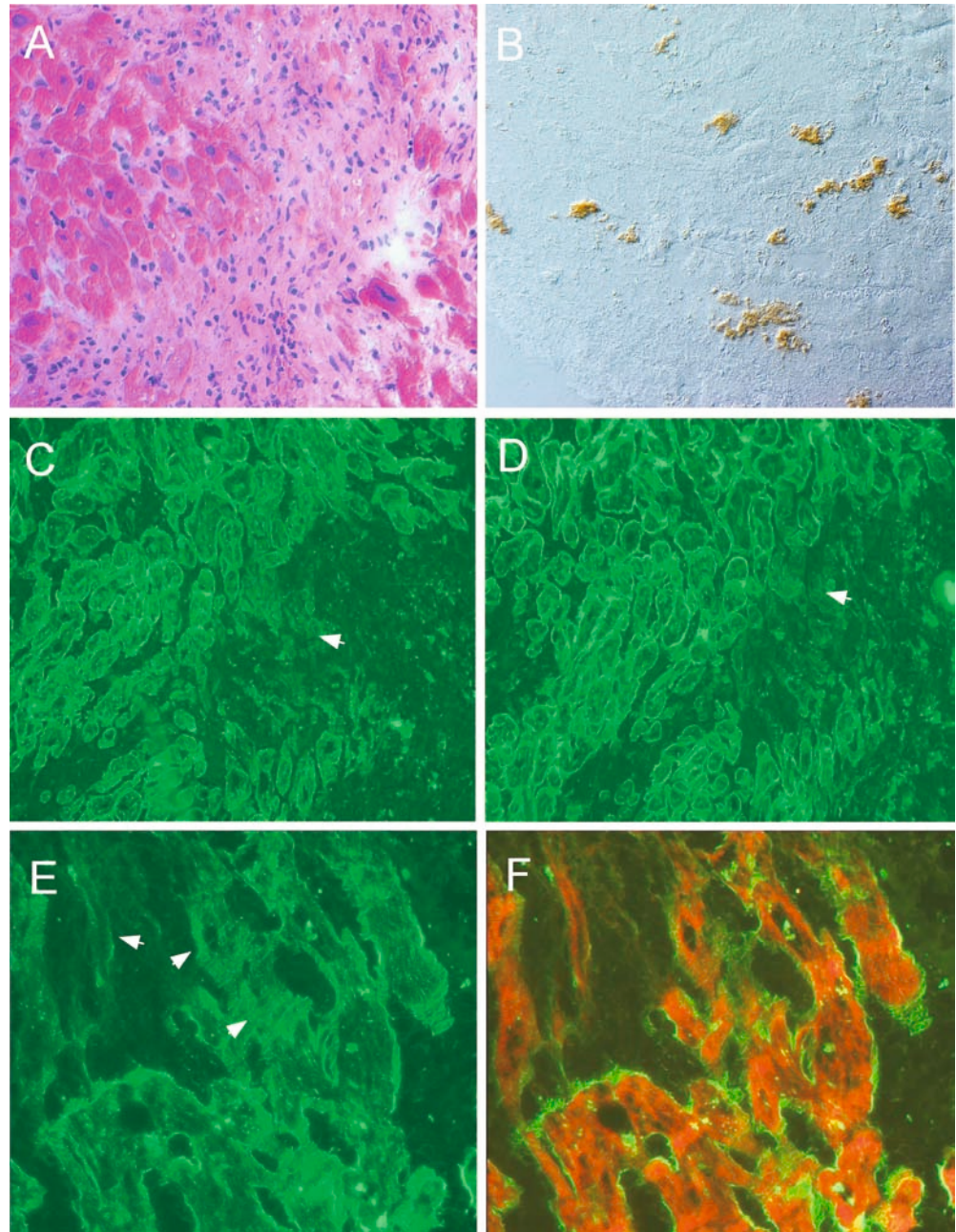
### Dystrophin disruption in human enterovirus-induced cardiomyopathy

Acute and convalescent sera from the patient with acute-onset congestive heart failure and the clinical picture of dilated cardiomyopathy demonstrated seroconversion of the neutralizing coxsackievirus B2 antibody titer from negative (< 1:8) to positive (1:256). Viral titers for other viruses including the other five serotypes of coxsackievirus B, coxsackievirus A, herpes simplex 1 and 2, hepatitis C and cytomegalovirus, were repeatedly negative with the exception of varicella virus antibodies that were detected in the acute sera of the patient.

HE staining of endomyocardial biopsy specimens taken from this patient demonstrated acute myocarditis with a prominent inflammatory infiltrate and myocytolysis (Fig. 1A). Immunohistochemical staining with an enterovirus group-specific VP1 antibody identified foci of virus-infected myocardial cells (Fig. 1B). The acute onset preceded by fever, the seroconversion, the acute myocarditis according to the Dallas criteria, and the presence of enteroviral antigen in the heart all indicate that an acute viral myocarditis was the cause of this patient's dilated cardiomyopathy.

Immunostaining for dystrophin (Fig. 1C) and  $\beta$ -sarcoglycan (Fig. 1D) in the patient with acute myocarditis demonstrated foci of a disrupted staining with a loss of the physiological sarcolemmal localization of dystrophin and  $\beta$ -sarcoglycan (arrows). Double staining for  $\beta$ -sarcoglycan and myomesin (Fig. 1E, F) identified cells with a disrupted  $\beta$ -sarcoglycan staining pattern as cardiomyocytes. Several cardiomyocytes displayed intact myofibrillar striations (Fig. 1F) despite a disrupted  $\beta$ -sarcoglycan staining, indicating that the  $\beta$ -sarcoglycan alteration was not merely a result of myocytolysis. There was also evidence for focal  $\alpha$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan disruption in the same biopsy sample (data not shown). These abnormalities were similar to the disruption of the DGC previously observed in mouse hearts infected with coxsackievirus B3 [2, 13].

**Fig. 1** Dystrophin disruption in human enteroviral myocarditis. Endomyocardial biopsy specimens from a patient with enteroviral myocarditis stained for hematoxylin and eosin (A), enteroviral VP1 (B), dystrophin (C), or  $\beta$ -sarcoglycan (D). The section in E and F was double stained for  $\beta$ -sarcoglycan (green) and myomesin (red). Superimposed stains are in F. The section in E and F was double stained for  $\beta$ -sarcoglycan (green) and myomesin (red). Superimposed stains are in F. The *arrows* point to myocytes with a disruption of the typical sarcolemmal localization of dystrophin and  $\beta$ -sarcoglycan staining pattern. *Arrowheads* point to cells with relatively intact sarcolemmal staining for dystrophin and  $\beta$ -sarcoglycan. *Bar* 50  $\mu$ m



### Coxsackievirus B2 protease 2A cleaves human dystrophin in the hinge 3 region

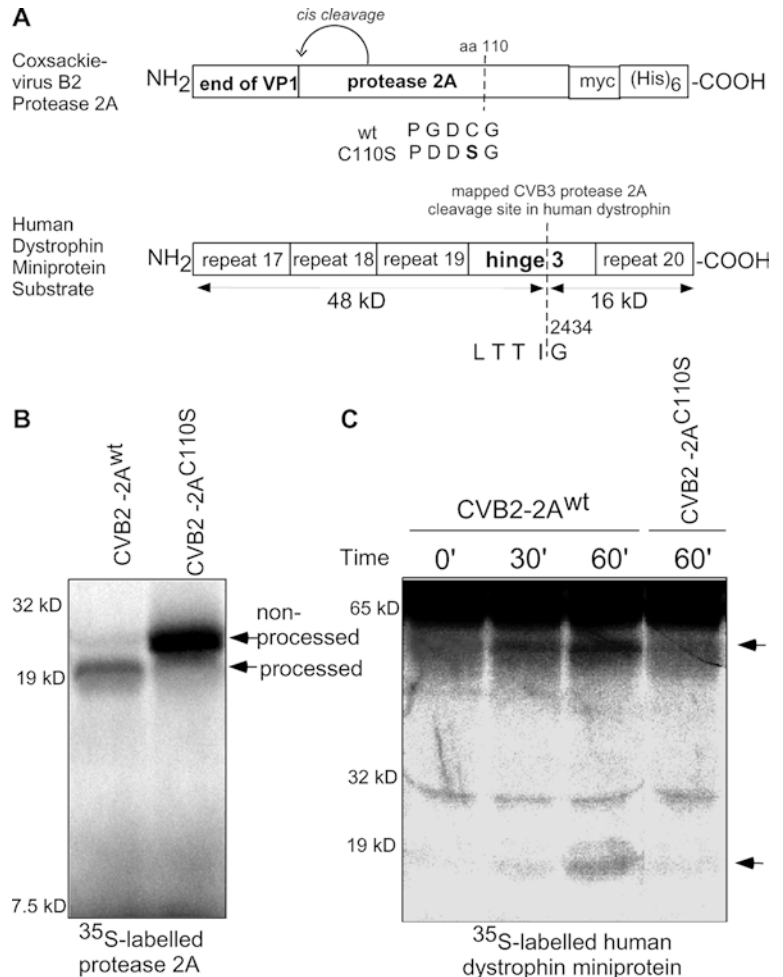
Since the serology identified coxsackievirus B2 as the etiological agent, we tested whether protease 2A from coxsackievirus B2 can cleave human dystrophin. Recombinant wild-type protease 2A and an inactive mutant (C110S) were cloned and expressed *in vitro* using the rabbit reticulocyte lysate system (Fig. 2A). Cleavage of a human dystrophin substrate was analyzed with a dystrophin miniprotein containing the previously mapped cleavage site in the hinge 3 region (Fig. 2A) [3]. As shown in Fig. 2B, only the wild-type,

but not the mutant (C110S) protease 2A was catalytically active and able to cleave itself *in cis* off the end of VP1, thus yielding a shorter and faster migrating protein.

Incubation of the radiolabeled human dystrophin substrate with *in vitro* translated, non-radioactive coxsackievirus B2 protease 2A led to a time-dependent appearance of two dystrophin cleavage fragments consistent with cleavage in the hinge 3 region (Fig. 2C). In contrast, there was no cleavage following incubation with the catalytically inactive protease 2A mutant, demonstrating the specificity of the dystrophin cleavage.

**Fig. 2** Cleavage of human dystrophin by coxsackievirus B2 protease 2A. **A** Schematic diagram of the recombinant coxsackievirus B2 protease 2A and the human dystrophin miniprotein substrate.

**B** Autoradiography of radiolabeled, *in vitro* translated recombinant wild-type coxsackievirus B2 protease 2A (CVB2-2A<sup>wt</sup>) or inactive protease 2A (CVB-2A<sup>C110S</sup>). Only the wild-type protease processes itself by *cis*-cleavage from the end of VP1, thereby generating a faster migrating protein. **C** Autoradiography of radiolabeled, *in vitro* translated human dystrophin miniprotein to which non-radioactive recombinant CVB2-2A<sup>wt</sup> or catalytically-inactive CVB-2A<sup>C110S</sup> has been added for various lengths of time. *Arrows* indicate the two cleavage fragments that occur in a time-dependent fashion following addition of protease 2A. Molecular masses for standard markers are on the *left*



## Discussion

The main finding of the present manuscript is that a focal, morphological dystrophin and sarcoglycan disruption—similar to our previous observations in murine coxsackievirus myocarditis models—was found in a patient with serologically documented coxsackievirus B2 myocarditis clinically presenting with dilated cardiomyopathy.

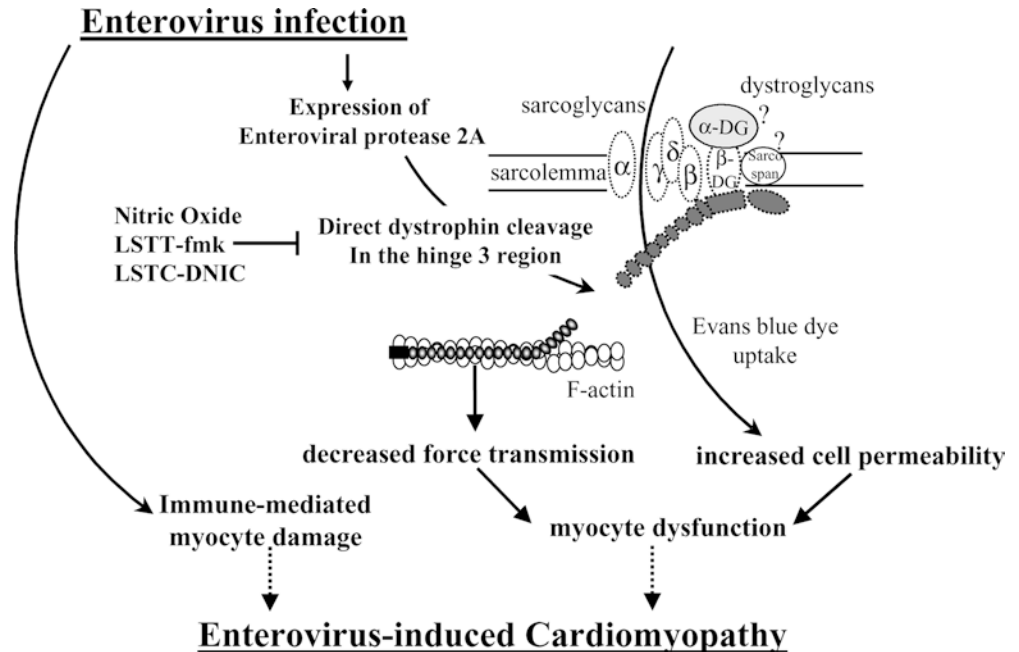
In the endomyocardial biopsy obtained from the patient with serologically documented coxsackievirus B2 infection and active viral replication in the heart, as evidenced by the presence of viral antigens, we demonstrate an absence of dystrophin from the sarcolemma with a diffuse cytoplasmic redistribution. This staining abnormality, also observed for  $\beta$ -sarcoglycan, was observed in numerous cardiomyocytes. Importantly, this abnormality in dystrophin staining was similar to our previous observations in mouse hearts infected with coxsackievirus B3 [2, 13]. The exact prevalence of the described morphological dystrophin disruption among patients with enterovirus-induced heart disease remains to be determined. Nevertheless, these data expand the evidence for a disruption of the DGC in enterovirus-

induced cardiomyopathy from experimental models to a patient with documented coxsackievirus B2 myocarditis, an extension from bench to bedside.

Importantly, we observed that the protease 2A from the disease-causing agent, coxsackievirus B2, was able to cleave human dystrophin *in vitro* at the previously mapped cleavage site in the dystrophin hinge 3 region. The low level of dystrophin cleavage observed in our assay likely results from the low concentration of protease 2A that was translated *in vitro*. Nevertheless, the dystrophin cleavage by coxsackievirus B2 protease 2A may represent the underlying mechanism for the dystrophin disruption detected in the patient with acute coxsackievirus B2 myocarditis.

These results suggest a pathogenetic link between virus-induced dystrophin abnormalities and genetic dystrophin deficiencies. A molecular model summarizing our experimental findings is depicted in Fig. 3. During enteroviral infection, the viral protease 2A is expressed and directly cleaves dystrophin *in trans* in the dystrophin hinge 3 region. This leads to a morphological disruption of dystrophin and other components of the DGC, such as the sarcoglycans and  $\beta$ -dystroglycan, whereas the effects on remaining components

**Fig. 3** Scheme summarizing the putative role of the dystrophin cleavage by the enteroviral protease 2A in the pathogenesis of enterovirus-induced cardiomyopathy



(sarcospan,  $\alpha$ -dystroglycan, syntrophins) are yet unclear. Based on what is known from muscular dystrophy studies, this directly impairs transmission of mechanical force and increases sarcolemmal permeability. These abnormalities trigger a cascade of events that ultimately contributes to the pathogenesis of enterovirus-induced dilated cardiomyopathy.

We further hypothesized that dystrophin deficiency would predispose to enterovirus-induced cardiomyopathy. Accordingly, we demonstrated a time-dependent increase in the severity of cardiomyopathy and an increase in viral replication in Coxsackievirus B2 infected dystrophin-deficient *mdx* mice when compared to Coxsackievirus B2 infected controls. This difference appears to be secondary to more efficient release of the virus from dystrophin-deficient myocytes. In addition, we found that dystrophin expression in cultured cells decreased the cytopathic effect of enteroviral infection [21].

Based on these results, the enteroviral protease 2A represents an attractive target for antiviral drug development. Based on the mapped dystrophin sequence recognized by and interacting with the enteroviral protease 2A [3], we generated peptide-based pseudosubstrate inhibitors of the enteroviral protease 2A by coupling the peptide to an agent that inactivates the catalytic cysteine residue of the protease. For this, we conjugated the tetrapeptide LSTT to fluomethylketone, which can form a covalent bond with the catalytic cysteine leading to protease inactivation [3]. As an alternative strategy, we have conjugated a similar peptide (LSTC) to the nitric oxide-releasing dinitrosyl iron complex [5] since nitric oxide inhibits the enteroviral protease 2A via S-nitrosylation of the catalytic cysteine residue (Fig. 3) [4]. However, the anti-viral properties of these compounds in animal models of coxsackievirus infection remain to be established.

In summary, we have demonstrated that disruption of the DGC can principally occur in human enterovirus-induced cardiomyopathy, a process that may contribute to the pathogenesis of this acquired form of heart failure.

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