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UNIVERSITY OF CALIFORNIA SAN DIEGO

New Therapeutic Approaches for Desmoplakin Mediated Myocarditis Using a Novel Animal Model

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Kimia Shojaee is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

تقدیم به بابای عزیزتر از جانم که با یاد و خاطره هاش زنده ام

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
ARVC	Arrhythmogenic Right Ventricular Cardiomyopathy
BW	Body weight
CRISPR	Clustered regularly interspaced short palindromic repeats
Cx-43	Connexin-43
DES	Desmin
DSC2	Desmocollin-2
DSG2	Desmoglein-2
DSP	Desmoplakin
ECG	Electrocardiogram
EDV	End-diastolic volume
EF	Ejection Fraction
ESV	End-systolic volume
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HW	Heart Weight
JUP	Plakoglobin
KO	Knockout
LV	Left ventricle
MRI	Magnetic Resonance Imaging
MUT	Mutant
N-Cad	N-cadherin
PBS	Phosphate-buffered saline
PKP2	Plakophilin-2
PVC	Premature ventricular contraction
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RV	Right ventricle
SCD	Sudden cardiac death
WT	Wild type

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VITA

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ABSTRACT OF THE THESIS

New Therapeutic Approaches for Desmoplakin Mediated Myocarditis Using A Novel Animal Model

by

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University of California San Diego, 2024

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Desmoplakin (DSP) is a critical protein component of desmosomes, specialized cell structures that function as intercellular junctions and are essential for maintaining the structural integrity of cardiac muscle cells. Mutations or loss of DSP are linked to arrhythmogenic right ventricular cardiomyopathy (ARVC), a genetic cardiac disease characterized by ventricular arrhythmias, fibrofatty myocardial replacement, and sudden

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cardiac death. ARVC patients with DSP mutations often show a unique form of cardiomyopathy associated with myocarditis, further complicating the clinical presentation of this disease.

In my studies, I have investigated the therapeutic potential of restoring Connexin-43 (Cx-43), a key gap junction protein, through gene therapy in a novel mouse model of myocarditis-driven DSP cardiomyopathy. Utilizing a cardiac-specific DSP heterozygous mice (DSP Het) invoked with inflammatory stress, I have assessed the effects of adeno-associated virus (AAV9) mediated Cx-43 gene therapy on electrical, structural and cardiac function. I hypothesized that restoring Cx-43 levels will reduce myocardial inflammation indirectly by stabilizing DSP levels, delaying the progression of ARVC, and improving the electrical and mechanical integrity of the heart.

My thesis studies have shown that Cx-43 restoration stabilizes electrical, mechanical, and structural function of the heart, and improves the levels of desmosomal proteins, particularly DSP. Electrocardiogram (ECG) and magnetic resonance imaging (MRI) analyses showed notable improvements in electrical conduction and cardiac function, respectively, in the treated compared untreated (diseased) group. Histological examinations revealed that treated mice showed no signs of fat or fibrosis, indicating a structural improvement. In conclusion, these findings suggest that Cx-43 gene therapy mitigates the adverse effects of inflammatory mediated DSP deficiency, providing a promising therapeutic strategy for myocarditis driven ARVC in patients with DSP mutations. This research advances our understanding of the molecular mechanisms linking DSP deficiency to myocarditis, highlighting the potential for developing targeted therapies for ARVC patients with DSP mutation

Chapter 1. Introduction

1.1 Desmosomes: Crucial for Maintaining Cardiac Muscle Integrity

Desmosomes are adhesive cell-cell junctions prominently found in epithelial and cardiac muscle cells [1]. They attach the intermediate filament cytoskeleton to the cytoplasmic membrane, playing a crucial role as cellular mechanical anchors and providing mechanical strength in tissues with high tensile stress, such as the heart [1]. Desmosomes in cardiac muscle are composed of a complex of different protein subfamilies [1]. The first subgroup, desmosomal cadherins, includes desmocollin-2 and desmoglein-2 and is regulated by calcium [1]. Another subgroup includes the armadillo proteins, which act as accessory proteins and consist of plakoglobin and plakophilin-2 [1]. Central to these structures is the plakin protein desmoplakin (DSP), which is most proximal to the intermediate filaments [1]. For desmosomes to function properly, desmoplakin must effectively connect desmosomal cadherins to the intermediate filament cytoskeleton [1]. Desmin, an intermediate filament protein specific to cardiac muscle cells, provides structural support and integrity by anchoring to DSP within desmosomes, linking the cytoskeleton to cell junctions and contributing to the mechanical resilience of heart muscle cells [1]. Additionally, fascia adherens junctions, serve as structural tethers within cells that link the actin cytoskeleton to membrane-bound cadherins, such as N-Cadherin, which connects to intracellular catenins and catenin-binding proteins [1, 2] . Fascia adherens junction and desmosomes, present at cardiac cell-cell junctions, work together to maintain cell-cell adhesion and signaling in cardiac tissue [1, 2]. Newly discovered desmosomal proteins, such as COP9 signalosome unit 6 (CSN6), interact with DSP, indicating new regulatory functions like protein degradation at the desmosome [3]. This emphasizes the complex nature of desmosomal function and highlights the central role of DSP in regulatory mechanisms at the cell-cell junction.

1.2 Desmoplakin a Central Linker Between Junctional Components and Cytoskeleton

Desmoplakin (DSP) is the central component of the desmosome complex. [1, 4-6]. DSP is important in transmitting force during cardiac contraction by bridging cardiac desmosomes and intermediate filaments [7]. DSP is found to be the most abundant protein in desmosomes within the heart, playing an important role in cell adhesion by anchoring the intermediate filaments to desmosomal plaques [8-11]. This is crucial because it helps anchor myocardial cells, providing strength for these cells and maintaining the structural integrity of tissues especially during high tensile stress [12]. The emerging role of CSN6 in desmosome function adds another layer of complexity, based on its interaction with DSP and its ability to regulate protein degradation at the desmosome [3]. These data highlight that there are intricate regulatory mechanisms mediated by DSP involved in maintaining cardiac muscle integrity. The importance of DSP extends to human cardiac disease, as mutations or loss of DSP have been linked to both autosomal dominant and recessive forms of the genetic cardiac disease called arrhythmogenic right ventricular cardiomyopathy (ARVC) [13]. Genetic mouse models have been instrumental in showing that homozygous loss of cardiac DSP or expression of DSP mutation is sufficient to drive recessive forms of ARVC [6, 13-17].

1.3 Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC)

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a genetic heart disease that is inherited through autosomal dominance in more than 50% of cases [18]. While ARVC affects the right ventricle of the heart, the disease can progress to the left ventricle as well result in left ventricular failure and biventricular forms of ARVC [14]. To better describe these conditions, the term “Arrhythmogenic cardiomyopathy” was suggested by the scientific community, although this

has yet to be fully adopted [19-21]. ARVC has been the cause of sudden death, especially among young individuals and athletes under the age of 40 years old, often triggered by physical exercise [21]. ARVC case numbers vary in different geographic locations affecting 1 in 2000-5000 individuals [22-24]. Early stages of ARVC are marked by cardiac arrhythmias, prior to structural changes becoming visible later in the progression of the disease [25]. Structural characteristics of ARVC include replacement of lost cardiac muscle by fibro-fat or fibrosis, inflammation and ventricular dysfunction [19, 20, 26]. ARVC can be asymptomatic during its early phase, making diagnosis challenging due to the heterogenous clinical presentation of affected patients [27]. Clinical diagnosis of ARVC involves identifying the electrical abnormalities, especially the ventricular arrhythmias during the early phases, and then the structural and functional changes in the right ventricle, which can include fibrofatty replacement through endomyocardial biopsy [28, 29]. There are no treatments for ARVC. Management approaches mostly focus on symptomatic relief, such as anti-arrhythmic drugs and beta-blockers [30, 31]. Patients are also recommended to avoid competitive sports to reduce their risk of sudden cardiac death. Exercise restriction is also important to keep in mind for affective individuals and their families to prevent sudden heart failure [30]. In severe and late stages, implantable defibrillators and heart transplant may be necessary [30]. These data altogether highlight a cardiac disease with a high unmet need as well as the need to identify early triggers and therapeutic targets for this disease.

1.4 Desmoplakin-Mediated Cardiomyopathy with a Unique Relationship to Myocarditis

Growing evidence highlights that cardiac inflammation, in the form of myocarditis, is thought to be an early component of ARVC [32]. Myocarditis is a term used for inflammation of the heart muscle [33]. It can be triggered by infections, toxic substances, and autoimmune

processes [33]. DSP-related cardiomyopathy has been identified as its own unique condition within ARVC, due to its strong and early association with myocarditis and myocardial inflammatory episodes [34]. It is also considered its own unique condition within dilated cardiomyopathy (DCM), because of its association of arrhythmias with left ventricular dominant nature of the disease [35]. Patients with DSP mutations present with recurrent myocarditis and worsened cardiac outcomes (worse cardiac dysfunction (especially in women) and lethal arrhythmias, mortality) [36], making these patients one of the most vulnerable populations within ARVC. Despite, the link between mutations and/or loss of DSP and inflammatory episodes in heart tissue, there are limited animal models and mechanistic insights into therapeutic targets for DSP-related myocarditis which results in cardiomyopathy in this patient population.

1.5 Generation of an Animal Model of Myocarditis Driven Desmoplakin-Mediated Cardiomyopathy

In cardiac biology, mice have become the preferred models for genetic manipulation [37]. This approach offers a direct path to investigate the molecular mechanisms that drive genetic based cardiomyopathies [38]. Even though the technologies for assessing cardiac function in mice is well established, there are still some limitations in depicting cardiac phenotypes in genetic mouse models with heterozygous loss or mutations in desmosomal genes [14, 37]. This is particularly important as autosomal dominant genetics govern desmosomal mutations and/or loss, mirroring observations in ARVC in humans [14].

Addressing these limitations, the Sheikh lab has developed a pre-clinical mouse model to study myocarditis driven DSP-mediated cardiomyopathy. This model utilizes their previously published cardiac-specific *DSP* heterozygous (*DSP* Het) mice [14], which show 50% reduction in cardiac DSP protein levels, replicating cardiac DSP “haploinsufficiency” seen in patients with

DSP mutations (data not shown). These mice remain asymptomatic for at least one year (data not shown), allowing for the assessment of inflammatory stress in the absence of pre-existing cardiac phenotypes. Utilizing a previously published inflammatory stress model that includes myocarditis induction combining alpha-MHC peptide and pertussis toxin [[39], **Figure 1A**], eight to ten week old *DSP* Het and wild type (WT) littermate mice were acutely exposed to this inflammatory protocol. At day 0, the initial injection of alpha-MHC peptide emulsified in Complete Freund's adjuvant (CFA) is administrated to induce an autoimmune response targeting cardiac myosin, followed by an injection of pertussis toxin on the same day to enhance the immune response by facilitating the entry of immune cells into the heart muscle [40]. On day 7, another injection of alpha-MHC peptide is given to reinforce and sustain the immune response initiated on day 0 [41]. This second dose is necessary for the development of chronic myocarditis symptoms. This cardiac myosin-specific autoimmune myocarditis model is an established mouse model in the field (used for decades) that is essential to understand the role of the immune cells in inflammatory cardiomyopathy [40]. As shown in **Figure 1**, *DSP* Het mice develop chronic cellular immunity-related cardiac toxicity (presence of inflammatory infiltrates) that is reminiscent of myocarditis (**Figure 1B**). This inflammatory response drives ARVC related deficits including right ventricular enlargement (**Figure 1C**), epicardial fat deposition (**Figure 1D**), fibrosis deposition (**Figure 1E**), cardiac arrhythmias as shown as premature ventricular beats (**Figure 1F**) and ventricular dysfunction with most prominent in the right ventricle (**Figure 1G**) when compared to WT littermate controls (**Figure 1G**). By generating this mouse model of DSP-related myocarditis, we have the potential to further investigate the role of molecular mediators in reducing myocardial inflammation and improving cardiac function.

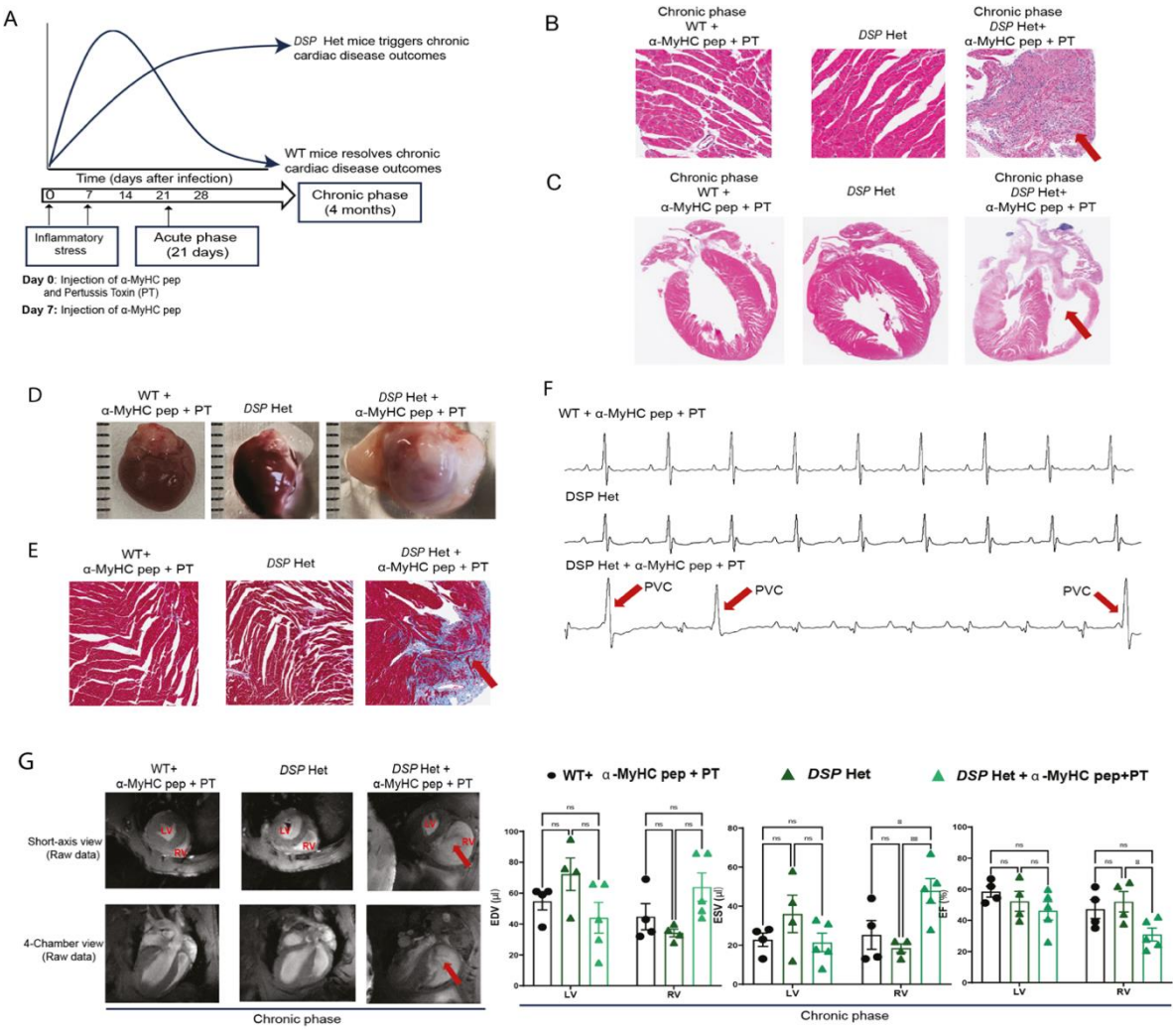

Figure 1. DSP Het mice are predisposed to myocarditis-driven cardiomyopathy following "acute inflammatory stress". (A) Diagram of inflammatory stressors applied on Day 0 (alpha-MHC peptide + pertussis toxin) and Day 7 (alpha-MHC peptide) in 6–8-week-old mice to induce myocarditis, with chronic endpoint assessments at 4 months post-treatment. (B-C) Hematoxylin & Eosin-stained cardiac sections exhibit significant lymphocytic infiltration (red arrow) and an enlarged right ventricular chamber (red arrow). (D) Representative cardiac gross morphology shows fat deposition and fibrosis in DSP Het mice subjected to "acute inflammatory stress".(E):Representative Masson trichrome stain displays Fibrosis (red arrow). (F) Surface ECG analysis displays premature ventricular contractions (PVC) in DSP Het mice subjected to "acute inflammatory stress". (G) MRI analysis of left ventricular (LV) and right ventricular (RV) dimensions, including end-diastolic volume (EDV), end-systolic volume (ESV), and ejection fraction (EF), highlighting RV dilation and dysfunction in DSP Het mice subjected to "acute inflammatory stress".

1.6 Unique Relationship Between Desmoplakin and Connexin-43 In Heart Muscle Cells

Desmoplakin mutations or loss in ARVC patients and mouse models have a direct impact on neighboring gap junctions at the cell-cell junction [1, 5, 6, 14, 16]. Gap junctions are essential for rapid electrical transmission between cells [1]. These junctional proteins are primarily composed of connexin proteins, where six connexin monomers form hemichannels in the membrane [42]. One of the most important gap junctions in the heart is connexin-43 (Cx-43), which serve as pore-like channels that allow for the exchange of small molecules and ions between adjacent cells [43-45]. This gap junction, especially Cx-43 is essential for facilitating electrical communication between cells and ensuring synchronized contraction in the heart muscles [42].

To further highlight this relationship the Sheikh lab has shown dose-dependent assessments following DSP loss in neonatal ventricular cardiomyocytes and revealed a primary reduction in Cx-43 levels, phosphorylation, and function [14]. This reduction occurs independently of the molecular dissociation of the mechanical junction complex, indicating that DSP is essential for stabilizing Cx-43 integrity. Importantly, the downregulation of Cx-43 appears to be the most sensitive (amongst all cell-cell junction proteins) to DSP loss in cardiac muscle as observed in DSP-cKO hearts [14]. Additionally, another study conducted by a different group used blinded immunohistochemical analysis of heart-biopsy samples from patients, revealed that Cx-43 signal levels were significantly diminished in subjects with DSP deficiency [46]. These observations suggest that the remodeling of Cx-43 is closely tied to DSP levels, supporting the notion that loss of DSP has primary consequences on Cx-43 levels and gap junction integrity, highlighting a unique and primary relationship between DSP and Cx-43.

1.7 Rationale, Aim, Hypothesis:

Desmoplakin (DSP) loss is uniquely linked to connexin-43 (Cx-43) loss in cardiomyocytes [14, 47]. Connexin-43 is a crucial component that classically facilitates electrical communication between adjacent cardiomyocytes, ensuring synchronized contraction of the heart muscle [48]. Disruption of Cx-43 due to DSP deficiency impairs electrical conduction and increases the risk of arrhythmias [14]. For instance, studies have reported a 50% reduction in Cx-43 expression significantly affects electrical conduction and contributing to heart failure progression [49]. Studies performed in a cardiac conduction-specific desmoplakin knockout model also show this unique relationship as cardiac conduction-specific DSP loss resulted in loss of SAN-related connexins and dysfunction of the sinus node, which included defects in the dominant pacemaker activity, causing increased sinus pauses in both mice and humans [50]. To further highlight this relationship supporting evidence from high-pacing-induced heart failure (HF) models shows that the remodeling of desmosomes and gap junctions, including changes in protein abundance and location, is fundamental to severe arrhythmogenicity [49]. In these models, the lateralization and reduced expression of Cx-43, which are a result of DSP deficiency, contribute to the uncoupling of gap junctions. This uncoupling results in slowed conduction and facilitates the development of arrhythmias [49]. This interconnection is further suggested by findings in models of pressure overload-induced pulmonary hypertension, where desmosome junctions were found to be distributed parallel to the direction of myocardial fibrosis similar to lateralization pattern observed for Cx-43 [49]. These findings suggest a significant interconnection between the structural integrity of desmosomes and proper localization of Cx-43.

Patients harboring DSP mutation and cardiomyopathy have shown episodes of recurrent myocarditis and worsen outcomes [34]. Myocarditis, characterized by inflammation of the heart

muscle, is commonly observed in DSP-related cardiomyopathies [34]. Emerging evidence suggests that viruses can exploit host cell junctions, such as gap junctions, to spread and amplify toxic factors and "damage signals" to adjacent uninfected cells [51]. Studies have shown that connexins, specifically Cx-43, can be utilized by viruses to facilitate the spread of infection and amplify pathogenic effects [51]. This interaction suggests that targeting specific cell junction proteins, such as connexins, at the point of cellular entry could be a potential therapeutic strategy. Although the exact mechanisms triggering myocarditis in DSP-related cardiomyopathy are not fully understood, the correlation between DSP loss and Cx-43 mis localization suggests a potential intermediate link. The disruption of desmosomes directly leads to the rearrangement of Cx-43 distribution, which is important for understanding the progression of ARVC and myocarditis [14]. However, there are limited studies that explore the specific impact of restoring Cx-43 levels and arrhythmias in DSP-related cardiomyopathy. Therefore, in this study, we aimed to assess whether restoring Cx-43 levels in the Sheikh lab's DSP-related myocarditis model will also restore levels of desmosome proteins, primarily DSP, and thereby reduce myocardial inflammation, arrhythmias and cardiomyopathy.

Given the inter-related mechano-electrical regulatory nature between DSP and Cx43 as well as studies that suggest that gap junctions can be vulnerable points for virus entry and amplification, we hypothesize that the gap junction mediator, Cx43, may be an important checkpoint and mediator of DSP-related myocarditis and subsequent ARVC pathogenesis. DSP mutations and/or loss will trigger loss of Cx43 and render the cardiomyocyte vulnerable to inflammatory stress and ensuing ARVC progression and pathogenesis. Thus, restoring Cx43 expression will improve cell communication, thereby enhancing electrical and mechanical

integrity of the heart. This approach could potentially provide therapies and improved prognosis for DSP patients exhibiting myocarditis in the future.

Chapter 2. Materials and Methods

All animal procedures were in full compliance with the ethical guidelines approved by university of California San Diego Animal Care and Use Committee.

2.1 Previously Developed Cardiac-specific DSP Heterozygous (DSP Het) Mice via MLC2v-Cre Cross-breeding

Previously, DSP heterozygous (DSP Het) mice were developed with the genotype DSP flox/+ (Flox; +). The Sheikh lab developed a breeding strategy to achieve ventricular-specific loss of DSP by cross-breeding these mice with MLC2v-Cre mice. This cross-breeding strategy, as previously published, has allowed for the creation of ventricular cardiomyocyte-specific homozygous DSP-cKO [DSPflox/flox: MLC2vCre (+)], heterozygous DSP-cKO [DSPflox/+: MLC2vCre(+)], and corresponding littermate controls [DSPflox/flox: MLC2vCre(−)]. These mice and their littermates were maintained on a congenic C57BL/6J background to ensure minimal variability in experimental results due to genetic differences between individual mice [14].

2.2 Myocarditis induction & Adeno-associated virus (Human- AAV9) treatment

At seven weeks of age (Day -1), mice received human AAV9-Cx-43 virus via retro-orbital injection using 31-gauge needle and syringe (Monoject,8881600800). Each injection delivered 1 $\times 10^{12}$ viral particles per mouse, 5 E13 gc/kg. Mice were induced with myocarditis with *of* alpha-MHC peptide + pertussis toxin via intraperitoneal (IP) injection at 8 weeks of age (Day 0), followed by a second injection with alpha-MHC peptide at 9 weeks of age (Day 1).

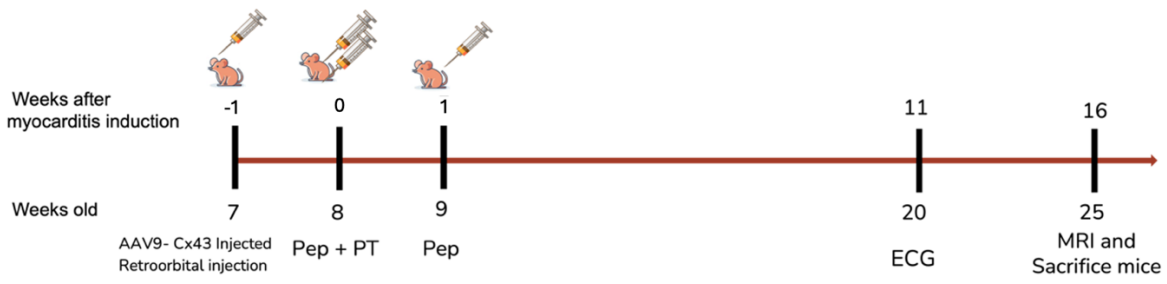

Figure 2. Timeline of the protocol followed to assay the effect of AAV9-Cx43 gene therapy in a myocarditis model in DSP het mice.

2.3 Surface Electrocardiogram

Adult (20 weeks old) mice were anesthetized with 1% isoflurane during the procedure. Needle electrodes (30 gauge) were inserted subcutaneously into the right forearm and left leg. Electrical activity was recorded for 5 minutes. ECG signals were analyzed for 100 consecutive beats, with specific wave parts identified manually to calculate heart rates, PR intervals, and QRS intervals. Abnormal heart beats were identified throughout the entire 5-minute recording for further analysis. The number of PVC's were analyzed using the LabChart version v8.1.27.

2.4 Magnetic Resonance (MRI)

In vivo cardiac MRI was performed in a 7T horizontal bore MR scanner. Twenty-week-old mice were anesthetized at 1-2% isoflurane for the scan. A 1.9-mm custom built dual quadrature volume driven transverse electromagnetic coil was used for radiofrequency signal transmission and a rapid MRI, a two-channel surface array coil was used to measure the RF signal. Cardiac CINE images were taken with a special imaging technique, Gate (Bruker) which is retrospective two-dimensional gradient echo pulse sequence known as FLASH with the parameters of TE=3.1ms, TR=5.6ms, and a flip angle of 7° with about 300-400 repetitions and 20 frames. The images obtained has a field of view 2.0 x 1.5 cm and had a high resolution of 0.078 mm per pixel.

To analyze the MRI images, we manually chose the inner contours of the heart in the two-chamber view. The software segment CMR version 4.0 R12067 was used to calculate the volumes of the heart chambers during end diastole and end-systole phase for both the right and left ventricle. Sum of the volumes from multiple slices were taken to obtain the total chamber volumes. Ejection fractions were averaged across all slices to analyze the function of the heart.

2.5 Histological Analysis

Twenty-week-old adult mice were perfused in relaxation buffer consisting of 300mM KCl (Sigma-Aldrich, P9333) in phosphate-buffered saline and fixed with 4% paraformaldehyde (ThermoFisher Scientific, J61899.AK). To proceeded with histology, fixed heart tissues were put in Tissue-Tek OCT (Sakura, 4583). Heart tissues were cut between 7μm and 10 μm in thickness. The 2-chamber view hearts were stained with hematoxylin and eosin (Sigma-Aldrich, HT109-1SET) to detect fibrosis. Mason Trichrome (Sigma-Aldrich, HT15) stain was used to detect collagen deposition and used according to manufacturer's instructions. Images were captured using the Hamamtsu Nanozoomer 2.0 HT slide scanner.

2.6 RNA Analysis – Quantitative PCR (qPCR)

Total RNA was extracted from twenty-week-old mouse hearts using TRIZOL (Invitrogen). After RNA isolation, the first strand of cDNA was created using the iScript RT reagent kit by Biorad. Using specific primer sequences, to look at human Cx-43 genes, (Cx-43) primers (forward, AGCAGAGCCAGCAGTC) (reverse, GAGCAACATAGTTAAGAATACCAGTC), WPRE-2 primers (forward, GCTATGTGGATACGCTGCTTTA) (reverse,

AGAGACAGCAACCAGGATTTATAC). For analysis Luna universal qPCR master mix (New England BioLabs, M3003L) and was conducted using Bio-Rad Master cycler. PCR products were sequenced using Eton Biosciences.

2.7 Protein Analysis -Western Blot

Insoluble proteins were extracted from twenty-week-old mouse ventricles, using previously published protocol [14]. About 40 ug of cardiac tissue was for 2 rounds at 8 seconds in 300 ul of lysis buffer (10% NP40, 0.1 M EGTA, 0.5 M EDTA, 5M NaCl, 1M Tris HCl pH 7.5, MiliQ H2O). The homogenized samples were separated into 2 tubes, each containing 150 µl of the sample. One tube was centrifuged at 4°C for 10 minutes at 14,000 rpm. The supernatant, containing soluble proteins, was transferred to a new tube. The pellet was resuspended with an additional 150 µl of JNK complete lysis buffer to extract the insoluble proteins of interest. Protein lysates were prepared using 1x NuPAGE LDS sample buffer and 10% DTT. Further the insoluble protein samples were loaded onto 4-12% NuPAGE gel. Proteins were transferred onto polyvinylidene difluoride membranes at 35 mA for overnight incubation at 4° Celsius. Membranes were blocked in 5% milk in 1x TBST (1X TBS, 0.1% Tween) for 1 hour at room temperature with gentle shaking. Membranes were put in primary antibodies, such as DSP (mouse, 1:1000, Bio-Rad, 2722-504), desmoglein-2 (mouse, 1:1000, fitzgerald, 10R-D106a), plakophilin-2 (mouse, 1:1000, Sigma-Aldrich). Connexin-43 (Rabbit, 1:5000, Sigma-Aldrich, C6219) B-actin (mouse, 1:1000, Santa Cruz Biotechnology, sc-47778). After primary incubation, membranes were washed first for 10 minutes and two other times for 5 minutes with 1x TBST at room temperature with gentle shaking.

Incubation with Secondary antibodies, donkey anti-rabbit igG (H+L) HRP (1:3000, ThermoFisher Scientific, A16023), and donkey anti-mouse igG (H+L) HRP (1:3000, ThermoFisher Scientific, A16011) and donkey anti-goat IgG (H+L) HRP (1:3000, ThermoFisher Scientific, A15999) was performed for 1 hour and 30 mintures with gentle shaking in room temperature. For imaging, chemilumiscent substrates (SuperSignal West Femto chemiluminescent subraste, ThermoFisher, YJ375848) (SuperSignal West Pico Plus chemiluminescent substrate, ThermoFisher,34580) were used at 1:1 ration to detect protein levels of interest.

Chapter 3. Results

3.1 Restoration of Connexin-43 restores neighboring proteins in DSP-Het mice induced with myocarditis: Western Blot Analysis

Given previous studies showing that desmoplakin mutations or loss in ARVC patients and mouse models directly impact neighboring gap junction proteins [14], we investigated whether restoring Cx-43 would also help restore desmoplakin protein levels by performing proteins analysis . To further determine the impact of Cx-43 gene therapy on *DSP* heterozygous mice at the chronic stage, we examined the expression of Cx-43 in whole ventricles from DSP het mice induced with myocarditis, and DSP Het mice treated with AAV9-Cx43. We observed a reduction in Cx-43 and DSP levels in *DSP* het mice induced with myocarditis compared to WT and DSP het groups **(Fig.7).** Interestingly, *DSP*-het treated with AAV9-Cx43 demonstrated an increase in Cx-43 and DSP protein expression levels compared to the diseased group **(Fig.7**). This indicates that the AAV9-Cx43 treatment was effective in restoring Cx43 levels, which in turn helped to restore desmoplakin levels. Moreover, the increased protein expression observed in the treated DSP-Het mice confirms that the AAV9-Cx43 virus was effective in delivering the Cx43 gene and enhancing its expression. This finding highlights the potential role of Cx43 restoration in primarily restoring desmoplakin protein levels and suggests a promising therapeutic approach for future ARVC patients with DSP mutations harboring myocarditis.

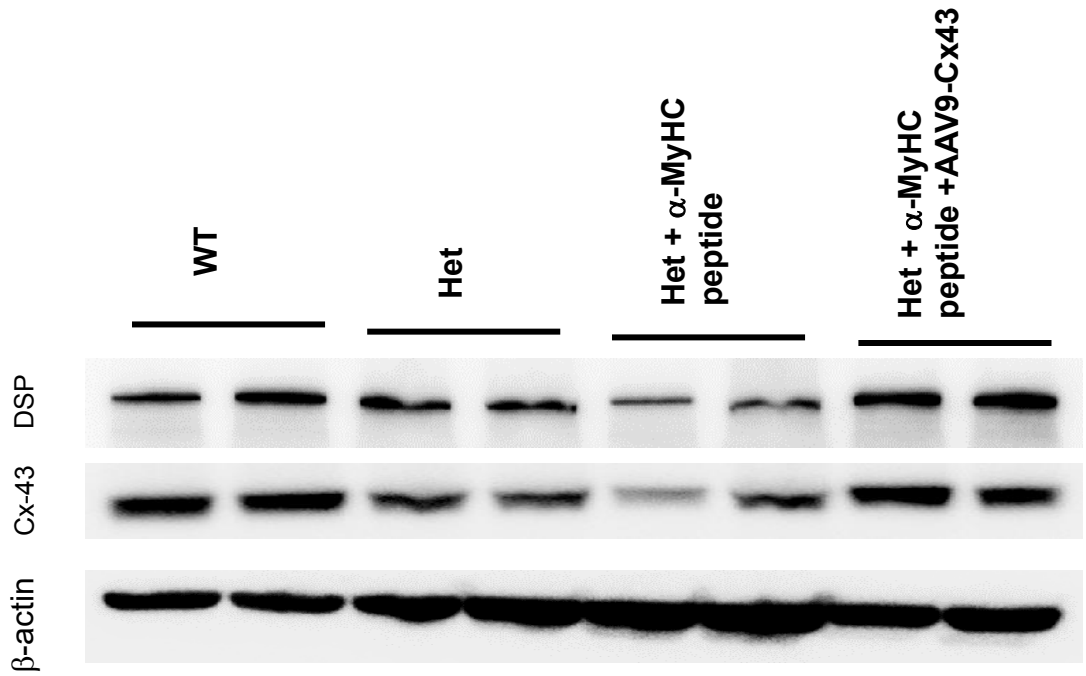

Figure 3. Representative western blot analysis (Insoluble) of Desmoplakin (DSP), and Connexin-43 (Cx-43) at 20 weeks of age. Beta-actin serves as the loading control.

3.2 Gene Expression Analysis Confirms Cx-43 Protein Restoration in DSP-Het Mice

To further assess the molecular impact of Cx43 restoration in DSP-Het mice induced with myocarditis, and mice treated with AAV9-Cx43 we performed quantitative PCR (qPCR) to evaluate the expression levels the Cx-43 gene associated with cardiac function. The qPCR analysis revealed significant differences in gene expression across the various experimental groups. The gene expression of Cx43 was reduced in DSP-Het mice induced with myocarditis compared to the wild-type (WT) **(Fig.7)**. However, DSP-Het mice treated with AAV9-Cx43 showed a significant increase in the gene expression levels of Cx43 compared to the untreated diseased group **(Fig.7)**. This indicates that the gene therapy was effective in restoring Cx43 expression. These qPCR results correlate with our Western Blot findings, further confirming the efficacy of AAV9-Cx43 treatment in restoring not only protein levels but also the gene expression of crucial cardiac proteins. The restored expression of these genes highlights the potential of Cx43 gene therapy in ameliorating the detrimental effects of DSP mutations in the context of myocarditis, paving the way for new therapeutic strategies for ARVC patients.

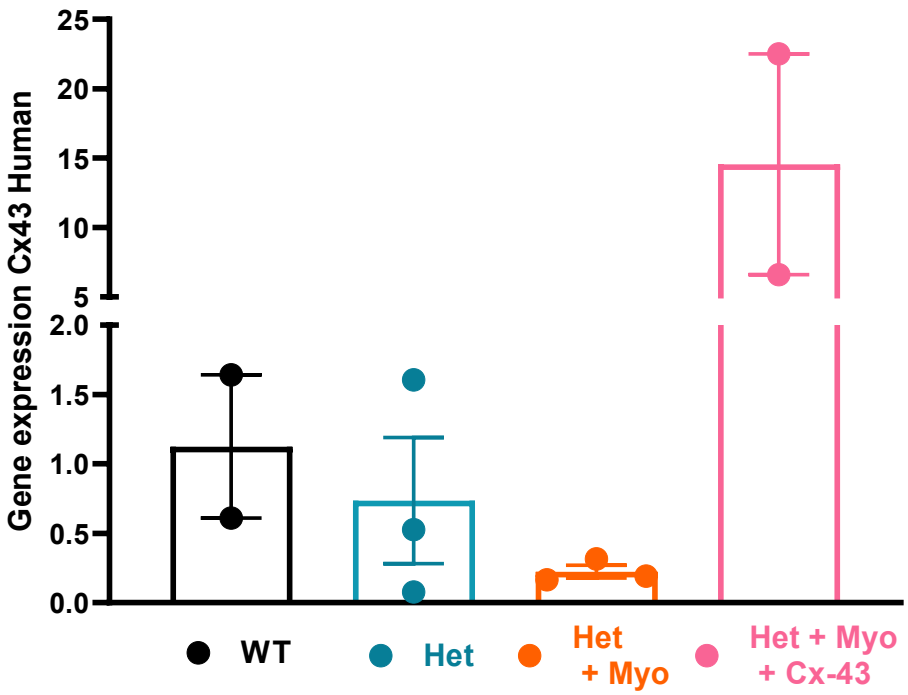

Figure 4. qPCR analysis of gene expression levels in WT, Het, Het + Myo, and Het + Myo + Cx-43 groups.

3.3 Electrical Dysfunction and Arrhythmias in DSP Het Mice Treated with AAV9-Cx43

Given with shown evidence that arrhythmias are the leading cause of sudden death in ARVC patients [52], I further investigated the role of Cx-43 gene therapy whether it improved the electrical function of the heart in 20-week-old mice. We analyzed the three groups; control group (DSP Het), diseased group (DSP Het + Myocarditis), and the treated group (DSP Het + Myocarditis treated with AAV9-Cx-43 virus).

After 11 weeks of myocarditis induction (20 weeks old), electrocardiogram (ECG) was performed to look deeper into the electrical defects in the DSP Het mice. The ECG tracings of the three groups showed no significant differences in their PR intervals and heartbeat **(Fig.3A)**. The diseased group showed evidence of prolonged QRS duration, and premature ventricular contraction (PVC) **(Fig.3B-C)** indicative of ventricular depolarization issues, which can be a primary factor for ventricular re-entry that led to premature ventricular contractions (PVCs) [14]. Meanwhile the treated group with AAV9-Cx43 showed noticeable improvements in both PVCs and QRS duration after Cx-43 gene therapy **(Fig.3C).** These results suggest that Cx-43 deficiency in DSP Het mice disrupts ventricular electrical conduction, and by restoring Cx-43 one can improve these conduction abnormalities.

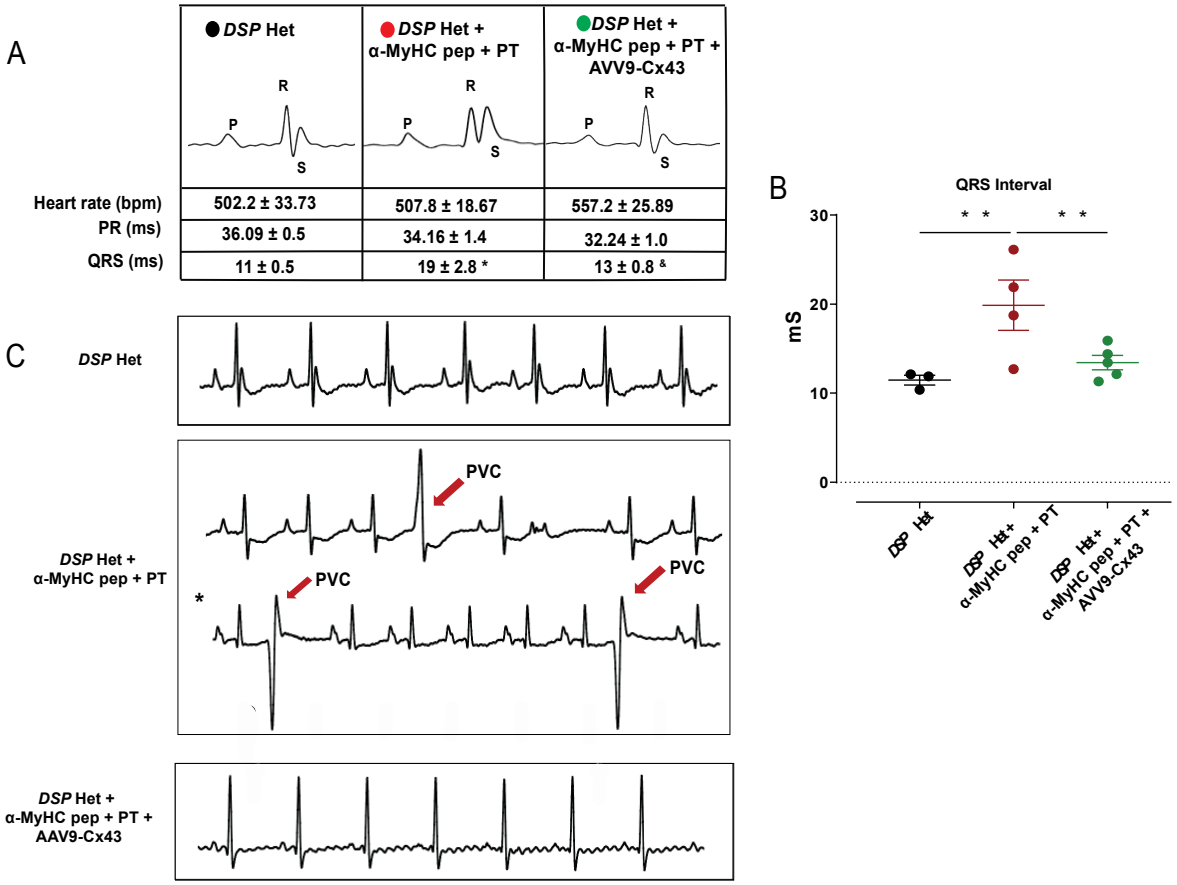

Figure 5. DSP het mice treated with AAV9-Cx43 show improvement in their cardiac electrical function. (A) ECG tracing from the control, diseased and treated DSP het mice with AAV9-Cx-43. Mathematical analysis of heart rate, PR, and QRS intervals from 100 ECG tracings per mouse was performed. (B) QRS intervals of the control, diseased and treated DSP het mice with AAV9-Cx-43. (DSP het, n=3; , DSP Het + alpha-MHC peptide + pertussis toxin, n=4; DSP Het + alpha-MHC peptide + AAV9 Cx-43, n=5 biologically independent animals). (C) Representative ECG tracings from DSP het, diseased group and treated DSP het with AAV9-Cx43 at 11 weeks after the first injection (20 weeks of age) and quantifications of mice showing premature ventricular contractions (PVCs) indicated in red arrows.

3.4 Mechanical Function in DSP Het Mice Treated with Cx-43

Given that ventricular abnormalities are one of the major characteristics of ARVC patients, DSP-Het mice were subjected to magnetic resonance imaging (MRI) at 20 weeks of age, 11 weeks post-injection. To better study the characterization of right and left ventricular dimensions and function, serial short-axis cine MRI views of the mouse heart were obtained. The endocardial borders at end-diastole and end-systole were measured to assess the volume and function of the heart. Physiological studies, such as MRI, are performed under isoflurane, which decreases heart rate and cardiac function [53]. Therefore, it is important that heart rates remain consistent throughout the experiment to ensure that functional differences are not driven by anesthesia. No significant differences were observed in the heart rate between the control, diseased, and treated groups. The diseased mice showed enlargement of the ventricles, especially the right ventricle, high end-systolic and end-diastolic volumes, low ejection fraction, and overall cardiac dysfunction compared to the control group **(Fig. 4B)**. Mice treated with AAV9-Cx43 demonstrated improved ejection fraction and better structural integrity, especially in the right ventricle, compared to the diseased group **(Fig. 4B)**. These results highlight the potential therapeutic effect of Cx-43 restoration in improving cardiac function and structure in DSP-Het mice with myocarditis.

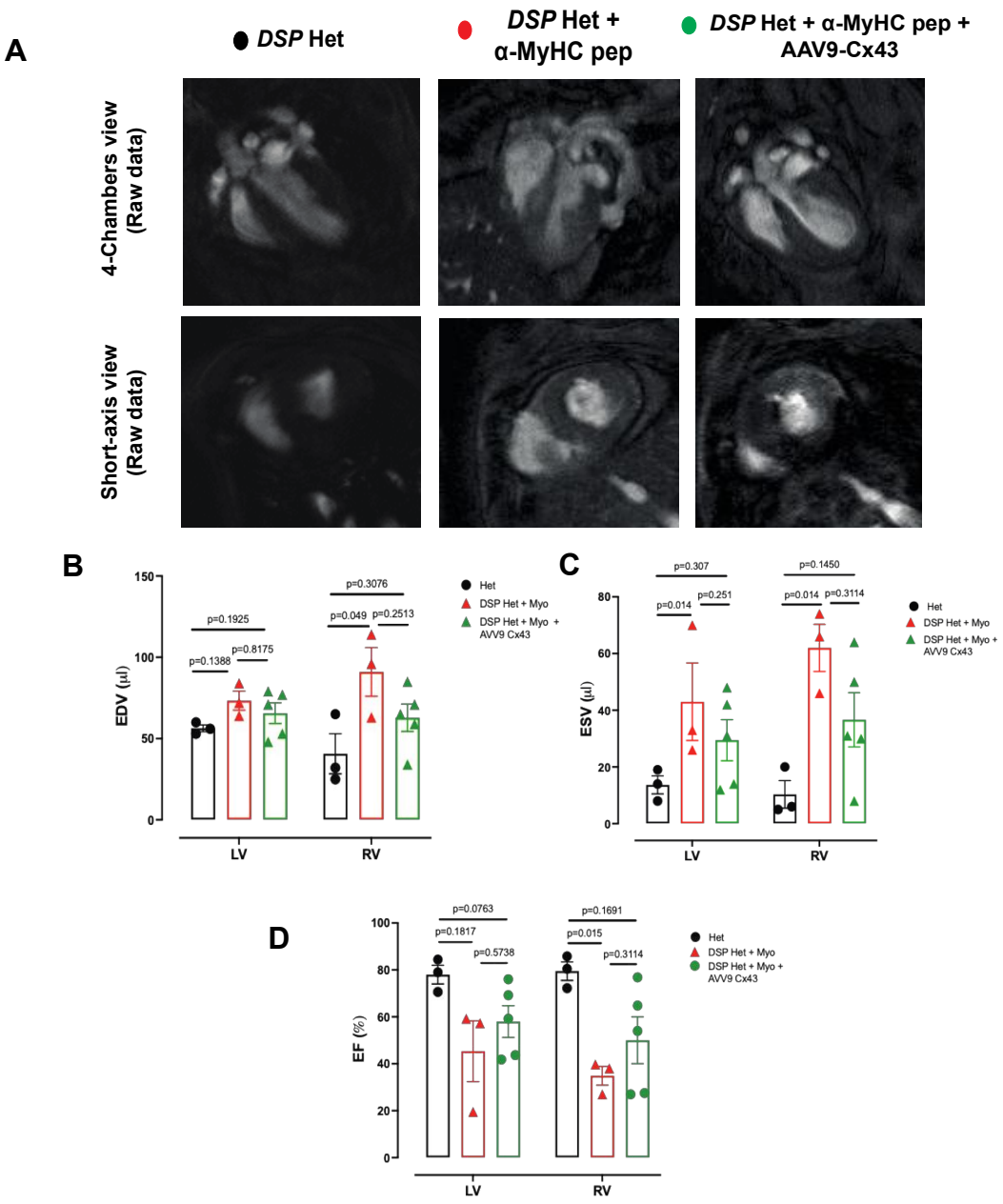

Figure 6. DSP Het mice treated with Cx-43 display improvement in their right ventricular. (A) Representative four-chamber and short axis cardiac MRI views from DSP het, DSP Het + alpha-MHC peptide + pertussis toxin) and DSP Het + alpha-MHC peptide + AAV9 Cx-43 at 20 weeks of age (12 weeks after the first injection). (B) end-diastolic volume (EDV). (C) End-systolic volume (ESV). (D) Ejection fraction (EF). DSP het, n=3; DSP Het + alpha-MHC peptide + pertussis toxin, n=3; DSP Het + alpha-MHC peptide + AAV9 Cx-43, n=5).

3.5 Restoration of Cx-43 Reduces Fibro-fatty Replacement in DSP-Het Mice induced with myocarditis

One of the clinical features of ARVC is the fibrofatty replacement of myocardium [54]. To investigate whether treated DSP het mice show improvement in fibrofatty replacement and to see if the electrical and functional improvements were reflected in the heart morphology, we looked at the whole heart of 20-week-old mice **(Fig.5A)**. The morphological analysis revealed that mice induced with myocarditis showed epicardial fat tissue and was grossly enlarged in size compared to the littermate controls **(Fig.5A)**. The heart also showed indication of fibrofatty replacement of the myocardium and around the atria, while mice treated with AAV9- Cx-43 were indistinguishable from the control groups **(Fig.5A)**.

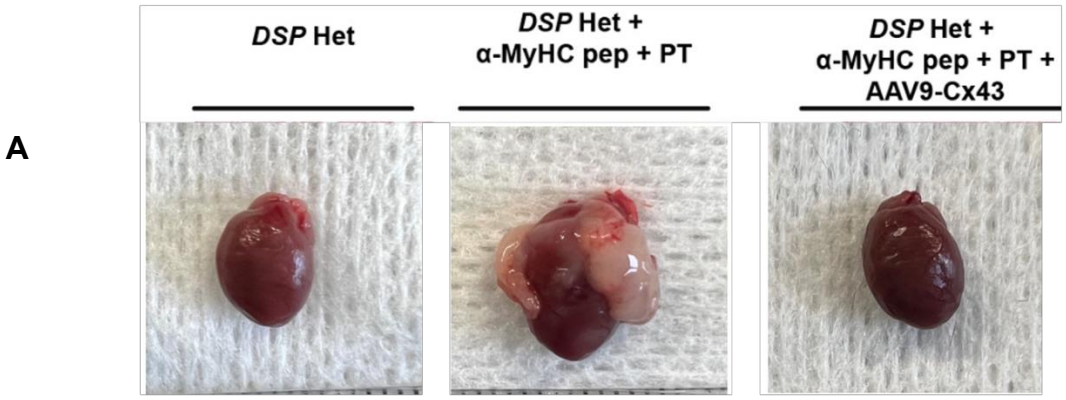

Figure 7. Morphological analysis of Hearts from DSP Het Mice with and without AAV9-Cx43 Treatment. (A) Whole-heart images of 20-week-old mice.

3.6 Histological Analysis: Restoration of Cx-43 Improves Cardiac Structure and Reduces Fibrosis in DSP-Het Mice

To assess the effect of Cx-43 gene therapy on cardiac remodeling and morphology in DSP-Het mice, we performed cardiac histology at 20 weeks of age. Hematoxylin and Eosin (H&E) staining, which marks different cell types and provides information on cell patterns and structure, showed enlargement of both the right and left ventricles, with cardiac dimensions and muscle integrity being affected in the diseased group **(Fig. 6A)**. There was noticeable disruption in the organization of myocardial fibers, along with the presence of inflammatory cell infiltration, indicating an inflammatory response **(Fig. 6A)**. The tissue architecture appeared disordered compared to the DSP-Het group alone, suggesting induced myocarditis and tissue damage due to the α-MyHC peptide and pertussis toxin (PT) treatment **(Fig. 6A).** Mice treated with AAV9-Cx43, however, were indistinguishable from control groups, indicating no evidence of disease **(Fig. 6A).** Masson's Trichrome staining, which marks collagen and fibrosis, showed extensive fibrosis (shown in blue) in the group injected with alpha-myosin-heavy chain peptide **(Fig. 6B)**. In contrast, the myocardial tissue in mice treated with AAV9-Cx43 appeared more organized compared to the DSP-Het + α-MyHC peptide + PT group **(Fig. 6B)**. There was a reduction in inflammatory cell infiltration, indicating that the AAV9-Cx43 treatment might have a protective or therapeutic effect. The overall tissue architecture of the treated group more closely resembled that of the DSP-Het group, suggesting improved cardiac integrity and reduced myocarditis-related damage. These findings confirm our observations in the electrical, functional, and morphological assessments of the heart.

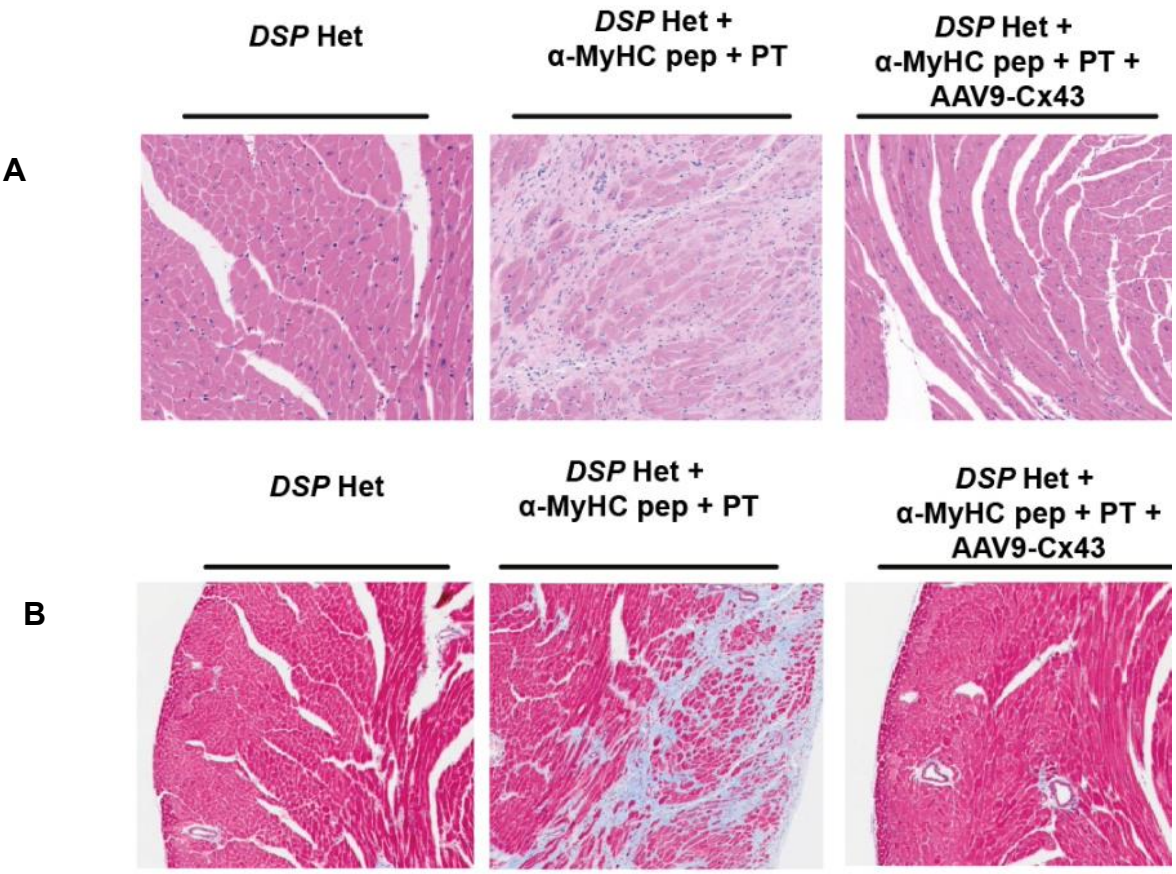

Figure 8. Histological analysis of DSP het mice. (A) Sections were stained for stained for nuclei and cytoplasm with hematoxylin and eosin (B) Masson trichrome stain highlighting fibrosis on cross-section hearts at 20 weeks.

Chapter 4. Discussion

4.1 Discussion

There are studies that highlight the relationship between desmosomal proteins, specifically desmoplakin (DSP), and the crucial gap junction protein Connexin-43 (Cx-43) [14] . However, there are limited studies focusing on the role of Cx-43 restoration in the context of desmosomal protein stabilization, particularly in DSP-induced myocarditis and ARVC patients at risk of sudden cardiac death. Based on the characterization of Cx-43 gene therapy, my thesis studies offer valuable insights into the impact of Cx-43 restoration on desmosomal proteins, particularly DSP. This research contributes to the understanding of how Cx-43 influences cardiac function and opens avenues for developing future therapies for patients with DSP-mutation-related cardiomyopathy.

4.1.1 Restoration of Connexin-43 is sufficient to restore the expression of desmoplakin protein

Restoration of Connexin-43 (Cx-43) in DSP-Het mice induced with myocarditis demonstrates that Cx-43 restoration is sufficient to restore the protein levels of other desmosomal proteins, particularly desmoplakin (DSP). This highlights the unique regulatory relationship between DSP and Cx-43. Prior Mechanistic studies in sheikh's lab demonstrated that DSP-deficient neonatal cardiomyocytes have shown that the loss of Cx-43 is primary caused by the absence of DSP even when other desmosomal and fascia adherens junction proteins remain unaffected [14]. Our study shows that restoring Cx-43 in DSP-Het mice prevents classic ARVC disease features, such as arrhythmias, mechanical dysfunction, and fibrofatty replacement of the myocardium. These findings provide compelling evidence that targeting Cx-43 can mitigate the adverse effects of DSP mutations in ARVC. This is significant because human genetic studies and pediatric populations have underscored the role of desmosomal protein loss in driving the disease,

particulary in recessive forms of ARVC [14, 55, 56]. My work provides the first in vivo evidence that restoring Cx-43 proteins can have a similar effect on desmosomal protein levels in myocarditis-induced mice, emphasizing the therapeutic potential of Cx-43 restoration. These findings align with studies linking desmosomal protein mutations, specifically desmoplakin, to reduced Cx-43 levels and gap junction remodeling, a common feature of ARVC [46]. Patients diagnosed with ARVC and DSP mutations often have inflammatory infiltrates in their hearts, with various cardiotropic viruses detected in ARVC cases [57]. This underscores Cx-43's role in inflammation as connexins, particularly Cx-43, can be used by viruses to spread infection and amplify pathogenic effects [51]. Additionally, Studies have shown that Cx-43 plays a beneficial role in inhibiting inflammation in other organs by protecting tissue integrity [58-60]. The importance of Cx-43 extends beyond cardiac function to immune responses and inflammatory stress. Blocking Cx-43 hemichannels has been shown to prevent tissue damage signals, reducing inflammation and fibrosis [61]. Moreover, Cx-43 protects neurons during stroke and reduces inflammation [62], forms gap junctions in the brain to protect against immune cell infiltration [63], and is essential for maintaining electrical conduction in the heart through cardiac macrophages [64, 65]. Implications of Cx-43 have also been observed in lung inflammatory conditions such as asthma and acute lung injury [66] .Therefore, recent attention has focused on the correlation between DSP loss and Cx-43 levels, serving as an intermediate link in the context of myocarditis, as myocarditis has been associated with DSP-related cardiomyopathy. This suggests that restoring Cx-43 levels can have a secondary effect on reducing myocarditis by restoring DSP levels, thereby decreasing the likelihood of myocarditis in DSP-related cardiomyopathy. My results add into the unique relationship between DSP and Cx-43 highlighting the therapeutic potential of targeting Cx-43. By restoring Cx-43, it may be

possible to mitigate the detrimental effects of DSP loss, reduce inflammation, and improve overall cardiac outcomes in ARVC patients. Our results pave the way for future studies to explore the mechanisms underlying this interdependence and to develop targeted therapies for DSP-related cardiomyopathies.

4.1.2 Cx-43 restoration improves electrical and functional abnormalities in DSP-Het induced with myocarditis

Electrical defects are a primary feature and driving force in the development of ARVC [67-69]. In this study, Electrocardiogram (ECG) analysis revealed significant electrical conduction abnormalities in DSP-Het mice induced with myocarditis, consistent with the known effects of Cx43 disruption on cardiac electrical function. Previous studies have shown that Cx43 is usually downregulated in cardiac remodeling processes, which decreases the electrical stability of the heart. A hallmark of these electrical changes is altered impulse conduction due to abnormal expression of Cx43-constituted gap junctions [70]. The loss of Cx43 has been associated with triggering cardiac arrhythmias underlying ARVC [14]. In our study, in vivo treatment with AAV9-Cx43 was sufficient to prevent the electrical arrhythmias that occurred in DSP-Het mice induced with myocarditis. The therapeutic potential of Cx43 restoration was evident as treated DSP-Het mice showed improvements in both QRS duration and the incidence of PVCs after receiving AAV9-Cx43 gene therapy. This suggests that restoring Cx43 levels can re-establish proper electrical conduction pathways, thereby reducing arrhythmogenic potential. Our findings are consistent with previous research highlighting the critical role of Cx-43 in cardiac electrical coupling and its implications in arrhythmias. In a guinea pig model with chronic aortic stenosis, researchers observed a decline in Cx43 expression accompanied by remodeling of intercalated discs (IDs) and changes in myocyte morphology during the transition from compensated to

decompensated left ventricular hypertrophy [71]. Similarly, in a rat model of pressure overload induced by ascending aortic banding, reductions in Cx43 expression, increased dephosphorylation of Cx43, and slowed conduction velocity were evident in the advanced stages of hypertrophy. These findings underscore the progressive nature of Cx43 alterations in response to cardiac stressors. Studies involving patients with dilated cardiomyopathy (DCM) have also demonstrated a consistent downregulation of Cx43 expression. Also, the hearts of DCM patients who experienced sudden death showed reduced and altered distribution of Cx43 expression in both ventricles [72]. Furthermore, in a pig model of DCM, researchers demonstrated a gradual decrease in Cx43 levels as heart failure progressed. These observations in various animal models and clinical studies emphasize the role of Cx43 dysregulation in the pathophysiology of cardiac diseases, contributing to electrical instability and arrhythmias [73]. In ischemia/reperfusion models, such as isolated perfused rabbit and rat hearts, progressive dephosphorylation of Cx43 during ischemia was observed, alongside electrical uncoupling and reductions in total Cx43 immunofluorescent signal [74, 75]. These observations suggest that ischemia-induced uncoupling may be associated with the translocation of Cx43 from gap junctions to intracellular sites [76]. By exploiting MRI, we have been able to see that AAV9-Cx43 restoration led to improvements in physiological cardiac abnormalities in DSP-Het mice induced with myocarditis, particularly in the right ventricle. In ARVC patients, MRI findings often show abnormalities in the right ventricle, with 96% of patients displaying issues such as basal inferior wall dyskinesia and basal anterior wall dyskinesia [77, 78].The improvements seen in the right ventricle of our treated DSP-Het mice likely reflect a reduction in these types of abnormalities, highlighting the effectiveness of Cx43 restoration in ameliorating disease symptoms. This is particularly relevant as the right ventricle is often more affected in ARVC due to its thinner wall, which poses challenges for accurate tissue

assessment but underscores the significance of the observed therapeutic benefits. My studies suggest s that cx-43 restoration in DSP het mice induced with myocarditis can open the door for new therapeutic strategies I the context of heart failure associated with arrythmias and ventricular remodeling, particulary in ARVC.

4.1.3 A Molecular Trigger for Reducing Fibrosis and Improving Cardiac Function

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is characterized by progressive structural changes, including right ventricle dilation, regional wall motion abnormalities, fibrosis, and fatty infiltration. These changes are part of the underlying molecular mechanisms of ARVC [52, 77]. These structural defects lead to the unique histopathological feature of ARVC, which is the replacement of myocardial tissue with fibrous and fatty tissue. Our experimental findings in DSP-het induced with myocarditis revealed morphological and histological changes in DSP-Het mice induced with myocarditis. The morphological analysis demonstrated that myocarditis-induced DSP-Het mice exhibited grossly enlarged hearts with epicardial fat deposition and fibrofatty replacement, consistent with the clinical hallmarks of ARVC. Previous studies have linked decreased levels of Cx-43 with cardiac fibrosis in various models, including a pig model of dilated cardiomyopathy [70]. Similarly, our mice showed fibrosis and structural disorganization in the myocardium. The diseased mice induced with myocarditis showed enlargement in their ventricles with evident disruption in cardiac muscle integrity. Furthermore, fibrosis was highlighted in the myocardium of the diseased mice. However, the fibrosis and structural disorganization were markedly reduced in the group treated with AAV9-Cx43, highlighting the crucial role of Connexin-43 (Cx-43) restoration in improving these changes.

Clinical studies have underscored the significance of Cx-43 redistribution in areas of fibrosis within the myocardium of patients with dilated cardiomyopathy whereas in other areas with no indication of fibrosis, Cx-43 was normally distributed [72, 78, 79]. This is particularly relevant as fibrosis is a key contributor to the progression of heart failure and arrhythmias in ARVC. Previous studies have proposed that inflammation in response to immune triggers and viral infections can lead to damage of the cardiac myocytes and necrosis, further leading to fibrotic remodeling in patients diagnosed with ARVC [57]. This is supported is by studies showing the presence of inflammatory infiltrates and different cardiotropic viruses in 2 out of 3 ACM hearts [57]. In my studies, I have shown that Cx-43 restoration results in a reduction of fibrotic tissues and improvement in cardiac morphology, accompanied by functional enhancements as indicated by improved electrical and mechanical heart functions in the treated group. Our data suggest that structural improvements in the treated group with AAV9-Cx43 were accompanied by functional enhancements. Several studies indicate that as fibrosis increases, the electrical propagation perpendicular to the fiber direction becomes asynchronous due to the complex pathways created by collagen fibers acting as electrical barriers. This increased complexity forces the electrical activation to travel longer paths, resulting in slower conduction perpendicular to the fiber direction [80]. . Therefore, targeting fibrosis through Cx-43 restoration could be a key strategy in improving electrical conduction and reducing the risk of arrhythmias.

4.2 Conclusions

In conclusion, by restoring Cx-43 through AAV9-Cx43 gene therapy we achieved restoration of desmoplakin (DSP) levels, leading to substantial improvements in both structural and functional cardiac parameters. Specifically, our data indicate that Cx-43 and DSP restoration

reduces myocardial fibrosis, enhances myocardial integrity, and reduces inflammatory cell infiltration in DSP-Het mice induced with myocarditis. These improvements correlate with enhanced electrical and mechanical heart functions, underscoring the multifaceted role of Cx-43 in cardiac health. We further highlighted that hat treated DSP-Het mice exhibited organized myocardial tissue architecture, and minimized inflammatory responses compared to the pronounced structural disarray and fibrosis in untreated DSP-Het mice. These findings highlight the therapeutic efficacy of Cx-43 restoration. Regarding molecular mechanisms, our study underscores the critical role of Cx-43 in maintaining cardiac tissue integrity and function. The observed improvements in cardiac morphology and function upon Cx-43 restoration suggest that Cx-43 acts as a crucial regulator of myocardial homeostasis, potentially through its interactions with other desmosomal and gap junction proteins. These findings align with previous studies that have implicated Cx-43 in the regulation of cardiac remodeling and arrhythmogenesis.

4.3 Future Studies

While my studies have defined the therapeutic potential of Cx-43 restoration in DSP-mediated myocarditis, there are still several avenues for future research to optimize this therapeutic approach. One important direction is to gain further insight into the precise molecular pathways through which Cx-43 exerts its protective effects. Investigating the interactions between Cx-43 and other desmosomal and gap junction proteins will provide a deeper understanding of its role in maintaining myocardial integrity and function. High-throughput sequencing and proteomic analyses could be employed to identify key signaling pathways and molecular interactions influenced by Cx-43 restoration. Additionally, longitudinal studies are essential to assess the long-term efficacy and safety of AAV9-Cx43 gene therapy. Monitoring the progression of cardiac pathology and overall health outcomes in treated DSP-Het mice over extended periods will help

determine the durability of the therapeutic effects. Evaluating potential off-target effects and the immune response to AAV9-Cx43 will also be crucial for translating this therapy into clinical practice. Future studies should explore the effects of administering AAV9-Cx43 gene therapy at different stages of development. Performing the AAV9-Cx43 injection during the neonatal stage, as opposed to in adult mice as done in the current study, could provide insights into the optimal timing for therapeutic intervention. Early administration might offer benefits in preventing or mitigating the onset of disease symptoms more effectively. Conducting experiments in neonatal cardiomyocytes to assess the dose-dependent effects of Cx-43 restoration will be essential to determine the optimal dosing strategy. These studies will help understand how different doses of Cx-43 impact cardiac structure and function, potentially leading to more refined and effective therapeutic protocols. Given the role of inflammation in DSP-mediated myocarditis, future studies should include experiments specifically targeting inflammatory pathways. Assessing the impact of Cx-43 restoration on inflammation-related markers and immune cell infiltration will provide a deeper understanding of its anti-inflammatory effects and contribute to a more comprehensive therapeutic strategy. Moreover, future research could investigate the therapeutic efficacy of starting AAV9-Cx43 treatment after inducing myocarditis, rather than preventing. This approach would more closely mimic clinical scenarios where patients receive treatment after disease onset, providing valuable insights into the timing and potential benefits of delayed intervention.

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