Effect of mitofusin 1 and mitofusin 2 deficiency on electrophysiology parameter following stimulated ischemia reperfusion

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**EFFECT OF MITOFUSIN 1 AND MITOFUSIN 2
DEFICIENCY ON ELECTROPHYSIOLOGY
PARAMETER FOLLOWING STIMULATED ISCHEMIA
REPERFUSION**

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SCHOOL OF BIOLOGICAL SCIENCES

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KWEK XIU YI

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for
the degree of Masters of Science

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Prof Lu Lei

Date: 19 Mar., 19

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ABSTRACT

Background

Acute myocardial infarction (AMI) and the arrhythmias which often follow are among the leading causes of death and disability in Singapore and worldwide. As such novel treatments are required to prevent cardiac arrhythmias following AMI. In an AMI, acute myocardial ischemia and reperfusion can disturb the electrical conduction system of the heart, impact conduction velocity (CV), and result in atrial and ventricular arrhythmias, some of which may be fatal. The gap junction (GJ) protein, connexin 43 (Cx43), is a key determinant of CV, and modulation of Cx43 can affect arrhythmia susceptibility. Changes in mitochondrial shape can impact on mitochondrial function and production of reactive oxygen species (ROS), which may impact Cx43 distribution/phosphorylation, and ultimately affect CV. In this thesis, we investigate the effect of the mitochondrial fusion proteins, Mitofusins 1 and 2 (Mfn 1 and Mfn 2, which are known to modulate mitochondrial shape and function) on Cx43 distribution/phosphorylation and CV in the heart following AMI.

Hypothesis

Hearts deficient in Mfn 1 and Mfn 2 will have disturbances in Cx43 distribution and Cx phosphorylation resulting in a reduction in CV, thereby increasing susceptibility to cardiac arrhythmias following AMI.

Methodology

We used conditional cardiomyocyte-specific Mfn 1 and Mfn 2 Double Knock Out (MfnDKO) mice (n=3) and wild-type littermate (WT) mice (n=3) to investigate

the effect of genetic ablation of Mfn 1 and Mfn 2 on CV (measured by multi-electrode array) at baseline and following stimulated acute ischemia and reperfusion (IR). CV data was analyzed by Clampfit Software. We also performed initial studies investigating the effect of genetic ablation of Mfn 1 and Mfn 2 on Cx43 distribution (measured by immunohistochemistry) and Cx43 phosphorylation (at Ser368 measured by western blotting) in MfnDKO mice (n=1), when compared to WT mice (n=1).

Results

CV in heart ventricular tissue from MfnDKO mice was significantly lower ($p < 0.05$) than WT at baseline and following simulated acute IR, a condition which would increase susceptibility to cardiac arrhythmias, suggesting Mfn 1 and Mfn 2 play an important role in the regulation of CV. Our initial data has also suggested that Cx43 localization within cardiomyocytes is altered in MfnDKO mice when compared to WT mice, with partial lateralization of Cx from the GJ to the cytosol, a feature which would affect CV and increase susceptibility to cardiac arrhythmias. Finally, preliminary data has also suggested that phosphorylation of Cx43 at Ser368 is increased in MfnDKO mice when compared to WT mice (n=1 for each group), a feature which would decrease CV and increase susceptibility to cardiac arrhythmias.

Conclusions

In summary, in heart ventricular tissue deficient in both Mfn 1 and Mfn 2, CV was reduced, a finding which was associated with partial redistribution of Cx43 away from the GJ, and phosphorylation of Cx43 at Ser368, findings which may be expected to increased susceptibility to cardiac arrhythmias post-AMI. This

initial data highlights the Mitofusins as potential therapeutic targets for preventing cardiac arrhythmias post-AMI.

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LIST OF ABBREVIATIONS

AP	Action potential
APD	Action potential duration
AMI	Acute myocardial infarction
ARP	Absolute refractory period
ATP	Adenosine triphosphate
AV	Atrioventricular
CKO	Conditioned knock out
Cre	Cyclization recombination
CV	Conduction velocity
CVD	Cardiovascular diseases
Cx	Connexins
Drp1	Dynamin-related protein 1
EP	Electrophysiology
ERP	Effective refractory period
Fis1	Fission protein 1
GJ	Gap junctions
ID	Intercalated discs
IHC	Immunohistochemistry
IHD	Ischemic heart disease
IRI	Ischemia reperfusion injury
LV	Left ventricle
MEA	Microelectrode array
Mer	Modified estrogen receptor
Mfn 1	Mitofusin 1
Mfn 2	Mitofusin 2
MfnDKO	Mfn double knock out
MI	Myocardial infarction
OMM	Outer mitochondria membrane

Opa1	Optic atrophy 1 protein
PCR	Polymerase chain reaction
RRP	Relative refractory period
SA	Sinoatrial
SDS-PAGE	Sodium dodecylpolyacrylamide gel electrophoresis
WB	Western blot
WT	Wildtype

INTRODUCTION

Cardiovascular diseases (CVD) is a disorder of the heart and blood vessels and includes coronary heart disease, cerebrovascular disease, rheumatic heart disease and other conditions. WHO reported that CVD is the leading cause of death globally, and an estimated 17.9 million people died from CVDs in 2016, representing 31% of all deaths worldwide¹.

In Singapore, about 17 people die from CVD (heart diseases and stroke) every day, and CVD accounts for 30.1% of all deaths in 2017². Of these, ischemic heart disease (IHD) comprised a 61% death rate among CVD, accounting for 16.7% of total deaths in 2015 to 18.5% in 2017.

Factors like age, gender, genetics and lifestyle such as smoking and an unhealthy diet are important risk factors that can contribute to the development of CVD^{2,3}. On top of that, diseases like obesity, hypertension, insulin resistance and diabetes are also major risk factors, which can increase the risk of developing of CVD.

IHD results from the reduction of coronary blood supply to the myocardium, resulting in the development of acute myocardial ischemia. Ischemia is associated not only with insufficient oxygen supply, but also with reduced availability of nutrients and inadequate removal of metabolic end products. The high morbidity and mortality have seen doctors focus on restoring blood flow to the ischaemic tissue, known as reperfusion, to recover the heart function⁴.

Recently, however, it has become evident that reperfusion can place the organs at risk of further cellular necrosis and thereby limit the recovery of function, a phenomenon known as ischemia reperfusion injury (IRI)⁵.

The deprivation of oxygen and nutrient supply results in a series of abrupt biochemical and metabolic changes within the myocardium. The absence of oxygen stops oxidative phosphorylation, leading to mitochondrial membrane depolarization, ATP depletion, and inhibition of myocardial contractile function⁶. The cellular metabolism also switches to anaerobic glycolysis, resulting in the accumulation of lactate, reducing intracellular pH⁷.

Cardiac arrhythmias are the consequence of acute myocardial IRI, and are common in the setting of acute myocardial infarction (AMI). 90% of patients with AMI have shown cardiac rhythm anomalies while 25% will suffer from cardiac conduction disturbance within a day of the onset of infarct⁸. Heart muscle contraction depends on an intrinsic electrical system of orderly activation and recovery of electrical excitation through the myocardium⁹, and the disruption of this orderly initiation and conduction of the electrical signal can lead to arrhythmias.

There are many types of cardiac arrhythmias or abnormal heart beating. In a normal heart, the heart rate ranges from 60-100 beats/min. Tachycardia has more than 100 beats/min while bradycardia has less than 60 beats/min, and both may lead to arrhythmias¹⁰. While atrial fibrillation is the most common sustained cardiac arrhythmia, ventricular fibrillation is lethal if not stopped within minutes, and is the main reason arrhythmias is one of the leading cause of death in the world^{11,12}.

The pathogenesis of cardiac arrhythmias has three basic mechanisms; abnormal automaticity, triggered activity and re-entry¹³, of which re-entry is the most common. Re-entry is defined as a persisting electrical impulse that

reactivates an area of tissue that are no longer in refractory, resulting in a circus movement of activation and sustains cardiac arrhythmias¹⁴.

Conduction velocity (CV) and the refractory period of the myocardium in the circuit are the main factors that will lead to the maintenance of re-entrant arrhythmias, combining to determine the “wavelength” of the arrhythmia with the formula as below:

$$\text{Conduction velocity (m/s)} \times \text{Refractory period (s)} = \text{Wavelength (m)}$$

Both factors are critical to determine the occurrence of re-entry, and re-entry cannot occur if the calculated wavelength of arrhythmia is more than the path length of the circuit, and is sustained if the wavelength is less than the path length¹⁵.

Normal heart electrical impulse propagates from the sinoatrial (SA) node, atria, atrioventricular (AV) node, His bundle, bundle branches, purkinje network respectively, ending at the ventricle¹⁶. To study the electrophysiology (EP) of the heart, it is necessary to understand the various EP parameters such as action potential duration (APD) and CV¹⁷.

Action potential (AP) is an electrical stimulation generated by the sequential activation and inactivation of ion channels conducting depolarizing Na^+ and Ca^{2+} , and repolarizing K^+ currents (Figure 1)^{18,19}. AP consist of 5 phases and the activity of each phases are described respectively in Table 1.

Figure 1. A typical action potential recorded from a ventricular myocyte.

Phase	Activity
0	Rapid Na^+ influx through open fast Na^+ channels
1	Transient K^+ channels open and K^+ efflux
2	Influx of Ca^{2+} through L-type Ca^{2+} channels is electrically balanced by K^+ efflux through delayed rectifier K^+ channels
3	Ca^{2+} channels close but delayed rectifier K^+ channels remain open
4	Na^+ , Ca^{2+} channels closed, open K^+ rectifier channels

Table 1. 5 phases of action potential

Slow conducting re-entry circuits will lead to impaired myocardial AP conduction that can predispose to arrhythmias. The propagation of myocardial electrical activity depends on AP junctions mediated electrical coupling, which ensures the coordination of the electromechanical functioning of the working myocardium²⁰.

All excitable cells enter a period known as refractory period, during which repeat activation is impossible. During the refractory period, the heart is allowed to relax, filling the chambers with blood. There are 3 types of refractory period,

absolute refractory period (ARP), effective refractory period (ERP) and relative refractory period (RRP) (Figure 2)²¹. Table 2 showed the responses for each refractory period.

Figure 2. Different degrees of refractoriness during an action potential.

Refractory period	Response
ARP	Cells do not response to new stimulus.
ERP	A stimulus may cause the cell to depolarize but will not result in a propagated AP.
RRP	It will cause an action potential.

Table 2. Different refractory period and their responses.

APD is determined by the balance of inward currents (Ca^{2+}), and outward currents (K^{+}) during the plateau phase of the AP, while CV is the speed at which the AP passes through the cardiac tissue²².

CV can be measured by microelectrode array (MEA) that has an arrangement of 60 electrodes which allows extracellular recording and stimulation (Figure 3).

The biological sample is positioned directly on the MEA recording area, where signals are amplified by a filter amplifier and sent to a data acquisition computer²³. The numbering format of MEA electrodes follows the standard numbering scheme where the first digit is the column number while the second digit is the row number. Electrode 15 is missing in MEA and is replaced by a big internal reference electrode, connected to the amplifier at pin 15.

Figure 4. GJ structure. A, Cx. B, six Cx to form connexon. C, two connexon to form a GJ.

GJ channels are located at the intercalated discs (ID), enabling communication and connections between cells by allowing the movement of ions and small molecules such as ATP²⁶. They mediate the electrical coupling between cardiomyocytes to propagate the orderly wave of electrical excitation of the AP responsible for organized contraction, and maintenance of a regular beating rhythm²⁷. Thus, an impairment in GJ communication has been implicated in the development of electric conduction defects leading to several cardiac disorders.

In the normal adult heart, there are three main isoforms of Cx: Cx40, Cx43 and Cx45. Cx43 is the most abundant and is expressed in ventricular cardiomyocytes²⁸. Studies have shown that after a myocardial infarction (MI), Cx43 redistributes away from ID to the cytosol, a phenomenon known as “lateralization”. Changes in Cx43 distribution and expression have also been observed following acute ischemia²⁹. Importantly, the lateralization of Cx43

away from the GJ affects the transmission of conduction across the cells, leading to a reduction in CV.

The phosphorylation status of Cx43 at the various serine residues, eg. Serine 262, 279, 282, and 368 is a key mechanism for regulating the gating of GJ channels, leading to a functional remodelling of connexins and altering GJ localization^{30,31}. During ischemia, there will be a modification in the phosphorylation status of Cx43, including dephosphorylation of Serine 325, 328, 330 and 365, and phosphorylation of 373 and 368³².

Phosphorylation at serine 368 (S368) is an important Cx43 event that increases during protein kinase C (PKC) activation or ischemia. Phosphorylation at S368 Cx43 is regulated by PKC ϵ and can only be found located at ID³³. Using dye transfer assay, it was reported that phosphorylation of Cx43 on S368 will lead to a decline of cells transferring dye, showing the disruption of GJ communications³⁴. During ischemia, there was an increase of phosphorylation on the S368 as there was an increase of PKC ϵ level. Phosphorylated S368 Cx43 remained at ID despite the dephosphorylation and relocation of large amounts of Cx43 to lateral borders, and has been implicated to alter CV during stress^{33,35}. We have investigated S368 for the purpose of this study, as we would like to see if ablation of Mfn 1 and Mfn 2 will affect the phosphorylated S368 Cx43 level in MfnDKO model.

In the heart, mitochondria are a key generator of adenosine triphosphate (ATP), energy that are required to maintain constant rhythmic contraction of the heart³⁶. The heart requires large amounts of ATP as a source of energy since up to 50%

is consumed for ion channel and transporter homeostasis, which are both mandatory for proper electrical activity³⁷.

Mitochondria are also a major source of ROS generation via oxidative phosphorylation. Oxidative phosphorylation generates toxic byproducts such as superoxide anion radical ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot\text{OH}$)³⁸. In physiological condition, although harmful ROS species are generated, they are balanced by antioxidant defense³⁹.

A group of mitochondrial fusion and fission proteins, known as mitochondrion shaping protein, manages the morphology to maintain a healthy mitochondrial network. Fusion proteins consist of mitofusin 1 (Mfn 1), mitofusin 2 (Mfn 2) and optic atrophy 1 protein (Opa1), while fission proteins consist of dynamin-related protein 1 (Drp1) and its outer mitochondrial membrane adaptor fission protein 1 (Fis1)⁴⁰.

Fusion and fission functions as a quality control system, allowing a damaged mitochondrial to compensate for damaged contents and repair its function by fusing to, and receiving components from, a healthy mitochondrion⁴².

The main role of Mfn 1 and Mfn 2 is to mediate the fusion of the outer mitochondrial membrane (OMM) of adjacent mitochondria. While both are large GTPases, Mfn 1 is a profusion protein while Mfn 2 is a tethering protein. Despite this, they share a similar molecular architecture with an approximate 65% similarity at the amino acid level, and have in several contexts even exhibited functional overlap or complementation in mediating ONM fusion⁴³.

To address the functional significance of Mfns, several laboratories have pursued the conditional inactivation of Mfn 1 and Mfn 2 in the skeletal and cardiac muscle of mice. Both Mfn 1 and Mfn 2 have been found to be necessary for the maintenance of the mitochondrial genome in skeletal muscle⁴⁴. In the heart, both Mfn 1 and Mfn 2 have also shown to be important during early embryonic cardiac development, and protects against long-term cardiac dysfunction in adulthood⁴³.

Previous studies have shown that Mfn Double Knock Out (MfnDKO) mice result in mitochondrial dysfunction and cardiac impairment, leading to reduced cardiac contractility, and eventually sudden death within 8 weeks of genetic knockout. It was reported that mutation of fusion protein in mitochondria results in mitochondrial dysfunction and increases the ROS level⁴⁵. Since ROS acts directly on PKC ϵ , we can conclude that knocking out of Mfn 1 and Mfn 2 will promote mitochondrial dysfunction, leading to an increase of ROS level which eventually increase PKC ϵ levels⁴⁶. Phosphorylation at S368 Cx43 will increase and disrupt the GJ communication, leading to a decrease in CV (Figure 5).

Figure 5. Reduced CV in MfnDKO mice model.

In this study, we first characterized the MfnDKO mouse model, knocking out Mfn 1 and Mfn 2 in order to perform downstream experiments to study the potential cause of cardiac arrhythmia susceptibility.

AIM AND HYPHOTHESIS

We propose a link between the mitofusins, mitochondrial dysfunction and arrhythmia susceptibility. In our study, we would like to gain new insights to the novel role of Mfn 1 and Mfn 2 as targets for preventing cardiac arrhythmias following acute MI. It is important to assess the function of these proteins since their role is to maintain mitochondrial health and a reduction in their expression has been implicated in a range of cardiac diseases associated with an increased susceptibility to MI and arrhythmias, and they may be a potentially novel therapeutic target to prevent cardiac arrhythmias.

We hypothesize that mice deficient in Mfn 1 and 2 are susceptible to arrhythmias under baseline conditions following IR. We also hypothesize that mice deficient in Mfn 1 and 2 are susceptible to lateralization of Cx43 at baseline, as slowed myocardial conduction velocity is associated to lateralization of Cx43, which predominantly regulates CV.

The following are the specific aims required to prove the stated hypothesis:

To characterize the MfnDKO animal model. First, we had to ensure that the mice carry both Mfn 1 and Mfn 2 floxed gene. The next step was to ensure that the mice are Cre+. This generated a MfnDKO model upon injection of Tamoxifen. Genotyping and western blotting were the main laboratory techniques used to ensure that both mitofusins are knocked out, before further experimentation can be carried out.

To investigate ex vivo, in ventricular slices, the effect on mitofusin deficiency on CV under baseline conditions and/or following IR.

Mouse hearts from MfnDKO and WT group were harvested and sectioned for MEA experiments. MEA allows us to investigate the effect of cardiac-specific ablation of Mfn 1 and Mfn 2 in ex-vivo ventricular tissue using electrophysiological parameters which are known to predispose cardiac tissue to arrhythmias. In our study, CV was assessed under baseline and following simulated IR. All the stimulation threshold and stimulation protocol used had been optimised by David Montaigne, for the atrial model in mice.

To investigate the localization and phosphorylation of connexin 43 in MfnDKO and WT mice at baseline level, before subjecting to ischemia and reperfusion.

Immunohistochemistry and western blot were used to investigate the difference in the level of Cx43 localization and phosphorylation, between MfnDKO and WT mice at baseline levels.

METHODS

1. Animal Model

1.1 Animal

We obtained MfnDKO mice courtesy from Gerald W. DornII for this study. Previous results from Dorn's lab showed that Mfn 1/Mfn 2 double cardiac knockout mice are lethal between e9.5 and e10.5⁴⁷. Hence, cardiomyocyte specific Mfn 1 and Mfn 2 were ablated using floxed allele mice crossed with tamoxifen-inducible modified estrogen receptor (Mer) cardiac-specific Myh6-cyclization recombination (Cre) transgene (Figure 6)⁴⁸.

Cre-lox technology is one of the most widely used tools for genetically engineering mice for human disease modelling⁴⁹. The experiments in this study utilise Myh6-MerCreMer mouse that has a cardiac-specific α -myosin heavy chain promoter directing the expression of a tamoxifen-inducible Cre recombinase (MerCreMer) to the cardiomyocytes. Figure 7 showed the breeding scheme for MfnDKO mice to generate Cre⁺ and Cre⁻ MfnDKO mice.

Cre recombinases is a site-specific enzyme that catalyze DNA recombination between two different loxP sites, which can be activated by the administration of tamoxifen to the animal. Upon tamoxifen injection, this is expected to result in deletion of both Mfn 1 and Mfn 2 in the cardiomyocytes, creating a MfnDKO model⁴³. Non-transgenic C57BL/6 mice were used as the wildtype (WT) group.

Figure 6. Schematic representations adapted from reference 47, on the conditional Cre-Lox strategy for combined cardiomyocyte-specific deletion of Mfn 1 and Mfn 2 using Myh6-driven modified estrogen receptor-Cre and tamoxifen.

Figure 7. Breeding scheme for MfnDKO mice to generate Cre⁺ and Cre⁻ MfnDKO mice.

1.2 House maintenance

Both groups, Myh6-MerCreMer and C57BL/6 mice were housed at Duke-NUS vivarium under the care of the veterinarian. The study was carried out in strict accordance to the standards as described in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animal and approved by

National University of Singapore Institutional Animal Care and Use Committee (NUS IACUC) at Duke-NUS Medical School. IACUC number is 2015/SHS/1049. Animals were maintained in SPF2 room, under pathogen-free conditions, a 12 hours day and 12 hours night rhythm and were fed with standard chow food pellets, with sterile water available ad libitum.

1.3. Tamoxifen treatment

Myh6-MerCreMer mice (aged 5 weeks) received intra-peritoneal injection of tamoxifen (Sigma, Cat. # T5648) at the dose of 100 μ l of 5 mg/ml daily for 5 days to genetically ablate Mfn 1 and Mfn 2 genes. 25mg of tamoxifen was dissolved in 0.5 ml of 100% ethanol then topped up with 4.5 ml of groundnut oil. Animals were sacrificed 4 weeks later and experiments were performed using WT and DKO littermate mice (aged 10 weeks).

1.4 Injection of anaesthetic and heparin

Intraperitoneal injection (65 mg/kg Ketamine (100 mg/ml) and 20 mg/kg Xylazine (20 mg/ml)) immediately prior to sacrifice. Depth of sedation was checked periodically for paw pinch reflexes. At the respective endpoints for sacrifice and following anaesthesia, a single intraperitoneal injection of heparin (100 IU) was administered prior to isolation of heart to prevent clot formation.

1.5 Heart isolation from mice

A midline thoracotomy incision was performed and the heart was removed and rinsed with ice-chilled PBS solution for histology experiments and Tyrode's solution for MEA experiments. Both left and right atria and apex were removed to isolated ventricular tissue. For histology, the heart sample, without both atria,

was incubated in 10% neutral buffered formalin (NBF), while for MEA, the heart ventricular tissue was embedded in a 4% agarose gel to cut the heart slices using vibratome.

2. DNA analysis

2.1 Extraction of genomic DNA from ear biopsy

Ear biopsies were genotyped from mice at 4 weeks of age for Mfn 1, Mfn 2 and Cre identification. Samples were incubated overnight at 55 °C in lysis buffer (DirectPCR lysis reagent, Viagen, Cat. #401-E) and 0.3 mg/ml proteinase K (Sigma, Cat. # P6556-100mg). The ear biopsies were incubated at 85 °C for 45 minutes. The hairs were precipitated by centrifuging for 1 minute at 1000 g and the supernatant was aspirated for DNA quantification and placed in a fresh tube kept on ice.

2.2 DNA concentration

The concentration of DNA was measured using the NanoDrop ND-1000 spectrophotometer, at an absorbance of 260 nm and 280 nm. The OD260/OD280 ratio was used to evaluate protein contamination. A clean DNA solution has a quotient between 1.8 and 2.0. 1 µL of elution buffer was used as a blank for Nanodrop. 1 µL of DNA was loaded onto the nanodrop to measure the concentration of the DNA.

2.3 Amplification of DNA

Polymerase Chain Reaction (PCR) is used to make many copies of a DNA fragment. For genotyping we used the TaqPCR core kit (Qiagen, Cat. #201223) to amplify the DNA exponentially. For standard amplification of DNA, 10ng of

template DNA were used for 50µL reaction, combined into a PCR tube together with 5 µL 10X buffer, 1 µL dNTP, 1 µL from each of the 3'- and 5'-primers (10 mM), 0.25 µL Taq DNA polymerase and 36.75 µL RNase free water (Table 3). Primers were diluted from 100mM to 10mM with RNase free water.

Primer sequence:

Gene	Primer	Sequence	Product size (bp)	Gel percentage (%)
Cre	Forward	ATC CGA AAA GAA AAC GTT GA	650	1
	Reverse	ATC CAG GTT ACG GAT ATA GT		
Mfn 1	Forward	TTG GTA ATC TTT AGC GGT GCT C	Flox/flox-450, wildtype-350	3
	Reverse	AGC AGT TGG TTG TGT GAC CA		
Mfn 2	Forward	GAA GTA GGC AGT CTC CAT CG	Flox/flox-180, wildtype-145	3
	Reverse	AAC ATC GCT CAG CCT GAA CC		

Table 3. Primer sequence of Cre, Mfn 1 and Mfn 2.

For DNA amplification, the T100 thermal cycler from Bio-Rad was used and the thermal cycler condition was 94 °C for 5 minutes to ensure that the DNA template as well as the primers were melted, before starting each replicating cycle at 94 °C for 1 minute (denature), 56 °C for 1 minute (annealing) and 72 °C for 1 minute (extension). This cycle was then repeated 35 times. The last cycle had the final extension adjust to 72 °C for 5 minutes. Annealing temperature will be different, depending on the length of the primers used (Table 4,5).

	Cre	Time
Initialization	94 °C	5 mins
Denaturation	94 °C	1 min
Annealing	56 °C	1 min
Extension	72 °C	1 min
Number of cycles	35	-
Final extension	72 °C	5 mins

Table 4. Cre PCR cycle.

	Mfn 1	Mfn 2	Time
Initialization	94 °C	94 °C	30 secs
Denaturation	94 °C	94 °C	30 secs
Annealing	59 °C	61 °C	1 min
Extension	72 °C	72 °C	1 min
Number of cycles	35	35	-
Final extension	72 °C	72 °C	1 min

Table 5. Mfn 1 and Mfn 2 PCR cycle.

2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA strands by size. The size of the separated strands can be estimated by comparing to a known DNA ladder mix, GeneRuler (Thermo Scientific, Cat. # SM0333). A 6X DNA Loading buffer (Thermo Scientific, Cat. # R0611) was added to the DNA in order to allow the sample to sediment in the gel well and to visualize the DNA fragments. For visualisation of the gel, GelRed nucleic acid stain (Biotium, Cat. # 41002) was used. The buffer used was 1X TAE, diluted from 50X TAE buffer, with a pH of

8.0 (1st Base Cat. # BUF-3000-50X1L) and consists of a mixture of 40 mM Tris-acetate and 1 mM EDTA. The usage of different concentration of agarose (1 – 3%) allows for different sizes of DNA fragments to be resolved. Agarose powder (1st Base, Cat. # BIO-1000-500g) was mixed with the electrophoresis buffer (TAE) in a glass beaker and dissolved at the desired concentration in 1X TAE buffer and boiled in the microwave. 1 µL of GelRed per 10mL 1X TAE buffer was added into the mixture once the agarose powder has completely dissolved in 1X TAE buffer. The mixture was then mixed well before it was poured into the 15x10 cm horizontal gel rack. The gel was allowed to cool in order for the gel to polymerize. Once polymerized, the gel tray was loaded into the electrophoresis tank filled with 1X TAE buffer. 10 µL of DNA sample was mixed with 2 µL of 6 X loading dye, and 10 µL of the mixture was loaded into each well. The agarose gel electrophoresis was performed at 90 V for 1 hour. Once the run was completed, the image of the gel was captured using ChemiDoc MP imaging system from Bio-Rad.

3. Protein Analysis

3.1 Protein extraction

30mg of heart sample was weighed out and 200 µL of lysis buffer, consisting of RIPA buffer (Thermo Scientific, Cat. No # 89900) and Protease Inhibitor (Thermo Fisher, Cat. # 87786) in the ratio of (95:5) were added into the lysing matrix D tube (MP, Cat. #6913100) with 1.4 mm ceramic spheres for homogenizing (MagNA Lyser, Roche) for 30 sec. The tube was spun at 14,000 rpm at 4 °C for 10 min and supernatant was kept for protein concentration quantification.

3.2 Bicinchoninic acid (BCA) protein assay for measuring protein concentration

Pierce BCA protein assay (Thermo Scientific, Cat. # 23225) is detergent-compatible formulation based on BCA for the colorimetric detection and quantification of total protein. Protein standards were prepared accordingly (Table 6), ranging from 20-2000 $\mu\text{g/mL}$, and diluted with lysis buffer consisting of RIPA buffer and protease inhibitor. As the concentration of the protein extracted from the heart exceeds the standard (2000 $\mu\text{g/mL}$), the protein was diluted to 1:20 before proceeding to BCA quantification.

Vials	Volume of diluent (μL)	Volume and source of BSA (μL)	Final BSA concentration ($\mu\text{g/mL}$)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = blank

Table 6. Preparation of BCA standard.

The following formula was used to determine the total volume of working reagent (WR) required:

$$\begin{aligned} & (\# \text{ standards} + \# \text{ unknowns}) \times (\# \text{ replicates}) \times (\text{volume of WR per sample}) \\ & = \text{total volume WR required} \end{aligned}$$

WR was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1, Reagent A:B). 25 μL of each standard or sample and 200 μL of WR were added into 96 well plate (Thermo Scientific, Cat. # 15041) and placed on the shaker for 30 seconds to mix well before incubation at 37 °C for 30 minutes. The plate was allowed to cool to room temperature and the absorbance was measured at 562 nm on the plate reader, SpectraMax M3 (Molecular Devices). A linear standard curve was established by plotting protein concentration ($\mu\text{g/mL}$) against net absorbance (562 nm) (Figure 8).

Figure 8. Plot of BCA protein assay.

3.3 Sample preparation

With the information from the linear standard curve, the concentration of proteins was calculated. The original concentration of the protein was obtained by multiplying the protein concentration derived from the BCA assay with the dilution factor (20X). The concentration of protein from different heart samples were normalized with lysis buffer to ensure that the protein concentration was

uniform across the samples. 1 part of reducing agent, 2-mercaptoethanol (ThermoFisher, Cat.# 21985-023) was added into 19 parts of 2X laemmli sample buffer (Bio-Rad, Cat. #161-0737). 2-mercaptoethanol was is used to reduce disulfide bond between tertiary structure. Preparation of sample was done by diluting 1 part of sample with 1 part of 2X laemmli sample buffer, and boiled at 95 °C for 5 minutes.

3.4 SDS-polyacrylamide gel electrophoresis

Proteins were separated according to their molecular weights by sodium dodecylpolyacrylamide gel electrophoresis (SDS-PAGE). SDS-protein complexes are anionic and gives a negative charge to protein. In an electric field, the proteins will migrate towards the anode, and the electrophoretic mobility of the proteins depends on their molecular weights. In this study, stacking gel buffer, resolving gel buffer and 5 % stacking gel, and a 10 % resolving gel were prepared as follows (Table 7-9):

Chemical	Stacking gel buffer	Resolving gel buffer
Tris base (Sigma, Cat. # T1503-1kg)	30.25 g in 200 ml H ₂ O	91 g in 300 ml H ₂ O
pH adjustment	6.8	8.8
20% SDS (Bio-Rad, Cat. # 161-0418)	10 ml	10 ml

Table 7. Preparation of stacking and resolving gel buffer.

	5% Stacking gel
DI water (mL)	3
30% Acrylamide/Bis solution (mL)	0.66
Stacking gel buffer (mL)	1.26
10% APS (μ L)	50
TEMED (μ L)	10

Table 8. Preparation of 5% stacking gel.

	10% Resolving gel
DI water (mL)	3
30% Acrylamide/Bis solution (mL)	0.66
Stacking gel buffer (mL)	1.26
10% APS (μ L)	50
TEMED (μ L)	10

Table 9. Preparation of 10% resolving gel.

When the gel polymerizes between the 1.0 mm spacers, the whole system is fixed into a vertical gel electrophoresis unit filled with 1X running buffer. Samples and Protein Dual Colour Standards (Bio-Rad, Cat. # 1610374) were resolved on SDS-PAGE at 70 V for 40 minutes then 100 V for 2.5 hours.

After separation of proteins SDS gel, it was transferred onto the PVDF membrane (Bio-Rad, Cat # 1620177) that was activated by 100% methanol (Merck, Cat. # 106009). Transfer is performed using Trans-Blot® Turbo™ Transfer System (Bio-Rad, Cat # 1704150EDU). After transfer was completed, the membrane was stained with ponceau S (Sigma, Cat. No # 09189-1L) for 1 minute to check the transfer efficiency.

Blocking of membrane with 5% milk (Bio-Rad, Cat. # 170-6404) or 5% Bovine Serum Albumin (BSA) (Sigma, Cat. # A7030) was performed to minimize any background that may develop by nonspecific binding of the antibodies. The blocking solution was prepared with 1X TBS (Bio-Rad, Cat. # 170-6435) and 0.1X Tween 20 (Bio-Rad, Cat. # 170-6531) (TBST). Selection of blocking solution, 5% milk or 5% BSA is based on the primary antibody. Membrane was blocked for 1 hour at 4 °C. After blocking, a primary antibody specific to the protein of interest was diluted in the blocking solution and the membrane was incubated overnight at 4 °C. The membrane was washed 3 x 10 minutes with 1x TBST solution the next day and it was probed with species specific secondary antibody for 1 hour at 4 °C. After that, the membrane was washed with TBST at 3 x 10 minutes. Dilutions used to prepare primary and secondary antibodies are stated below (Table 10,11).

Primary antibody	Concentration	Species	Diluted in	Company, Cat. #
Mfn 1	1:1000	Mouse	5% milk	Abcam, Ab57602
Mfn 2	1:1000	Rabbit	5% milk	CST, 9482S
Cx 43	1:6000	Rabbit	5% BSA	CST, 3512S
Phospho Cx 43	1:1000	Rabbit	5% BSA	Invitrogen, 48-3000
GAPDH	1:5000	Mouse	5% milk	Protein tech, 60004-1-1g

Table 10. Preparation of Mfn 1, Mfn 2, Cx43, Phospho Cx43 and GAPDH primary antibody for WB.

Secondary antibody	Concentration	Company, Cat. #
Anti-mouse	1:1000	CST, 7076S
Anti-rabbit	1:1000	CST, 7074S

Table 11. Preparation of anti-mouse and anti-rabbit secondary antibody for WB.

3.5 Signal detection and immunoblot analysis

For the signal detection, Amersham ECL select western blotting detection reagent (GE Healthcare, Cat. # RPN2235) was prepared by adding 1 part of solution A and 1 part of solution B. The signal on the membrane was detected using the ChemiDoc MP imaging system from Bio-Rad. Densitometry was performed following acquisition using Image Lab software from Bio-Rad.

4. Microelectrode Array (MEA)

4.1 Tyrode solution preparation

Tyrode solution was prepared according to Table 12.

Chemical	Concentration (mM)	per 1L	Cat. #
NaCl	155	9.058 g	S7653-1kg
KCl	5.4	0.403 g	P5405-500g
NaH ₂ PO ₄	0.33	0.04 g	SLBL3354V
Glucose	10	1.802 g	G8270-100g
HEPES	10	2.383 g	H3375-100g
BDM	30	3.033 g	B0753-100g
MgCl ₂	1	1 mL	M1028-100ml

Table 12. Preparation of Tyrode solution.

Hydrochloric acid was added to adjust the pH of tyrode solution to pH 7.4, filtered with Nalgene rapid-flow filter (Thermo scientific, Cat. #596-4520) and stored in 4 °C fridge for usage the following day.

4.2 Preparation of normoxic and hypoxic tyrode solution

Normoxic and hypoxic tyrode solution were prepared and used on the same day using the following method. 500ml of tyrode solution were bubbled with pure O₂ gas for at least 15 minutes and divided according as below (Figure 9, Table 13):

Figure 9. Uses of Tyrode solution.

Solution preparation	Tyrode solution required (mL)	CaCl ₂ to be added (µL)
0.9mM Tyrode solution	150	135
1.8mM Tyrode solution	80	144
Hypoxic solution	80	144

Table 13. Preparation of different concentration of CaCl₂ Tyrode solution.

The remaining tyrode solution was placed in -80 °C freezer for vibratome usage. 4% of agarose gel (Invitrogen, Cat. # 16520-050) was prepared with tyrode solution and left in water bath at 40 °C till usage. In the meantime, 80mL of non-bubbled tyrode solution with 1.8 mM CaCl₂ was prepared as hypoxic solution by gassing the solution with N₂ gas for 30 minutes.

4.3 Vibratome sectioning

Mouse hearts were removed from the anaesthetised MfnDKO or WT mice, depending on the experimental schedule and rinsed with ice-chilled tyrode solution, cut transversely and moulded in 4% agarose. The agarose with heart sample was mounted on the vibratome stage (Figure 10). Heart slices were cut at 250 μm in ice crushed, Ca^{2+} free tyrode solution using vibratome (Leica, VT1000S).

Figure 10. Vibratome sectioning of heart.

The slices were transferred to 0.9 mM CaCl_2 tyrode solution for 30 minutes incubation, subsequently to 1.8 mM CaCl_2 tyrode solution for another 30 minutes for equilibrium.

4.4 Setup of MEA station

The MEA chip (MultiChannel Systems, Pedot MEA) consists of 59 electrodes and one reference electrode, arranged in an 8 x 8 matrix. Each electrode is 30 μm in diameter, with an interelectrode distance of 200 μm . The electrodes are made of PEDOT-CNT carbon nanotube – poly 3,4-ethylene-dioxythiophene, gold tracks and contact pads have very low impedance values of approximately 20 k Ω . The chip was loaded onto the amplifier connected to a powerpack

(MultiChannel System, Cat. # PS40W) and the whole system was continuously perfused with oxygenated tyrode solution at 37 °C (2 mL/minute). The heart slice was placed gently on the MEA chip and a nylon weighing harp was placed over the tissue to improve contact with the underlying electrodes and to ensure that the heart slice do not float off during perfusion. For consistency, the placement of the ventricle issue was orientated in the same orientation with right ventricle on the left and left ventricle (LV) on the right and the edge of LV are always in contact with electrode 62 and 72 by looking under the microscope (Figure 11A-C). Electrode 62 and 72 were chosen as the stimulation electrode so as to keep all the experiments consistent.

Figure 11. (A) Layout of MEA chip. (B) Location of electrode 62 and 72 on actual MEA chip that was used for stimulation. (C) Nylon weighing harp was placed on the top of sample tissue to ensure maximum contact.

The perfusion solution was maintained at 37 °C by a perfusion cannula (MultiChannel System, PH01) and a 2-channel temperature controller (MultiChannel System, TC02). The consistent peristaltic flow was maintained by the mini-peristaltic pump (Harvard apparatus, MP11).

4.5 Stimulation threshold definition and stimulation protocol

After 15 minutes of stabilization, electrical stimulation (Table 14) was applied to the heart slice at two electrodes, 62 and 72 using a software, MCStimulus (MultiChannel System), and the heart slice was observed for response. In the event that no response was observed after pacing a stimulus, the previous threshold value was recorded. The final stimulation was subsequently determined by multiplying the captured threshold by 2 (Table 15,16). A multiplication factor of 2 was chosen to ensure that the stimulus given to the tissue is enough to capture the feedback throughout the whole experiment. If no feedback was captured during the experiment, the experiment was voided as no data was collected for CV calculation.

	Pulse	value	Unit	time	Unit	value	Unit	time	Unit	value	Unit	time	Unit	Row re
1	rectangular	-100	μA	1000	μs	100	μA	1000	μs	0	μA	248000	μs	25
2	rectangular		μA		μs		μA		μs	0	μA	2500000	μs	
3	rectangular	-80	μA	1000	μs	80	μA	1000	μs	0	μA	248000	μs	25
4	rectangular		μA		μs		μA		μs	0	μA	2500000	μs	
5	rectangular	-70	μA	1000	μs	70	μA	1000	μs	0	μA	248000	μs	25
6	rectangular		μA		μs		μA		μs	0	μA	2500000	μs	
7	rectangular	-60	μA	1000	μs	60	μA	1000	μs	0	μA	248000	μs	25
8	rectangular		μA		μs		μA		μs	0	μA	2500000	μs	
9	rectangular	-50	μA	1000	μs	50	μA	1000	μs	0	μA	248000	μs	25
10	rectangular		μA		μs		μA		μs	0	μA	2500000	μs	
11	rectangular	-40	μA	1000	μs	40	μA	1000	μs	0	μA	248000	μs	30
12	rectangular		μA		μs		μA		μs	0	μA	2500000	μs	
13	rectangular	-30	μA	1000	μs	30	μA	1000	μs	0	μA	248000	μs	35
14	rectangular		μA		μs		μA		μs	0	μA	2500000	μs	
15	rectangular	-20	μA	1000	μs	20	μA	1000	μs	0	μA	248000	μs	40
16	rectangular		μA		μs		μA		μs	0	μA	2500000	μs	
17	rectangular	-10	μA	1000	μs	10	μA	1000	μs	0	μA	248000	μs	50
18	rectangular		μA		μs		μA		μs	0	μA	2500000	μs	
19	rectangular	-5	μA	1000	μs	5	μA	1000	μs	0	μA	248000	μs	50
20	rectangular		μA		μs		μA		μs	0	μA	3000000	μs	

Table 14. List of electrical stimulation threshold applied to ventricular tissue.

No.	Stimulus threshold (μA)	Observation	Stimulation protocol (μA)
1	100	✓ response	
2	80	✓ response	
3	60	✓ response	✓ ($60 \times 2 = 120 \mu\text{A}$)
4	40	X response	

Table 15. Final electrical stimulus protocol.

	Pulse	value	Unit	time	Unit	value	Unit	time	Unit	value	Unit	time	Unit	Row re
1	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	248000	μs	9
2	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	248000	μs	
3	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	500000	μs	
4	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	248000	μs	9
5	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	238000	μs	
6	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	500000	μs	
7	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	248000	μs	9
8	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	228000	μs	
9	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	500000	μs	
10	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	248000	μs	9
11	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	218000	μs	
12	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	500000	μs	
13	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	248000	μs	9
14	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	208000	μs	
15	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	500000	μs	
16	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	248000	μs	9
17	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	198000	μs	
18	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	500000	μs	
19	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	248000	μs	9
20	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	188000	μs	
21	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	500000	μs	
22	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	248000	μs	9
23	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	183000	μs	
24	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	500000	μs	
25	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	248000	μs	9
26	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	178000	μs	
27	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	500000	μs	
28	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	248000	μs	9
29	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	173000	μs	
30	rectangular	-120	μA	1000	μs	120	μA	1000	μs	0	μA	500000	μs	

Table 16. Example of an electrical stimulation protocol at 120 μA .

All the stimulation threshold and stimulation protocol were optimised by David Montaigne. A list of stimulation protocols were pre-set into the software and was chosen based on the stimulation threshold.

Once the setup for stimulation protocol is ready, the whole experimental condition commences as below (Figure 12):

Figure 12. Experimental protocol.

Baseline was recorded for 6 minutes with oxygenated tyrode solution at 37 °C then LV was challenged with hypoxic condition by replacing the oxygenated tyrode solution with oxygen-deprived solution (37 °C; N₂) for 8 minutes and then re-exposed to oxygen-enriched tyrode solution for 32 minutes. During this baseline and ischemia reperfusion (IR) simulated protocol, readings were assessed at every 2 minutes interval starting from 0 min to 46 minutes (Figure 13). We were able to look at the detailed signal generated from each individual electrode by clicking on the respective box, eg. Electrode 76, with point of stimulus at A and feedback at B (Figure 14). Field potential data were acquired simultaneously from all 60 microelectrodes and feedback was recorded by MC_Rack (MultiChannel System).

Figure 13. Real time stimulus and feedback on MCStimulus software.

Figure 14. A close up of stimulus and feedback at electrode 76.

4.6 Statistical analysis

Analysis was done by using a software, Clampfit (Molecular device) (Figure 15). The time difference between point of stimulus and feedback were measured consecutively for the first two stimulus at every 2 minutes interval (Figure 16). CV was calculated by using maximum distance across LV divided by time. Data for both MfnDKO and control mice were collected and used to plot a graph for comparison. All statistical analysis was performed with Microsoft Excel 2016. All results were expressed in terms of mean $\pm$ standard deviation (SD) values, unless otherwise indicated. Differences between groups were compared using the two-tailed test; $p < 0.05$ was considered statistically significant.

Figure 15. Analysis of stimulus and feedback on specific electrode.

Figure 16. Time difference between point of stimulus (A) and feedback (B).

5. Immunohistochemistry

5.1 Preparation of paraffin-embedded tissue

Heart tissue were processed as shown in Table 17.

Step	Reagent	Time (hrs)
1	10% NBF	48
2	70% Ethanol	3
3	90% Ethanol	Overnight
4	100% Ethanol	2
5	50% Ethanol:50% HistoClear	1
6	100% HistoClear	1
7	100% Paraffin wax	1
8	100% Paraffin wax	1
9	100% Paraffin wax	1

Table 17. Heart tissue processing protocol.

After processing, the tissue was embedded in paraffin wax and stored until required. Paraffin sections were cut using a microtome (Leica RM2255) at 4 μ m, placed onto 30% alcohol solution and floated out onto warm water. The sections were picked up on superfrost slides and left to dry at 37 °C for 48 hours.

5.2 Immunohistochemistry staining on paraffin-embedded tissue

Immunohistochemistry (IHC) was performed on both MfnDKO and WT mice together, to ensure consistency in the IHC techniques. Slides were left in 70 °C incubator for 20 minutes. Sections were deparaffinized in three changes of histoclear for 7 minutes, followed by rehydration using graded alcohols (100%,

95% and 70%). Slides were washed in PBST for 3X 5 minutes then it was incubated in 0.1% triton X-100 (Sigma, Cat. # X100) for 15 minutes. Slides were washed in PBST for 3X 5 minutes. Antigen retrieval was performed by incubating the slides in bull's eye decloaker at 100 °C for 10 minutes. Slides were washed in PBST for 3X 5 minutes. Endogenous peroxidase activity was blocked using 0.3% H₂O₂ in PBS for 30 minutes followed by washes in PBST for 3X 5 minutes. Sections were blocked in 5% BSA for 1 hours in room temperature. The BSA was then replaced with the Cx 43 primary antibody (1:100) diluted in 5% BSA (Table 18) and incubated overnight at 4 °C.

Primary antibody	Concentration	Species	Diluted in	Company, Cat. #
Cx 43	1:100	Rabbit	5% BSA	CST, 3512S

Table 18. Preparation of Cx43 primary antibody for IHC.

The following day, the slides were washed in PBST for 3X 5 minutes. Incubation with the Anti-rabbit secondary antibody (1:100) (Table 19) was carried out for 1 hour at room temperature, followed by washing in PBST for 3X 5 minutes.

Secondary antibody	Concentration	Company, Cat. #
Anti-rabbit	1:100	CST, 7074S

Table 19. Preparation of Cx43 secondary antibody for IHC.

Sections were incubated in ImmPACT DAB (VECTOR, Cat. #SK-4105) for 2 minutes followed by washes in PBST 3X 5 minutes. Sections were counterstained with haematoxylin (Sigma, Cat. # H3136) for 5 seconds, washed in distilled water and permanently mounted with DPX (Sigma, Cat. # 06522).

RESULTS

1. Characterizing MfnDKO animal model

1.1 Genotyping strategy for detection of MfnDKO

Genotype analysis was carried out, and it was observed that in Figure 17A, Mfn 1 floxed mice produced a 450bp product and WT mice produced a 350bp product, while in Figure 17B, Mfn 2 floxed mice produce a 180bp product and WT mice produced a 145bp product. No band was observed at NTC which was used as a negative control.

A

B

Figure 17. Gene expression of Mfn 1 (A) and Mfn 2 (B) in MfnDKO and WT mice. Agarose gel electrophoresis was performed to confirm the genotype of MfnDKO mouse. NTC was loaded with the same amount without DNA.

1.2 Genotyping strategy for detection of MfnDKO Cre+ and Cre- mice

Identification of Cre+ and Cre- MfnDKO for experiments was done by genotyping (Figure 18). Cre has a 650bp product. Hence, the presence of the

band at 650bp allow us to identify the Cre+ mice. The absence of band at 650bp identifies Cre- mice. No band should be seen at NTC.

Figure 18. Gene expression of Cre in MfnDKO mice. Agarose gel electrophoresis was performed to check the presence of Cre gene. NTC was loaded with the same amount without DNA.

1.3 Evaluation of cardiac-specific Mfn 1 and Mfn 2 deletion in MfnDKO mice upon tamoxifen injection

To validate that Mfn 1 and Mfn 2 are knocked out after tamoxifen injection, western blot was performed on 10 weeks old mice. The expression of a band at both 84kDa for Mfn 1 and 80kDa for Mfn 2 significantly decreased in MfnDKO group compared to WT group (Figure 19) which demonstrated that the tamoxifen administration did knockout cardiac-specific Mfn 1 and Mfn 2 in MfnDKO mice heart. GAPDH was used as the housekeeping gene for all western blot experiments. Densitometric analysis was done to show the significance of the difference in Mfn 1 and 2 protein expression between the 2 mice types (Figure 20).

Figure 19. Protein expression of Mfn 1 and Mfn 2 decreased in MfnDKO mice. Representative WB was performed using Mfn 1 and Mfn 2 primary antibody and anti-rabbit secondary antibody. WB was replicated in 3 biological replicates. GAPDH was used as the housekeeping protein.

Figure 20. Protein expression of Mfn 1 and Mfn 2 decreased in MfnDKO mice. Densitometric analysis of WB was performed (n=3). Statistical analysis using student T test, 2 tailed was performed. Data presented as mean $\pm$ SD. * $p < 0.05$.

2. MEA experimental setup characterization for ventricular tissues

Preliminary data from Dr Andrew Hall from University College London showed that there was a significant decrease in the CV of MfnDKO atrial tissue at baseline and following acute simulated IRI (unpublished work). In this thesis, we wanted to investigate if MfnDKO ventricular tissue has the same effect as the atria preliminary data. Characterization for MEA experimental setup for

ventricular tissue was mandatory as there were additional steps that were required to be performed before loading of ventricular tissue slice onto MEA. For atria MEA experiments, it was harvested directly from the heart and loaded on the MEA chip directly for selection of optimal stimulation protocol. However, for ventricular MEA experiments, before loading it on the MEA, the whole heart was harvested, with both left and right atria and apex removed. The additional steps that were required for ventricular tissue preparation are, embedding of ventricular tissue in 4% agarose and cutting of tissue using vibratome. We characterized all the steps to ensure that the tissue is in an optimal condition before proceeding to load onto the MEA chip.

2.1 Embedding of ventricular tissue in 4% agarose

The time taken to harvest the heart and embed the ventricular tissue into 4% agarose is critical as the tissue undergoes hypoxia if the duration is too long, indicated by pale tissue. If the heart is healthy, it will be red in color (Figure 21). If the tissue is whitish at this point of time, the experiment will be ceased. The purpose of the agarose is to hold the tissue in place while it is cut with a vibratome. Hence it is important to ensure that both ventricles are filled with agarose to prevent the tissue from falling off from the agarose block when the blade cuts through the tissue. Should the tissue detach from the agarose, the experiment will also cease.

Figure 21. Photographs of the embedded heart on vibratome showing pallor of the heart during prolonged hypoxia (left) and redness in healthy heart (right) under prompt embedding.

2.2 Vibratome optimization

Sectioning of heart tissue was optimized to ensure that the thickness of the heart is optimal for measurement of CV via MEA. The optimized thickness is 250 μm . Thin heart slices will cause the tissue to shrivel, causing the surface to not have full contact with the electrodes, resulting in the electrode being unable to apply stimulation at a specific point (Figure 22, left). Thick heart slices will cause the tissue to undergo hypoxia, resulting in no feedback upon stimulation. A healthy heart slice will show a flat surface with rounded edge (Figure 22, right).

Figure 22. Photographs of a shriveled heart slice sectioned at suboptimal thickness (left) and a heart slice that was at optimal thickness (right) loaded onto the MEA chip.

Under the microscope, we are able to tell from the heart slices phenotype if they are healthy. Slices with uneven surface or circumference will be eliminated (Figure 23, left). Only slices with smooth and flat surfaces will be loaded onto the MEA for CV measurement (Figure 23, right).

Figure 23. Photographs from an inverted microscope of an unhealthy heart slice (left) and healthy heart slice (right) emplaced onto the MEA chip.

2.3 MEA chip

A normal MEA chip will be free of air bubbles (Figure 24, left). It is important to ensure that there are no air bubbles around the electrodes (Figure 24, right) as this will interfere with grounding and results in increased noise level (Figure 25). It is important to keep the noise level as low as possible via grounding as this can help to avoid background noise. Both the MEA company and us could not determine the reason for the air bubbles trapping under the coating, and hence we exchanged for a new MEA chip.

Figure 24. Photographs of a normal MEA chip (left) and the appearance of tiny bubbles surrounding the individual electrodes (right).

Figure 25. Images of the background noise level showing minimal (left) and high noise level due to electrical disturbance (right).

2.4 Selection of optimal stimulation protocol and data collection

The selection of optimal stimulation protocol is crucial as the feedback of stimulation from the heart slice is based on the pacing current. If the current of the stimulation protocol is insufficient to pace the heart, no feedback will be observed after every stimulation (Figure 26).

Figure 26. Diagram of serial stimulation paces without ventricular feedback on Clampfit software.

It is necessary to increase the current of the stimulation protocol if there was no response from the tissue. The heart slices will follow the pacing systematically if the stimulation protocol is optimal (Figure 27).

Figure 27. Diagram of stimulation paces with corresponding ventricular feedback on Clampfit software.

Observations were made during the whole experiment to ensure that there was feedback throughout the baseline, ischemia and reperfusion. If there was any point of time that there was no feedback upon stimulation, we will cease the experiment.

Every step of the experiment, from harvesting the heart till the measurement of CV using MEA is of utmost importance, and should any step fail, the experiment would have to cease and be restarted with a new mouse.

3. Measurement of CV of MfnDKO and WT group using MEA

Utilizing the optimized MEA protocol as described above, our results showed that the CV of MfnDKO was significantly lower than WT at baseline and following simulated IR (Figure 28). At baseline (0 – 6 minutes) there were no significant changes in CV. However, when both groups of ventricular tissue were subjected to ischemia for 8 minutes (6 – 14 minutes), we observed a decline in CV. During the start of reperfusion at 14 minutes, the CV for both groups surged and remained plateau from 22 minutes till the end of the experiment (14- 46 minutes). Both groups had the same trend throughout the experiment (n=3 for each group).

Figure 28. CV of MfnDKO was significantly lower than WT at baseline and following simulated IR. MEA was used to measure the CV of the ventricular slices (n=3). Statistical analysis using student T test, 2 tailed was performed. Data presented as mean $\pm$ SD. * $p < 0.05$.

4. Localization and expression of Cx43 in MfnDKO and WT group

4.1 Evaluation of localization of Cx43 using IHC

IHC staining of ventricular tissue showed that localization of Cx43 in MfnDKO was partially redistributed away from ID to around the cell, while the localization of Cx43 in WT was confined at the ID (Figure 29) (n=1 for each group).

Figure 29. Lateralization of Cx43 in MfnDKO mice. Photomicrograph (20X magnification) of MfnDKO (top) and WT hearts (bottom) with anti-Cx43 (brown staining) by IHC). Note, Cx43 was confined at the ID in WT heart. Red arrow denotes ID.

4.2 Evaluation of Cx43 and Phospho S368 Cx43 level in MfnDKO and WT mice

Figure 30 shows that the expression of Cx43 and phospho S368 Cx43 was higher in MfnDKO mice when compared to WT mice. Densitometric analysis confirmed that Cx43 and phospho Cx43 levels were higher in MfnDKO when compare to WT (Figure 31) (n= 3 for each group).

However, the ratio of phospho S386 Cx43 to total Cx43 does not show much difference between MfnDKO and WT group (Figure 32). It might be due to low sample size.

Figure 30. Protein expression of Cx43 and phospho S368 Cx43 were higher in MfnDKO mice. WB was performed using Cx43 and phospho Cx43 primary antibody and anti-rabbit secondary antibody. (n=1). GAPDH was used as the housekeeping protein.

Figure 31. Protein expression of Cx43 and phospho S368 Cx43 decreased in WT mice. Densitometric analysis of WB was performed (n=1). Statistical analysis using student T test, 2 tailed was performed. Data presented as mean $\pm$ SD. * $p < 0.05$.

Figure 32. Ratio of phospho S368 Cx43 to total Cx43 was lower in WT mice. Statistical analysis using student T test, 2 tailed was performed. Data presented as mean $\pm$ SD. * $p > 0.05$.

DISCUSSION

In this study, we sought to study the link between Mfns, mitochondrial dysfunction and arrhythmia susceptibility. Earlier studies have discovered that Mfns plays key roles in maintaining mitochondrial health, and a reduction in their expression has been implicated in a range of cardiac diseases associated with an increased susceptibility to MI and arrhythmias⁵⁰.

It was established by other groups that myocardial ischemia will lead to lateralization of Cx43 and an increase of phosphorylation at Ser368⁵¹. However, no work was done on MfnDKO to study if there are any changes in the electrical conduction and localization of Cx43, and in total and phospho S368 Cx43 level. In this thesis, we investigated the CV of the ventricular slices isolated from cardiac tissue via MEA, to determine if Mfns deficient mice are susceptible to arrhythmias under baseline condition following IR. Also, we used IHC to show the localization of Cx43, and in conjunction with WB to show the expression level of Cx 43 and phospho S368 Cx43 in both groups.

To obtain CV, we optimized the MEA experimental setup for ventricular tissues and ran MEA and the data was analysed by Clampfit and a graph was plotted for comparison between MfnDKO and WT groups. Here, we showed that the CV of MfnDKO mice is observed to be significantly lower than WT at baseline and IR conditions. The CV for both groups decreased when the tissue was subjected to hypoxic Tyrode solution, and increased when subjected to normoxic Tyrode solution, mimicking ischemia and reperfusion respectively. This suggest that ischemia further damages the cells and leads to decreased CV, while reperfusion will restore it to the original state.

It had been reported that during ischemia, mitochondrial respiration is suppressed leading to a reduction in ATP production, eventually reducing cardiac ATPase efficiency and causing a further reduction of ATP that can result in the malfunctioning of transporters and ion channels. This results in ionic imbalances in Na^+ , Ca^{2+} , K^+ and H^+ , disrupting the cardiac rhythm and thereby affecting CV⁵².

Studies have shown that upon reperfusion, there is an increase in oxygen level which leads to an increase in ATP production, restoring ionic balance, cardiac rhythm and CV, which is consistent with the results we have observed during IR for both MfnDKO and WT mice. However, ROS may also be released from damaged cells, which may damage proteins, including those forming the electron transport chain⁵³. Due to ROS damage to the electron transport chain, mitochondrial dysfunction will become self-propagating.

CV of MfnDKO mice was observed to be significantly lower than WT at baseline and IR conditions, suggesting that both Mfn 1 and Mfn 2 play a significant role in the regulation of CV by mediating the fusion of adjacent mitochondria that are involved in mitochondria maintenance and repair.

Since CV is determined by GJ, we are interested to study Cx43, which is the most abundant and is expressed in ventricular tissue. Seeing that CV was significantly lower in MfnDKO and Cx43 is known to regulate CV, we decided to investigate the localization of Cx43. Cx43 is located at the ID and its lateralization will affect the electric conduction across the cells. The localization of Cx43 is determined by phosphorylation or dephosphorylation at different sites.

We have chosen to study phospho S368 Cx43 as it had been reported that phosphorylation at that site reduces GJ communication.

We ran IHC to see the localization of Cx43, utilizing the antibodies for the detection of a specific antigen, Cx43, to determine the distribution of antigen interest in tissue sections. Our IHC results showed that Cx43 are partially disorganized in the MfnDKO mice group. This could be a potential reason for the observed reduction in CV because it will take a longer time for electrical current to travel across the tissue. However, IHC is only able to detect the distribution and localization of Cx43, but it is unable to provide any information about functionality.

It was reported that the lateralization of Cx43 could have occurred due to it moving away from the cellular stress, or new Cx43 could have been located randomly in order while attempting to compensate for the closed channels due to increased acidity or other signalling events at the ID²⁸. The latter might also explain higher Cx43 protein level in MfnDKO mice. The knocking out of both Mfn 1 and 2 have affected the localization of the GJ, and perhaps even the functionality of the GJ. Hence, the cells may have attempted to produce more GJ.

On top of the structural remodelling of connexins, the phosphorylation status of the channels will also lead to a functional remodelling of connexins in the diseased heart. Although it had been reported that functional channels can be formed without phosphorylation, phosphorylation at multiple serine residues is required for regulating the gating of GJ channels^{54,55}.

Our WB results showed that the expression of Cx43 and phospho S368 Cx43 are significantly higher in MfnDKO compared to WT. Compensation of closed GJ might be the potential reason why there was an increase of Cx43 in MfnDKO group. In one of the studies, phosphorylation of Cx43 on Ser 368 was shown to lead to a decrease in GJ communication. In addition, the flow chart in Figure 5 explained the reason of elevated levels of phospho S368 Cx43 in MfnDKO group. Our WB results does support the correlation of MfnDKO and the increase of Cx43 and phospho S368 Cx43.

Changes in levels of both Mfns eventually leads to mitochondrial dysfunction, leading to an increase of ROS and PKC level. When PKC level elevates, phosphorylation at S368 Cx43 will increase and disrupt the GJ communication, leading to a decrease of CV. The movement of Cx43 from ID to lateral membrane is based on the phosphorylation status on a specific site on Cx43.

IR is a good example of how the phosphorylation and unphosphorylation of a specific site on Cx43 will lead to a redistribution of Cx43 (Table 20). In fact, the increase of Cx43 and phospho S368 Cx43 level in our MfnDKO model showed the same trend as in Lampe's ischemia study⁵⁶.

Phosphorylation site	Phosphorylation level	
	Normoxic condition	Ischemic condition
S262	Lower	Higher
S279/282	Lower	Higher
S325/328/330	Higher	Lower
S365	Higher	Lower
S368	Lower	Higher
S373	Lower	Higher

Table 20. Phosphorylation level of different phosphorylation site in different condition.

CONCLUSION

In conclusion, Cx43 lateralization occurs in many cardiac pathologies although the mechanisms for these changes are not known. In our study, CV in heart ventricular tissue from MfnDKO mice was significantly lower than WT at baseline and following simulated acute IR, a condition which would increase susceptibility to cardiac arrhythmias, suggesting Mfn 1 and Mfn 2 play an important role in the regulation of CV. Our initial data has also suggested that Cx43 localization within cardiomyocytes is altered in MfnDKO mice when compared to WT mice, with partial lateralization of Cx from the GJ to the cytosol, a feature which would affect CV and increase susceptibility to cardiac arrhythmias. Finally, our preliminary data has also suggested that phosphorylation of Cx43 at Ser368 is increased in MfnDKO mice when compared to WT mice, a feature which would decrease CV and increase susceptibility to cardiac arrhythmias.

To date, there are no comprehensive studies on the molecular mechanisms by which Cx43 lateralizes except in the case of IRI^{28,57}. Further studies can be carried out to further determine the main cause and its mechanism.

However, it is also important to note that this is only a pilot study, as the sample size for both IHC and western blot were only 1 and 3 respectively. Further studies, with a larger sample size, would be needed to support the results obtained here.

In summary, in heart ventricular tissue deficient in both Mfn 1 and Mfn 2, CV was reduced, a finding which was associated with partial redistribution of Cx43 away from the GJ, and phosphorylation of Cx43 at Ser368, findings which may

be expected to increased susceptibility to cardiac arrhythmias post-AMI. This initial data highlights the Mitofusins as potential therapeutic targets for preventing cardiac arrhythmias post-AMI.

FUTURE WORK

From our IHC results, we have shown that MfnDKO has a disorganized localization of Cx43 at baseline level compared to WT. It will be interesting to look at the location of Cx43 and phospho S368 Cx43 during ischemia and reperfusion for both MfnDKO and WT. We would also like to study the phospho S325/328/330 in MfnDKO model as it was reported that the expression level decreased during ischemia.

Further studies on Cx43 localization and lateralization in IR are needed to determine if the molecular mechanisms of lateralization are uniform across injuries or if differing pathologies cause different molecular events to occur which translate into the same phenotype, i.e. lateralized Cx43.

From our western blot results, we have also shown that MfnDKO has a higher level of phospho S368 Cx43 and total Cx43 compared to WT at baseline level. It will be interesting to look at the level of phospho S368 Cx43 and total Cx43 during ischemia and reperfusion for both MfnDKO and WT.

We have proposed a pathway in figure 5, and also showed that MfnDKO has a higher level of phosphorylated S368 Cx43 and a lower CV compared to WT. The next approach would be to look at ROS and PKC ϵ level in both groups, to determine the link between MfnDKO and the decreased CV via the pathway we proposed in fig. 5.

We have investigated the MfnDKO in *ex vivo*, using MEA technique, to show that ventricular slices isolated from cardiac tissue deficient Mfns are susceptible to arrhythmias under baseline condition following IR. MEA showed that MfnDKO has a lower CV across the experiment compared to WT. Moving forward, we

can investigate *in vivo* whether our previous observations, made *ex vivo*, validate the susceptibility of MfnDKO to cardiac arrhythmias at baseline and IR. Mice will undergo *in vivo* recovery MI by open chest surgery and occlusion of the left anterior descending coronary artery followed by reperfusion and recovery.

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