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Programvezető: Dr. Szökő Éva, egyetemi tanár

Témavezetők: Dr. Ferdinandy Péter, egyetemi tanár

Dr. Görbe Anikó, egyetemi docens

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# NOVEL CARDIOPROTECTIVE MECHANISMS IN TRANSLATIONAL MODELS OF THE ISCHEMIC HEART

**PhD thesis**

**András Makkos, MD**

Pharmaceutical Sciences Doctoral School

Semmelweis University

Supervisors:

Anikó Görbe, PhD

Péter Ferdinandy, DSc

Official reviewers:

Zoltán Jakus, PhD

Attila Tóth, DSc

Head of the Complex Examination Committee:

Zoltán Benyó, DSc

Members of the Complex Examination Committee:

Zsuzsanna Helyes, DSc

Mária Judit Molnár, DSc

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**List of abbreviations**

Akt – protein kinase B

AMI – acute myocardial infarction

ANP – atrial natriuretic peptide

ATP – adenosine triphosphate

CAD – coronary artery disease

cGMP – cyclic guanosine monophosphate

ERK – extracellular signal-regulated kinase

HF – heart failure

HIF1α – hypoxia-inducible factor 1-alpha

I/R – ischemia-reperfusion

IPreC – ischemic preconditioning

IPostC – ischemic postconditioning

GLP-1 – glucagon like peptide 1

LAD – left anterior descending (coronary artery)

MI – myocardial infarction

MMP – matrix metalloproteinase

MPTP – mitochondrial permeability transitions pore

mRNA – messenger RNA

miRNA - microRNA

NO – nitrogen monoxide

NOS – nitrate oxidative stress

$P_2Y_{12}$ – purinergic receptor $P_2Y_{12}$

PI3K – phosphoinositide 3-kinase

PPCI - primary percutaneous coronary intervention

RIC – remote ischemic conditioning

RISK – reperfusion injury salvage kinase

PKCα – protein kinase C-alpha

PKCε – protein kinase C-epsilon

PKA – protein kinase A

PKG – protein kinase G

RNA – ribonucleic acid

ROS – reactive oxygen species

SAFE – survival activating factor enhancement

SGLT-2 – sodium-glucose cotransporter 2

SI/R – simulated ischemia/reperfusion

SNAP – S-nitroso-N-acetyl penicillamin

STAT – signal transducer and activator of transcription

TNFα – tumor necrosis factor alpha

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# 1 Introduction

## 1.1 Ischemia/reperfusion injury

Despite the progress in our understanding of mechanisms and treatment options, cardiovascular diseases remain a leading cause of death and disability [1]. The dominant pathological entity among cardiovascular diseases is the ischemic heart disease, especially myocardial infarction [2]. Myocardial ischemia can manifest as acute occlusion of the coronary artery or as chronic stenosis of the coronary vessel, which leads to lack of blood flow, oxygen and nutrient supply of the affected myocardial area [3]. The ischemia results in an anaerobe shift of metabolism, as well as lowers the intracellular pH, depletes ATP and creatinine phosphate to impair ion pump function and contractility. All these changes lead to an increase in intracellular calcium ion concentrations and subsequently lead to cell death [4].

Currently, primary percutaneous coronary intervention (PPCI) is the first line treatment to restore blood flow and reduce the rate of the ischemic injury [5]. However, the induced reperfusion may not only lead to the recovery of ischemic heart tissue, but the restored circulation brings additional damage named “reperfusion injury” [6]. Reperfusion injury may account for as much as 50% of the total damage of the myocardium [7].

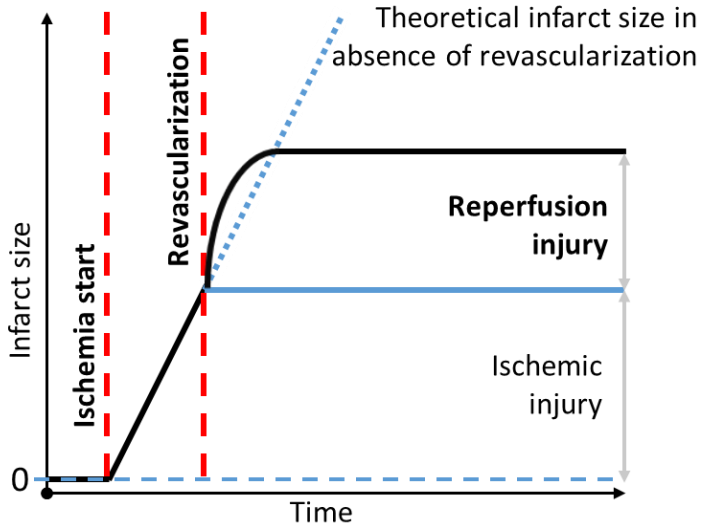


**Figure 1** Individual contributions of ischemic injury and reperfusion injury to the final damage of the ischemia-reperfusion injury (I/R) on the myocardium (expressed in arbitrary units). Based on Hausenloy et. al, 2013

In terms of these, the combined ischemia-reperfusion (I/R) injury can be manifested as reperfusion-induced arrhythmias, myocardial stunning, microvascular obstruction, and cardiac myocyte death. Oxidative stress, calcium overload, and irreversible hypercontracture of cardiac myocytes contribute to the loss of myocardium under I/R injury [8, 9].

### 1.2 Cardioprotection against ischemia/reperfusion injury

Protection of the ischemic heart is an unmet clinical need. There are several promising targets for cardioprotection, which were successfully tested in preclinical *in vitro* models and in small animal studies [10, 11]. However, their translation to clinical practice as treatment of acute myocardial infarction (MI) has failed so far. Therefore, there is an urgent need for effective cardioprotective therapy. From ischemic conditioning to pharmacological conditioning and new approaches, there are many possibilities for cardioprotection.

#### 1.2.1 Ischemic conditioning

Brief non-lethal cycles of ischemia and reperfusion (termed as “ischemic conditioning”) dramatically reduce the myocardial damage of acute MI [12].

When these ischemia-reperfusion cycles are applied before the long, potentially lethal ischemia, it provides powerful endogenous cardioprotection, this phenomenon is named **ischaemic preconditioning (IPreC)** [12]. Ischemic preconditioning has two windows: early and delayed protection. However, clinical application of an invasive cardioprotective manoeuvre before the insult is limited.

In case of **ischemic postconditioning (IPostC),** the non-lethal ischemia-reperfusion cycles are applied at the beginning of the reperfusion, but still invasively on the injured vessel [13, 14]. In contrast to IPreC, IPostC is a cardioprotective technique that can be implemented in clinical practice.

**Trigger of the ischemic conditionings** can be the physical stimuli directly [15] as myocardial stretch activates downstream signalling (e.g. ATP sensitive $K^+$ channel) or endogenously released chemical stimuli [16] (e.g. reactive oxygen and nitrogen species (ROS and NOS), and nitrogen monoxide [17]. Autacoids, neurotransmitters, hormones

(e.g. adenosine, bradykinin, acetylcholine, catecholamine, endothelin, opioids, adrenomedullin, natriuretic peptide), lipid mediators, growth factors and cytokines can also induce protection via their respective receptors [9].

**Intracellular mediators** of ischemic conditionings include multiple cytosolic kinases: PKC and its isoforms (PKCα and PKCε), PKA, mitogen activated protein kinases (p38, jun-activated kinase) and PKG [9]. RISK pathway, one of the canonical pathways in cardioprotection, includes the activation of PI3K, phosphoinositide-dependent kinase, Akt, and ERK in response to cardioprotective stimuli [18]. SAFE pathway, the other major canonical signal transmission direction includes TNFα receptor 2 activation and STAT proteins as intracellular mediators. SAFE pathway can also induce protection related gene expression changes, however also exerts acute protection on the mitochondria [19]. HIF1α also requisite for mitochondrial ROS formation and infarct size reduction by ischemic conditionings [20]. Formation of ROS have an ambivalent role. Distinct amount of ROS contribute to protection, however, excessive formation of ROS contributes to irreversible injury [16].

Altogether, mitochondria are the common point where cardioprotective signalling pathways converge. The cardioprotective signals prevent the opening of the mitochondrial permeability transitions pore (MPTP), a non-specific pore enables the release of mitochondrial molecules smaller than 1.5 kD (protons, ions, etc.) and leads to cell death [21].

**In preclinical studies**, despite the differences in the timing of the stimulus, there is an overwhelming agreement that ischemic pre- and postconditioning defend the heart resistant to lethal I/R injury [9, 10, 22, 23].

However, **the first clinical studies** showed reduction in infarct size with IPostC [24], the results of following clinical studies are controversial [25-27]. The contradictory results are probably in association with the selection of patients and the applied IPostC protocol itself. DANAMI-3, the largest clinical trial investigated the effectiveness of IPostC, found a non-significant reduction in major adverse cardiac events [28].

### 1.2.2 Pharmacological cardioprotection

Endogenous or exogenous pharmacological agents are also able to induce cardioprotection against I/R injury. Many potential cardioprotective agents successful in preclinical settings has failed after promising preliminary studies [29]. However, there are other targets or drugs still under investigation.

**Nitric monoxide (NO)** donors and nitrites are still in focus to manipulate tetrahydrobiopterin and particulate or soluble guanylate cyclase [30] to increase cGMP-PKG activity and exert cardiocytoprotective effect [31]. In NIAMI trial, sodium nitrite prior PPCI did not decreased infarct size [32]. Although, **atrial natriuretic peptide (ANP)** decreased cardiac necroenzyme release in J-WIND-ANP trial [33].

**GLP-1 and its analogue exenatide** also able to reduce the infarct size through the cGMP/PKG activation in mouse and porcine models [34, 35]. Also clinical trials with exenatide given in early reperfusion demonstrated increased myocardial salvage [36, 37].

**Adenosine**, an endogenous autacoid and its synthetic agonist AMP579 was investigated excessively [38-41]. AMISTAD-II [40] and PROMISE trial [41] reported protective effect when adenosine was given in early reperfusion and early PPCI was performed.

**Matrix metalloproteinase (MMP) inhibition** reduced MI size in experimental studies [42, 43]. Although, there are very few clinical trial conducted with MMP inhibitors in acute myocardial infarction. Non-selective MMP inhibition in high dose failed to exert protection [44]. However, doxycycline, which has also MMP inhibitor activity showed significant cardioprotective effect in AMI patients [45].

**Cyclosporine-A,** an inhibitor of the MPTP opening, proved infarct size reducing effect in large animal experiments [46] and clinical studies [47, 48]. However, in CIRCUS trial failed to reduce infarct size or improve 1-year clinical outcome [49, 50].

**$P_2Y_{12}$ inhibitors** are able to reduce MI size via 'conditioning' signalling beside their anti-thrombotic effect in preclinical animal models [51-53]. Based on the observations of small retrospective studied infarct size reduction can be transferred to the clinics [54, 55]. An additional retrospective analysis of $P_2Y_{12}$ inhibitors induced protection showed benefit with ticagrelor compared with other $P_2Y_{12}$ inhibitors [56].

**Metoprolol** was found to be cardioprotective in a large animal model when administered prior reperfusion [57]. This effect was confirmed in METOCARD-CNIC trial, when metoprolol was administered prior PPCI [47]. However, a later trial failed to show protection with similar protocol [58]. Metoprolol is one of the most promising clinically available agents to exert cardioprotection and there are several ongoing clinical trials.

Since most of the above mentioned drugs showed inconsistent results, further clinical trials and additional studies are required to define optimum dose, timing and assess long-term clinical outcomes.

### 1.2.3 Novel approaches to induce cardioprotection

Over the past 3 decades, many cardioprotective strategies have been proposed against I/R injury. Several pharmacological treatments have been failed, while ischemic conditioning strategies are promising, however, inconsistent in several studies. To achieve patients' benefit, there is a need for novel approaches to induce cardioprotection against I/R injury.

**Microvascular injury** due to the obstruction of the coronary microcirculation may amplify the damage caused by the obstruction of the epicardial arteries and diminish the result of reperfusion therapies in MI patients [59]. Inhibition of these microvascular damages can lead to sparing of final infarct size and loss of myocardium [10].

**Inflammation** targeted therapeutic approaches have been disappointing so far. However, new components of I/R induced inflammation are discovered, e.g. inflammasomes [60], extracellular nucleic acids (RNA, DNA) [61, 62] and neutrophil extracellular traps [63]. Approaches to inhibit the initial pro-inflammatory and facilitate the anti-inflammatory processes may provide more significant benefits.

**Extracellular vesicles** (exosomes and microvesicles) are players of intercellular signal transmission [64]. These intercellular or interorgan communications play role in ischemic conditioning [65]. They can carry proteins and short non-coding RNAs (e.g. microRNAs) from one cell to another [66]. In addition, exosomes are proven to mediate the therapeutic effects of transplanted stem cells. Therefore, novel cell-free therapies based on stem cell-secreted exosomes can provide a safe and effective alternative of stem cell therapies [67]. However, it needs to be established whether a therapy by extracellular vesicles may confer cardioprotection [68].

**Drugs can be repurposed** for cardioprotection. For example, sodium-glucose cotransporter 2 (SGLT2) inhibitors are originally a class of drugs for the management of type 2 diabetes. Clinical trials demonstrated that, SGLT2 inhibitors reduce cardiovascular mortality for heart failure patients [69-71]. Moreover, there are experimental evidences to demonstrate, SGLT2 inhibitors improved cardiac function during the ischemic episode, reduced the infarct size and attenuated heart failure development [72-74].

Most approaches until now used a single agent to target a component of myocardia I/R injury. However, there is a complex mechanism beyond I/R injury and cardioprotection, thus the approach to prevent or inhibit detrimental effects should also implement complex interventions.

**Combination therapy** is a possibility to apply a combination of drugs or stimuli that may have new or greater effect than components alone. The combination of I/R cycles on the limb with exenatide has synergistic effect in terms of reducing MI size in porcine model of MI [75]. Although, primary outcome results of COMBAT-MI clinical trial was largely disappointing, since neither limb ischemia nor exenatide, or its combination, were able to reduce infarct size [76]. Furthermore, combination of remote conditioning (RIC) and IPostC was successfully reduced MI size in clinical setting, but there was a lack of cardioprotection with IPostC alone [77].

**Multi-targeted therapy** or a multi-targeted approach can be directed to different cell types, since the damage caused by myocardial I/R occurs as a result of the combined action of multiple players (i.e. cardiomyocytes, microvasculature, fibroblasts, inflammatory cells and platelets). Optimal cardioprotection may be achieved via the combination of additive or synergistic multitarget therapies [78, 79]. Combination therapy can offer one kind of multi target effect where we combine two or more interventions, each with a distinct target. However, there are many examples of a single cardioprotective agent or single intervention having multiple targets and we would expect that these approaches would be more effective as single target therapies.

Endogenous cardioprotective strategies are known to protect the heart through multiple different signalling pathways [29]. Hybrid molecules, e.g. with two structural domains act as two distinct pharmacophores, can provide additive protective effects [80, 81].

Furthermore, a single microRNA or small interfering RNA effects a variety of different target mRNAs and may protect the heart [82].

### 1.3 Translational models for cardioprotection – role of co-morbidities and importance of large animal studies

The translation of cardioprotective approaches into the clinical setting for patient benefit has been largely disappointing so far [22, 23, 78, 83].

There could be multiple problems of clinical translation of the cardioprotective therapies. Major problems include (1) target discovery and validation miss known confounding factors and co-morbidities, (2) clinically relevant animal models are crucial before clinical development studies and (3) novel targets and approached to induce cardioprotection are also needed.

**The complexity of the preclinical test systems** in early phase of development is not satisfactory, often due to lack of concomitant co-morbidities. However, concomitant underlying diseases could result in remodelling of the myocardium and vasculature eventually preventing the effect of cardioprotective interventions [11, 84]. Metabolic heart diseases (e.g. myocardial dysfunction caused by obesity, hyperlipidemia, or diabetes mellitus) can alter myocardial energetics via mitochondrial dysfunction, nitro-oxidative stress, increased inflammation and collagen deposition [85-87]. These pathological alterations result in myocardial dysfunction (primary diastolic) and development of heart failure (HF) [88].

**Hyperlipidemia and hypercholesterolemia** are well established risk factors of cardiovascular diseases [89]. Hyperlipidemia is present in most patients with myocardial infarction [90]. In fact, diet-induced hypercholesterolemia in rabbits and rat was the first co-morbidity where loss of cardioprotection was observed [91]. *In vivo* experimental and clinical studies demonstrated that hypercholesterolemia can disrupt the cardioprotective signalling [11, 84]. Hyperlipidemia in rodents can deregulate cardioprotective cascade via the inactivation of salvage kinase cascade, modulation of ATP sensitive $K^+$ channels, impairment of NO utilization and reorganization of the connexin-43 localization in the cardiac myocytes [11, 84, 92].

**Diabetes mellitus and hyperglycemia** are major components of chronic metabolic disease. Diabetes and pre-diabetes are important risk factors of ischemic heart disease [93]. Diabetic heart is more susceptible to I/R injury and the outcome is worsened in diabetic patients [94]. Diabetes induces chronic activation of Akt, which cannot be further augmented in ischemic conditioning and impairs cardioprotective signal transduction [95]. Hyperglycemia can increase oxidative and nitrosative stress [96, 97] and impairs protein functions via glycation. Thus, hyperglycemia can inhibit cardioprotection by ischemic pre- and postconditioning by various pharmacological agents [11, 98-100].

Furthermore, opinion leaders of the scientific community emphasized recommendations in the recent IMPACT guideline to **include co-morbidities in early testing of a potential cardioprotective therapy** [83]. Thus, there is still a need to define preclinical models of hyperlipidemia and hyperglycemia to investigate the effectiveness of potential cardioprotective approaches in early stage of development.

Second, an additional factor to improve translation of cardioprotection to humans is the **application of data obtained from large animal models** with high translational value. Implication of large animals in the preclinical phase of drug development process before entering into clinical trials should be included according to regulatory guidelines and position papers [101].

**Domestic pigs** (sus scrofa domestica) are closest to humans in terms of the anatomy of the heart, haemodynamics, and temporal and spatial development of myocardial infarction [102]. Moreover, critical endpoints, including infarct size and microvascular obstruction as well as oedema, have been established allowing correlation of preclinical outcomes with future clinical endpoints [103, 104]. Meanwhile, large animal models offer opportunity to asses molecular mechanisms parallel with clinical endpoints.

Pigs are suitable for acute I/R studies as they have little or no collateral flow, so the severity of I/R injury and of the subsequent infarct sizes are more uniform [105]. Thus pigs are widely used and accepted in cardiovascular research. However, there are differences between different animal species in the spatial and temporal organization of cardioprotective signalling. The RISK pathway plays a major role in the development of cardioprotection in rats, however it has no role in pigs [106], where the SAFE axis is more important [107].

### 1.4 microRNAs – novel targets in cardioprotection

MicroRNAs are small non-coding RNAs with an average 22 nucleotide length. MicroRNAs are involved in the fine regulation of gene expression in cardiac physiology and pathology [108]. MicroRNAs are conserved regulators of gene expression through incomplete base-pairing with their target mRNAs leading to mRNA degradation or inhibited protein translation [82]. Functional [109] or structural [110] classes of microRNAs can regulate complex cellular signalling pathways. More than half of the human proteins coding genes are estimated to be post-transcriptionally regulated by microRNAs [111].

Over the last decade, miRNAs are extensively researched in the setting of acute myocardial infarction. One of the primary publications on miRNA expression changes after MI was performed by Rooij et al. [112] who investigated miRNA changes in the infarct border zone on rodent and human hearts. They described a large number of dysregulated miRNAs, including the upregulation of miR-15b, miR-21, miR-199 and miR-214 and downregulation of miR-29c and miR-150. Since then, dysregulation of miRNA expression profile after MI has been studied extensively and reviewed previously [113, 114].

Furthermore, increasing number of publications has presented that endogenous or exogenous miRNAs are able to mediate the protection against I/R injury [113, 115]. Our group and others have reported several miRNAs (e.g. miR-139-5p, miR-125b*, let-7 family, miR-487b, miR-144/451 cluster, miR-107 and miR-210) are involved in cardioprotection [116-118]. Moreover, miR-125b* and other microRNAs were reported previously as common effectors in ischemic pre- and postconditioning [116] and also miR-21 can play role in multiple forms of ischemic conditioning [119, 120].

Cardioprotective miRNAs, termed ProtectomiRs can be identified with the comparative analysis of their expression in MI and in ischemic conditionings showing protective phenotype [116]. MiRNAs with ProtectomiR expression pattern, when the miRNA is up- or downregulated by I/R injury and counter regulated by ischemic conditioning, can have potential therapeutic applicability.

The importance of microRNAs in I/R and in cardioprotection suggests their promising therapeutic potential [113, 121]. Different approaches can be used to silence detrimental miRNAs (e.g. antisense locked nucleic acid modified oligonucleotides or cholesterol conjugated single-stranded RNA analogues, so called “antagomiRs”) [122]. Preclinical studies have already demonstrated that, inhibition of such detrimental miRNAs (e.g. miR-15 [123], miR-92 [124] or miR-132 [125]) reduce infarct size or inhibit the development of post-MI heart failure in large animal model. Inhibition of miR-132 to prevent post-MI heart failure is now in clinical development phase [126].

### 1.5 Unbiased microRNA – mRNA network analysis – a novel approach

An additional problem of the clinical translation is the application of hypothesis-driven approaches. Most studies to identify novel cardioprotective targets follow a biased fashion focusing on previously well-studied molecular pathways. As an effect of this approach, important mediators of cardioprotection can be overlooked.

Omics-based approaches can avoid such bias, and enable the identification of new, possibly more relevant molecular pathways and mediators to induce cardioprotection [121, 127]. Transcriptomics is a relatively cheap method with decent coverage to measure the expression of RNA species. [128] Moreover, considering that there are only about 2000 microRNA (miRNA) encoded in human genome [129] compared to the number of messenger RNAs (mRNAs) around 20,000 [130], it is more cost-effective to measure the expression of all miRNAs. Nevertheless, by *in silico* evaluation, the most important mRNA mediators could be identified relying solely on the miRNA expression pattern [131].

MiRNA mediated RNA-interference contributes to the high complex regulation of the posttranscriptional regulatory system. MiRNAs can bind to a large number of target RNAs by the binding to the more or less complementary sequences. Also, RNAs can be regulated by multiple miRNAs, as they provide binding sites for multiple various miRNAs [82]. To describe and study these complex miRNA-mRNA interactions, we need specialized tools, which can present the complexity of possible interactions between miRNAs and their target mRNAs.

There are multiple experimentally validated and predicted miRNA-target mRNA interaction databases to collect which genes are targeted by which miRNAs. Experimentally validated databases (e.g. miRTarBase [132], miRecords [133], DIANA-miRTarBase [134]) collect records for miRNA-target interactions based on experimental evidences either by qRT-PCR, Western blot or luciferase reporter assay. However, these experimentally validated databases are highly incomplete [135]. MiRNA-target interactions not listed in these experimental databases could be found in other, predicted interaction database, in which a miRNA-target mRNA prediction algorithm is available (e.g. microRNA.org [136], miRDB [137], TargetScan [138]). Advantage of these collection is the higher or full coverage of miRNAs, but the proportion of false predictions can be high.

A network theoretic approach is necessary to integrate the available datasets with the results of the transcriptomic measurement, to select the highly relevant mediators for further experimental validation. This molecular network approach offers, beside the rigorous mathematical description, the possibility to visually identify mediators and functional clusters that are central and studied phenotype [139].

The network represented by a graph, which contains ordered pairs of a set vertices and a set of edges. Vertices, also referred as nodes are represent the building blocks of the complex system (e.g. miRNAs and their target genes). Edges, also referred as links symbolize the relation between a pair of the building blocks (e.g. interaction between a miRNA and its target gene). Based on the structure of a network, we can describe the centrality or the importance of nodes and edges of the network [140]. Target mRNA hubs which were selected based on the highest number of interactions with differentially expressed miRNAs are expected to be the most influenced by the changes in the miRNA expression profile. MiRNA-target hubs selected this way were successfully validated in our several previous publications [141-143].

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# 2 Objectives

Based on the above mentioned aspects, our aims were the followings:

## 2.1 Development of an *in vitro* combination model of hypercholesterolemia or metabolic disease condition with ischemia/reperfusion injury

The aim was to develop an *in vitro* medium throughput test system of primary isolated cardiac myocytes that mimics ischemia/reperfusion injury in presence of hypercholesterolemia and hyperglycemia.

## 2.2 Identification of microRNAs associated with cardiac adaptation and remodelling

The aim was to identify miRNAs associated with cardiac adaptation and remodelling induced by acute myocardial infarction in rat and porcine models.

## 2.3 Identification of key molecular targets of ProtectomiRs

The aim was to identify key molecular targets of cardioprotective microRNAs - ProtectomiRs and confirm their association with cardioprotection in pig model of acute myocardial infarction.

Methodologies are briefly described in the result section and are detailed in the publications, on which this thesis based [144-146].

# 3 Results

## 3.1 *In vitro* combination model of hypercholesterolemia or metabolic disease condition with ischemia/reperfusion injury

Neonatal rat cardiac myocytes were cultured in normoglycemic (5 mM glucose) medium supplemented with vehicle (normChol) or hypercholesterolemic supplementation (hiChol). HiChol supplementation was applied in three different – gradually increasing concentrations suitable for obtaining the responses by the cells (Table 1).

**Table 1** Composition of the applied *in vitro* supplementation (hypercholesterolemic medium/hiChol) of cardiac myocytes. Data are the amount (mg) per 500 mL of complete medium.

| | **HICHOL 1** | **HICHOL 2** | **HICHOL 3** |
|---|---|---|---|
| **CHOLESTEROL** | 1.93 | 4.83 | 9.67 |
| **OTHER LIPIDS** | 2.45 | 6.14 | 12.26 |
| **TOTAL LIPIDS** | **4.38** | **10.97** | **21.93** |

Cellular cholesterol content of normChol and hiChol treated cardiac myocytes was controlled to validate cholesterol uptake by cells (Figure 3 A).

NormChol and hiChol groups were kept under normoxic conditions or subjected to simulated ischemia/reperfusion injury (SI/R). Cell viability and oxidative stress levels (total ROS and superoxide levels) were measured at the end of the protocol (Figure 3 B).

As cytoprotective control S-nitroso-N-acetyl penicillamin, a NO donor compound was applied on normChol and hiChol treated cardiac myocytes under ischemia and reperfusion (Figure 3 C).

Additionally, to mimic metabolic disease condition hiChol supplementation was completed with high glucose concentration (25 mM). Each groups were subjected to normoxia or simulated ischemia/reperfusion injury (SI/R), cell viability and oxidative stress levels (total ROS and superoxide levels) were measured (Figure 3 D).

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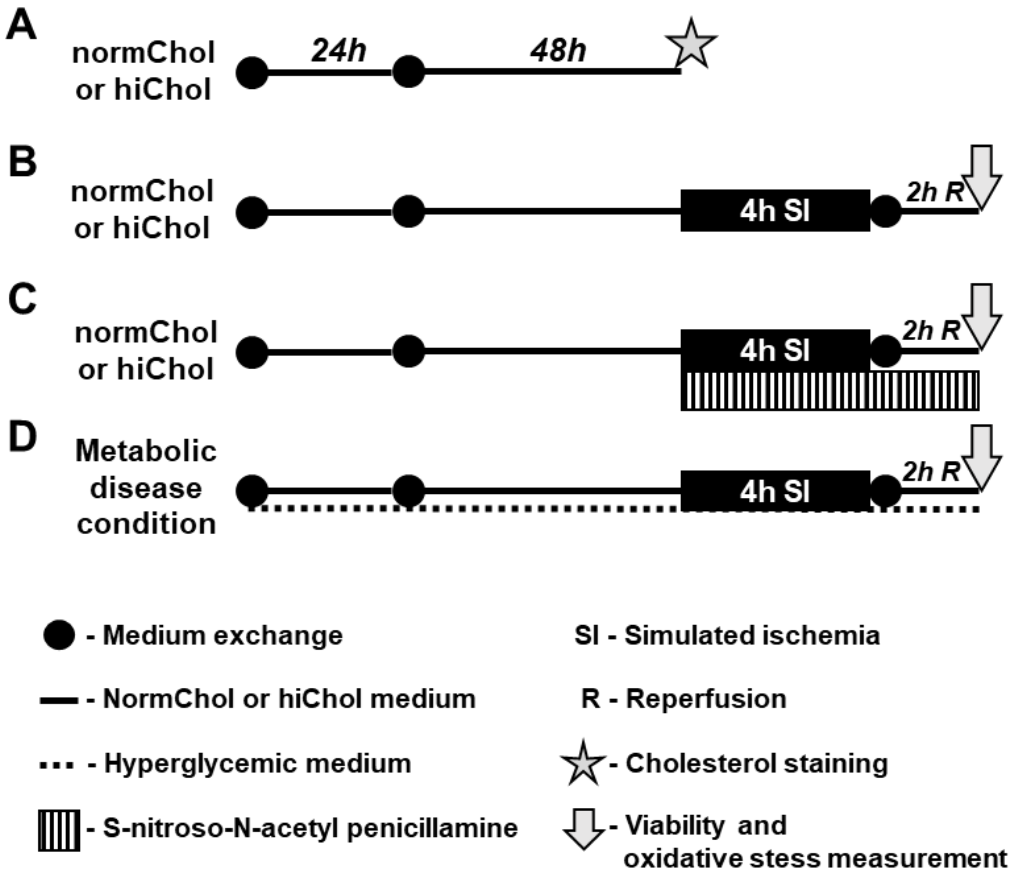


**Figure 3** Experimental design.

### 3.1.1 Rat cardiac myocytes taken up cholesterol in a concentration dependent manner

Filipin staining reflected the cholesterol content of the cardiac myocytes and propidium iodide counterstain reflected the total cell count (representative images Figure 4 A). Fluorescence signal analysis showed that cholesterol uptake from the hiChol supplements

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was efficient and cholesterol content increased in cardiac myocytes in concentration dependent manner (Figure 4 B).

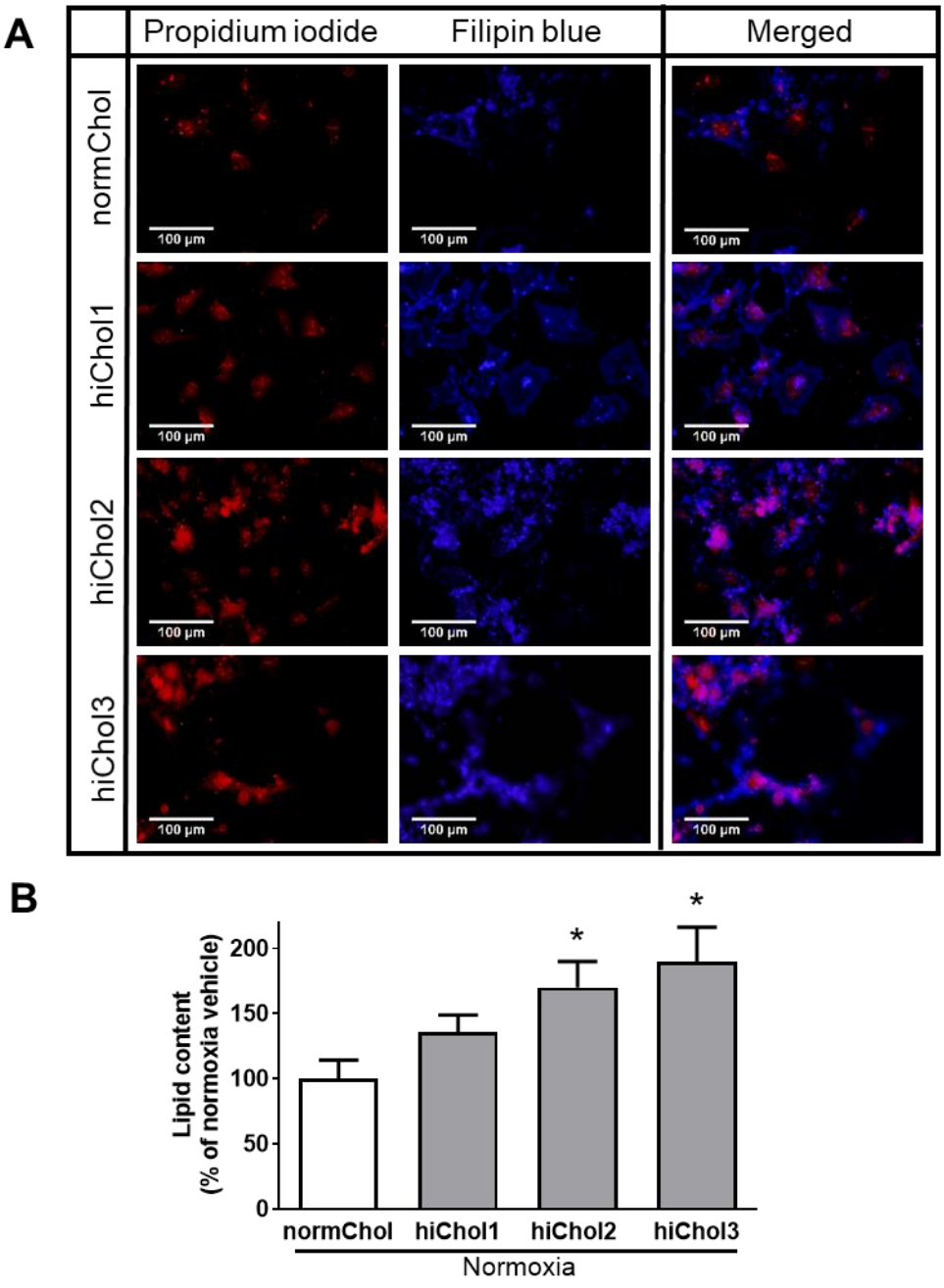


**Figure 4 (A)** Representative images of membrane cholesterol content in neonatal cardiac myocytes without (normChol) and with cholesterol supplementation (hiChol1, hiChol2, hiChol3) measured with Filipin staining (for cholesterol content) and propidium iodide staining (for total cell count). **(B)** Evaluation of membrane cholesterol levels. Data are expressed as mean ± SEM compared to vehicle control group (100%). *$p < 0.05$ vs. normoxia vehicle (one-way ANOVA, Tukey's posthoc), n=20-23.

### 3.1.2 Hypercholesterolemia aggravated simulated ischemia/reperfusion injury induced damage

Under normoxic condition, the cell viability and total ROS production of neonatal cardiac myocytes was not influenced by the hypercholesterolemic supplementation (Figure 5 A, B). However, superoxide levels were significantly elevated in all groups (Figure 5 C), reflecting some detrimental effect in presence of high level of cholesterol.

The applied simulated ischemia/reperfusion (SI/R) injury lead to significant cell death in normocholesterolemic group compared to the normoxic group. The viability of cardiac myocytes was significantly decreased in hiChol3 group (Figure 5 A). SI/R injury alone increased both total ROS and superoxide levels in normocholesterolemic (normChol) groups, which were further increased in hypercholesterolemic group (hiChol3) (Figure 5 B, C).

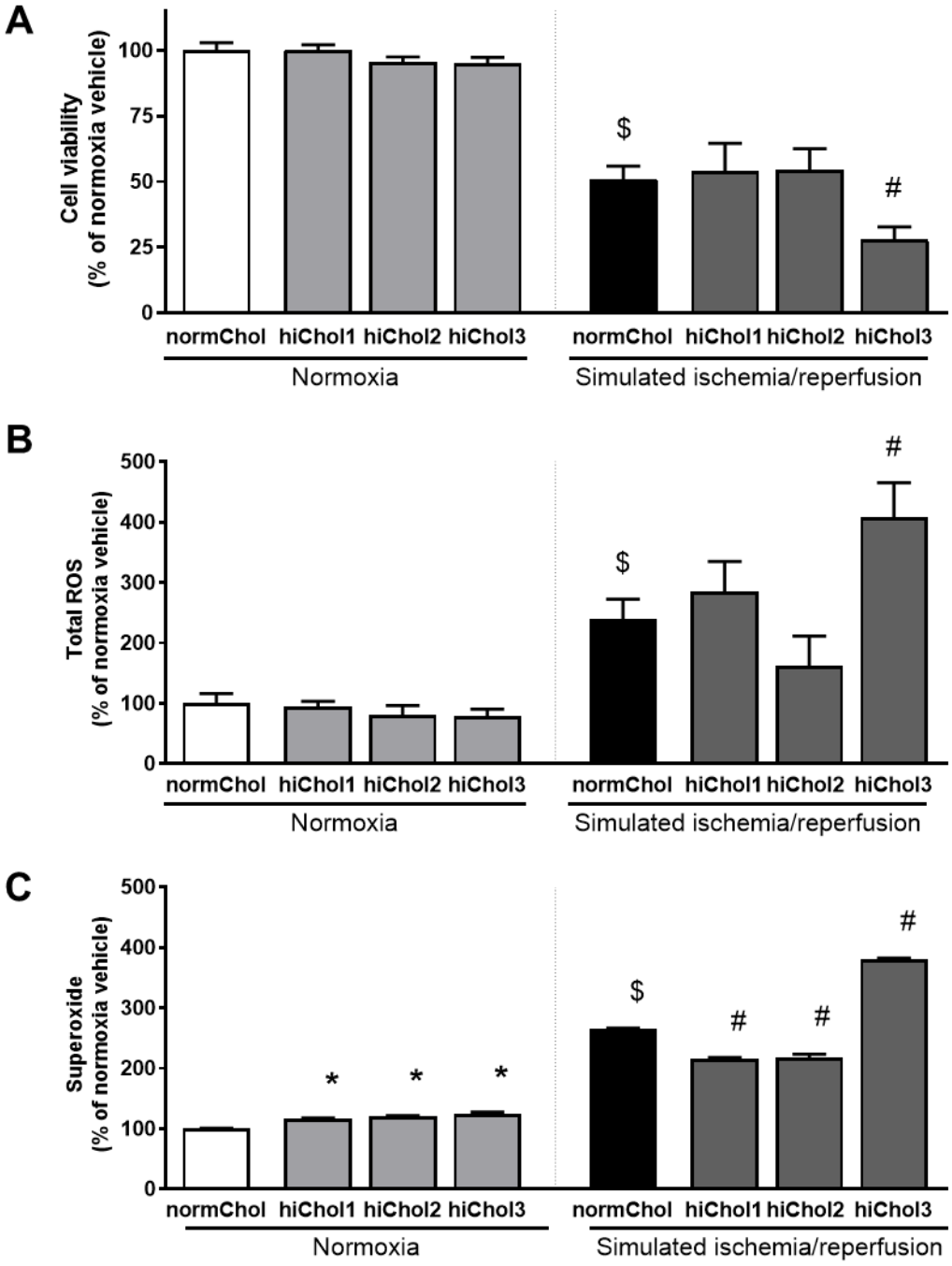


**Figure 5** Cardiac myocyte viability **(A)** was measured with calcein AM staining after treatment with or without hiChol supplements under normoxic conditions and after simulated ischemia/reperfusion injury (SI/R). Total ROS **(B)** was detected with 2,7-dichlorodihydroflourescein diacetate and superoxide **(C)** with dihydroethidium staining in each groups. Data are expressed as mean ± SEM, in comparison to normoxia vehicle control group (100%). $^{\$}p < 0.05$ normoxia vehicle vs. SI/R vehicle (t-test); $^{*}p < 0.05$ vs. normoxia vehicle (one-way ANOVA, LSD posthoc); $^{\#}p < 0.05$ vs. SI/R vehicle (one-way ANOVA, LSD posthoc); n=5-11.

### 3.1.3 Pharmacological cardiocytoprotection by NO donor against simulated ischemia/reperfusion injury was lost in hypercholesterolemia

The NO-donor S-nitroso-N-acetyl penicillamine (SNAP) significantly decreased cell death induced by SI/R injury in neonatal normocholesterolemic cardiac myocytes (Figure 6 A). The protective effect of SNAP was abolished in each hiChol supplemented groups (Figure 6 B).

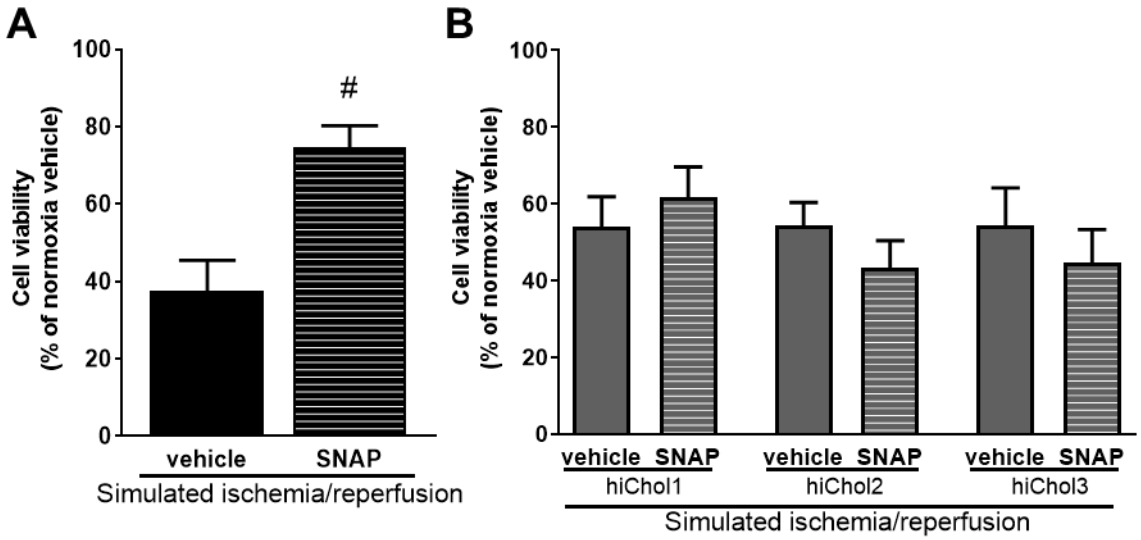


**Figure 6 (A)** S-nitroso-N-penicillinamine (SNAP) was able to increase the cell viability of neonatal cardiac myocytes, detected with calcein-AM. **(B)** Hypercholesterolemia inhibited the protective effect of SNAP in each concentration (hiChol1, hiChol2, hiChol3). Data are expressed as mean ± SEM, in comparison to normoxia vehicle control group (100%). $^{\#}p < 0.05$ vs. SI/R vehicle (t-test); n=13-15

### 3.1.4 Metabolic disease condition further aggravated simulated ischemia/reperfusion injury induced damage

In normoxic condition, when hypercholesterolemic supplementation was applied in combination with high glucose in medium to mimic metabolic disease condition, reduced cell viability was detected at higher concentration of cholesterol (hiChol2 and hiChol3) (Figure 7 A). In these groups, total ROS and superoxide levels increased correspondingly (Figures 7 B, C). Simulated ischemia/reperfusion further decreased cell viability in hiChol2 and hiChol3, while total ROS and superoxide levels increased (Figure 7).

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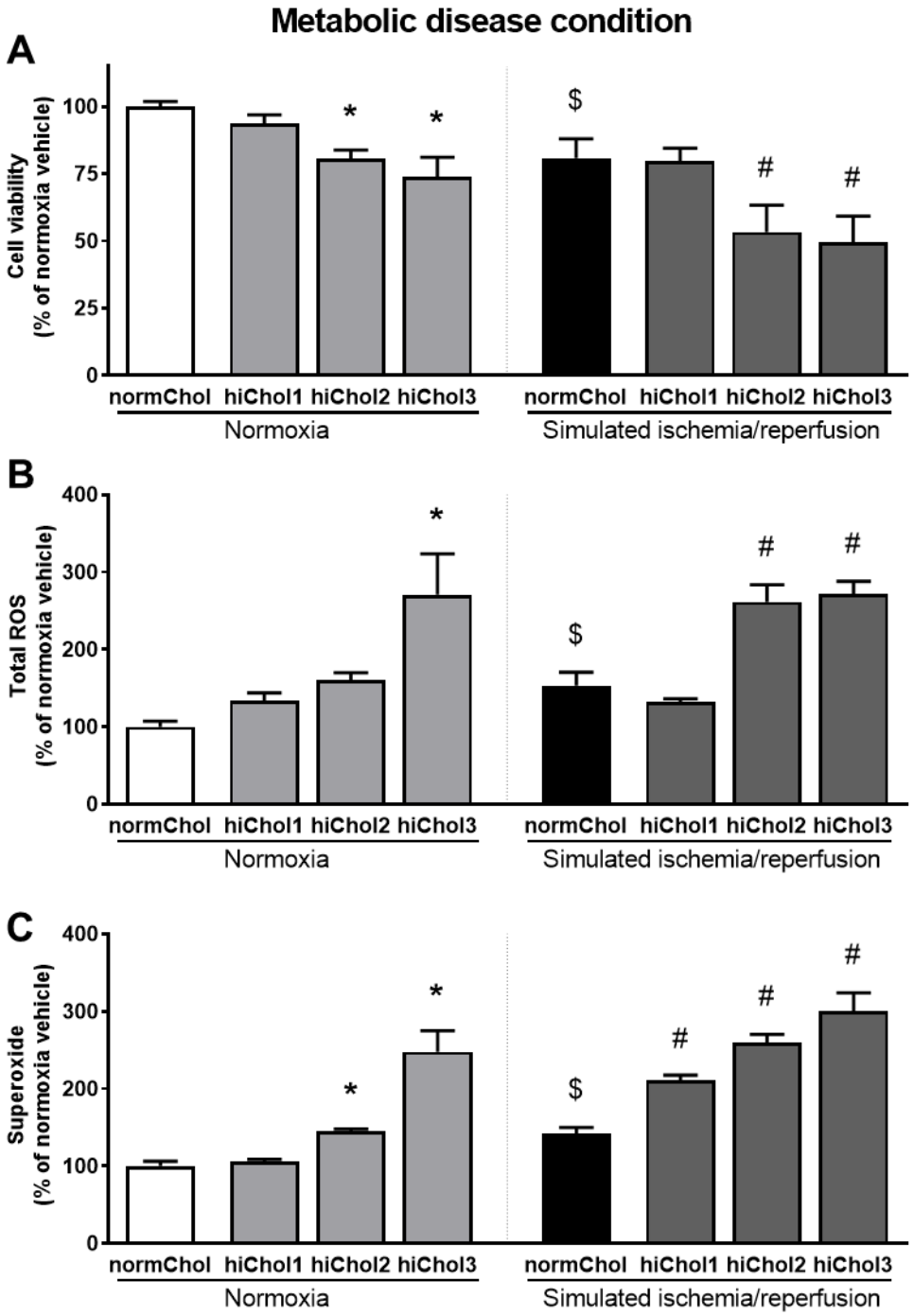



**Figure 7** Neonatal rat cardiac myocyte cells cultured in high glucose medium with or without hiChol supplements. Viability **(A)** was measured with calcein AM staining after treatment with/without hiChol supplements under normoxic conditions and after simulated ischemia/reperfusion injury (SI/R). Total ROS **(B)** was detected with 2,7-dichlorodihydroflourescein diacetate and superoxide **(C)** with dihydroethidium staining in each groups. Data are expressed as mean ± SEM, in comparison to normoxia vehicle control group (100%). $^{\$}p < 0.05$ normoxia vehicle vs. SI/R vehicle (t-test); $*p < 0.05$ vs. normoxia vehicle (one-way ANOVA, LSD posthoc); $^{\#}p < 0.05$ vs. SI/R vehicle (one-way ANOVA, LSD posthoc); n=6-12.

### 3.2 Identification of microRNAs associated with cardiac adaptation and remodelling

*Swiprosin-1* (EFhD2) was previously identified as a molecule that triggers structural adaptation of isolated adult rat cardiac myocytes, in a process that mimics aspects of cardiac remodelling [147, 148]. Here we analysed the expression of *Swiprosin-1* in rat hearts undergoing subsequent remodelling due to myocardial infarction with or without cardiac protection by ischemic pre- and postconditioning. Thereafter, we identified potential miRNAs, which expression associated with *Swiprosin-1* expression pattern and may be involved in the cardiac remodelling. Finally, we confirmed post-infarction expression changes of *Swiprosin-1* and associated miRNA in a translational porcine model [145] (Figure 8).

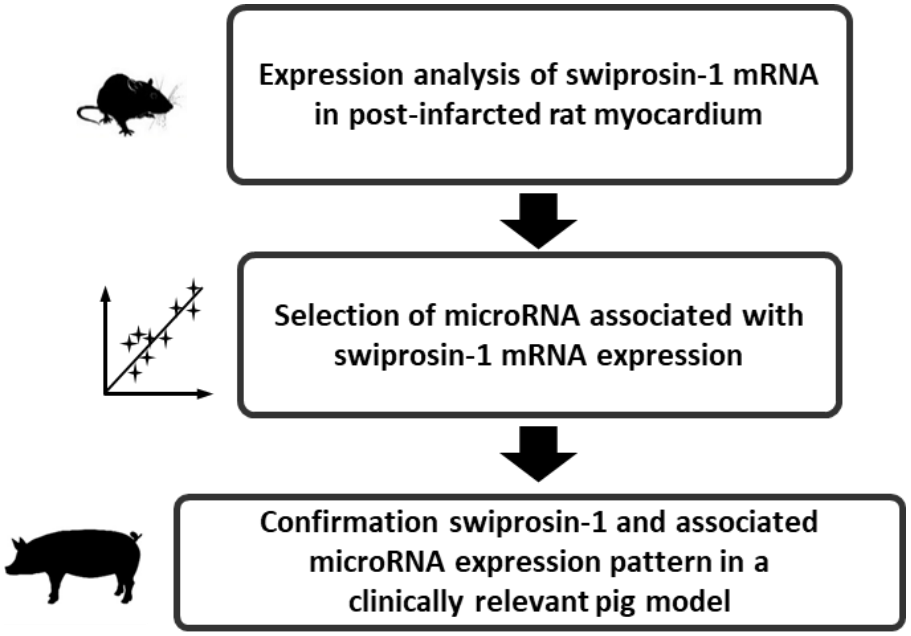



**Figure 8** Experimental flowchart.

We utilized samples from our previously described and well-characterized animal models:

Wistar-Hannover rats underwent 30 minutes left anterior descending (LAD) coronary artery occlusion with subsequent reperfusion for 7 days. Ischemic preconditioning (IPreC) was induced by 3 cycles of 3-minute ischemia and 5-minute of reperfusion before test ischemia, and postconditioning (IPostC) was induced after test ischemia by 3 cycles of 30-second of reperfusion and 30-second of ischemia. Both IPreC and IPostC reduced Troponin-I plasma levels 1 h after reperfusion. Moreover, IPreC improved left ventricular

systolic function 7 days following the reperfusion [149]. Left ventricular myocardium samples were collected after 7 days of reperfusion.

Domestic juvenile pigs underwent 90 minutes balloon occlusion of the left anterior descending (LAD) coronary artery with subsequent reperfusion for 3 hours or 3 days. Ischemic preconditioning (IPreC) was induced by 3 cycles of 5-minute ischemia and 5-minute reperfusion before test ischemia, and postconditioning (IPostC) was induced after test ischemia by 6 cycles of 30-second of reperfusion and 30-second of ischemia. IPreC reduced infarct size measured by histological staining after 3 hours of reperfusion or by cardiac MRI after 3 days reperfusion. IPostC did not reduce myocardial necrosis, however reduced myocardial oedema and microvascular obstruction measured by cardiac MRI [150]. Left ventricular myocardium samples were collected from parallel animals after 3 hours or 3 days of reperfusion.

### 3.2.1 *Swiprosin-1* expression correlated with myocardial protection in post-infarcted rat hearts

In rat hearts underwent 30 min LAD occlusion with subsequent reperfusion for 7 days Swiprosin-1 mRNA expression was induced by I/R. However, either ischemic preconditioning (IPreC) or postconditioning (IPostC) abrogated this induction (Figure 9).

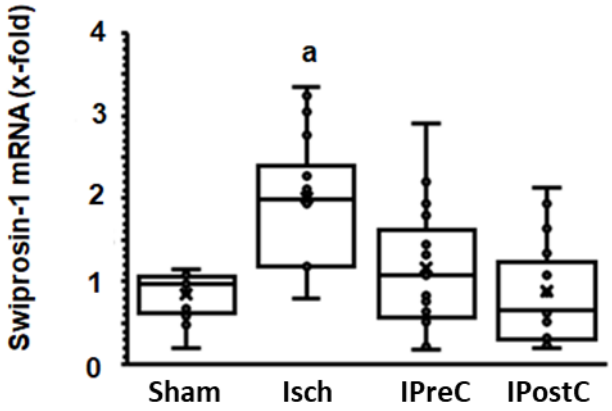



**Figure 9** Expression of *Swiprosin-1* mRNA in the left ventricle from rats 7 days after I/R. Box plots showing the level of expression and distribution of samples from sham surgery (Sham), ischemia/reperfusion (Isch), ischemic preconditioning (IPreC), and ischemic postconditioning (IPostC). $^{a}p < 0.05$ vs. all other groups (one-way ANOVA followed by Student–Newman–Keuls post-hoc test).

### 3.2.2 *Swiprosin-1* and rno-miR-34c expression correlated in post-infarcted rat myocardium

364 miRNAs were constitutively detected in rat heart tissue samples. Among them, 27 miRNAs were either positively (n = 15) or negatively (n = 12) associated with the mRNA expression of *Swiprosin-1* in all samples. Only 12 of 27 miRNAs showed an ischemia/reperfusion-dependent regulation such as *Swiprosin-1*, and only in two cases could the induction of miRNA be abrogated by IPreC or IPostC as found for *Swiprosin-1*. These two most likely candidates were rno-miR-32-3p and rno-miR-34c-3p (Figure 10).

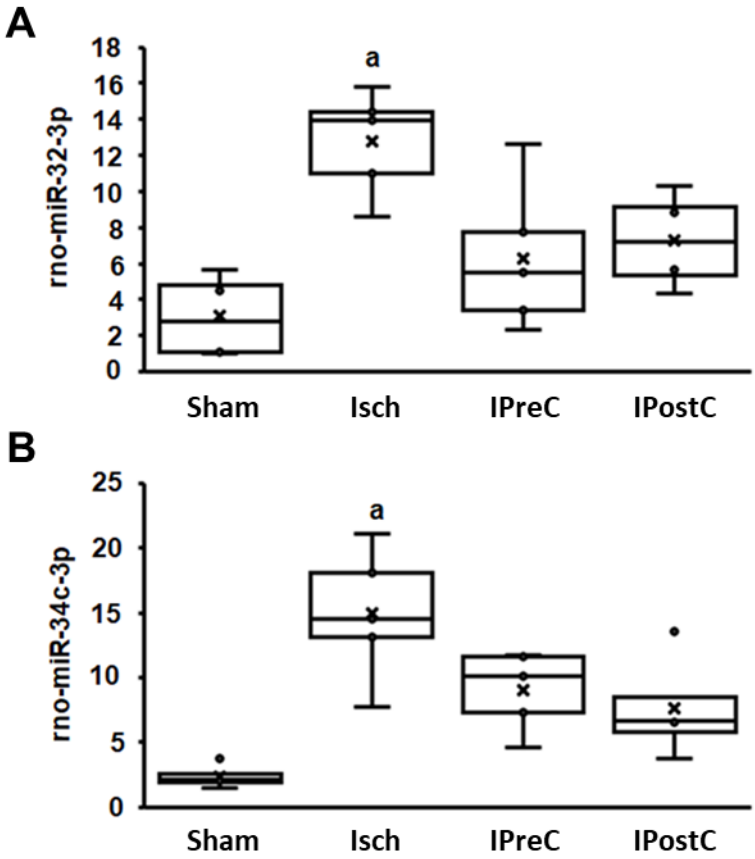



**Figure 10** Expression of rno-miR-32-3p **(A)** and rno-miR-34c-3p **(B)** in the left ventricle from rats seven days after I/R. Box plots showing the level of expression and distribution of samples from sham surgery (Sham), ischemia/reperfusion (Isch), ischemic preconditioning (IPreC), and ischemic postconditioning (IPostC). Data are analysed by One-Way-ANOVA followed by Student–Newman–Keuls posthoc analysis $^{a}p < 0.05$ vs. all other groups.

In the rat cardiac tissue, beyond *Swiprosin-1*, mRNA expression of 72 different genes related to cardiac biology have been analysed previously [151]. A detailed analysis of further co-regulated mRNAs with both miRNAs was performed by investigating a potential linear correlation between mRNAs and miRNAs. Interestingly, rno-miR-32-3p showed a positive correlation with genes associated with hypertrophy and fibrosis. These data suggested that the increased expression of rno-miR-32-3p contributes to the maladaptive phenotype of cardiac adaptation in these hearts. Therefore, miR-34c has been investigated in further steps.

### 3.2.3 *Swiprosin-1* and ssc-miR-34c expression correlated also in a clinically relevant porcine model

To investigate the relevance of *Swiprosin-1* and miR-34c expression changes for other species as well, the investigation was extended by analysis of pig hearts.

Pig myocardium samples were taken from the border zone (BZ) of the injured myocardium region, and from the injured myocardium (infarct zone - IZ) from the previously presented pig acute myocardial infarction and cardioprotection model. *Swiprosin-1* mRNA expression was induced at the late time-point (day 3) in the infarct zone (Figure 11 A). In the reperfused heart, ssc-miR-34c was again increased by ischemia/reperfusion, and this was again abrogated by IPreC and IPostC (Figure 11 B).

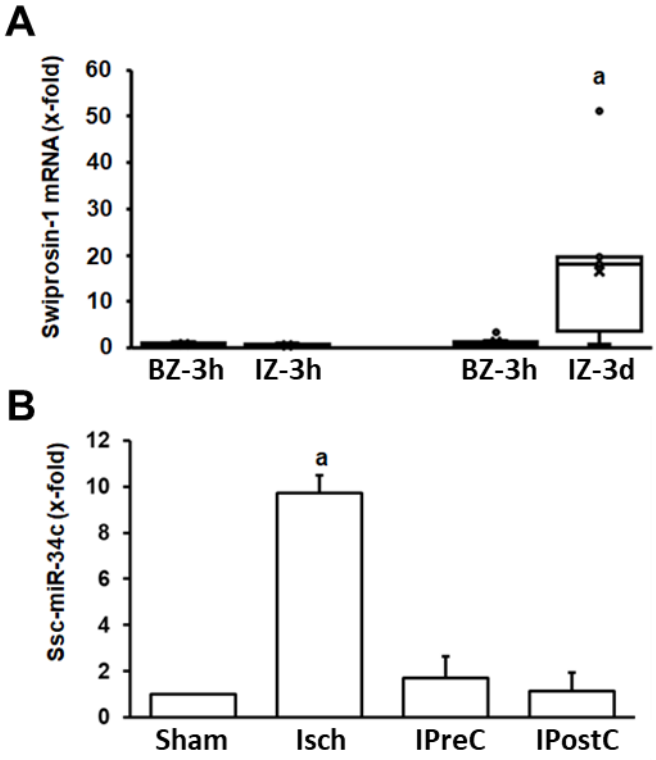



**Figure 11** Expression of *Swiprosin-1* mRNA and ssc-miR-34c in the border zone (BZ) or infarct zone (IZ) three hours or 3 days after reperfusion in heart utilized from pig model of MI and cardioprotection. The quantification for ssc-miR-34c was performed in the infarct zone 3 hours after reperfusion. Data are analysed by One-Way-ANOVA followed by Student–Newman–Keuls posthoc analysis $^{a}p < 0.05$ vs. all other groups.

### 3.3 Identification of key molecular targets of ProtectomiRs with an unbiased network approach

To identify potential mRNA targets of 18 different previously discovered and validated mimic and antagomiR protectomiR [116] were predicted by miRNAtarget.com software.

To express the number of microRNAs interacting with their predicted mRNA targets node degree was calculated for each mRNA target. Target node with the highest node degree values was considered as the most important target hub and was selected for further validation.

To validate the expression of rat ProtectomiRs and their central target experimentally, myocardial tissue samples were obtained from a previously published, well characterized, clinically relevant, closed-chest porcine model of reperfused acute myocardial infarction and cardioprotection which was detailed in the previous chapter of the thesis on page 25 [150]. Tissue samples were collected from parallel animals after 3 hour reperfusion.

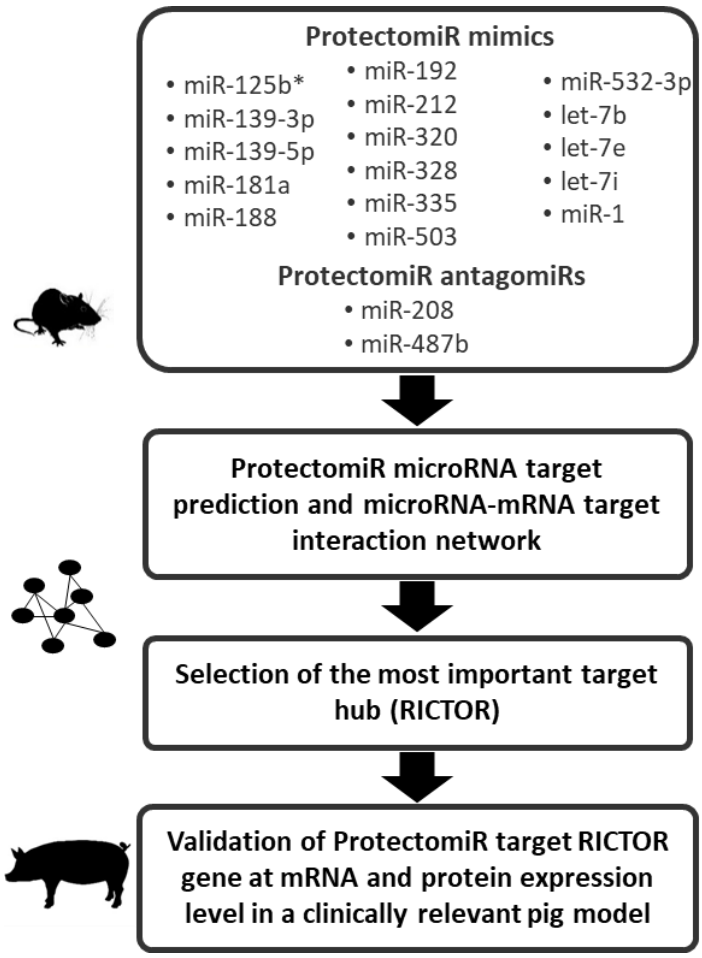



**Figure 12** Experimental protocol.

### 3.3.1 Prediction of targets of ProtectomiRs revealed *Rictor* as most central target hub of microRNA-mRNA target interaction network

The 18 different protectomiRs revealed 882 predicted target mRNAs by *in silico* target prediction. The microRNA-mRNA interactions were visualized to highlight the central hub of the mRNA targets (Figure 13). In this interaction network, 84 mRNAs had

interactions with more than one microRNA, and 15 mRNAs interacted with at least 3 microRNAs.

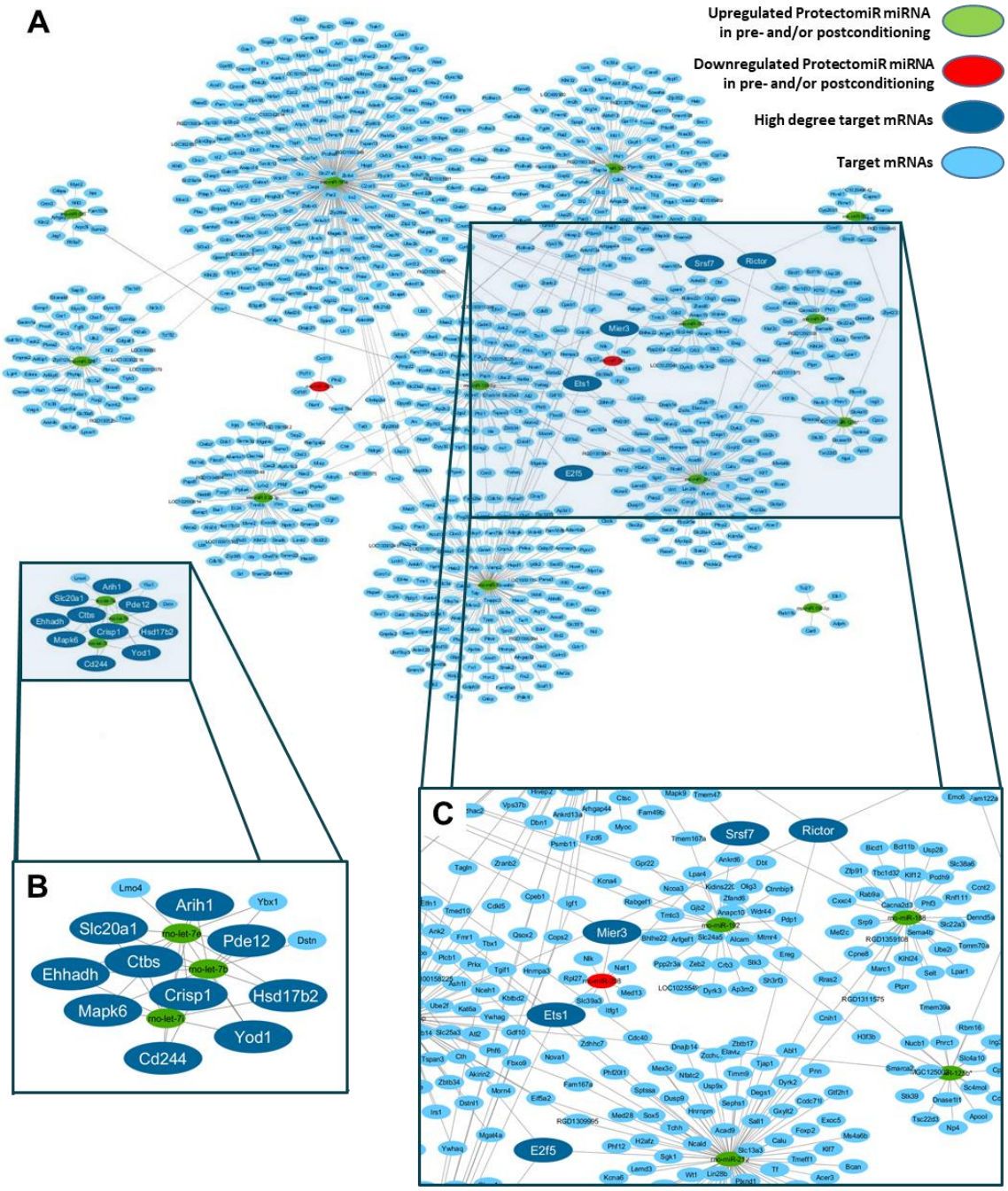


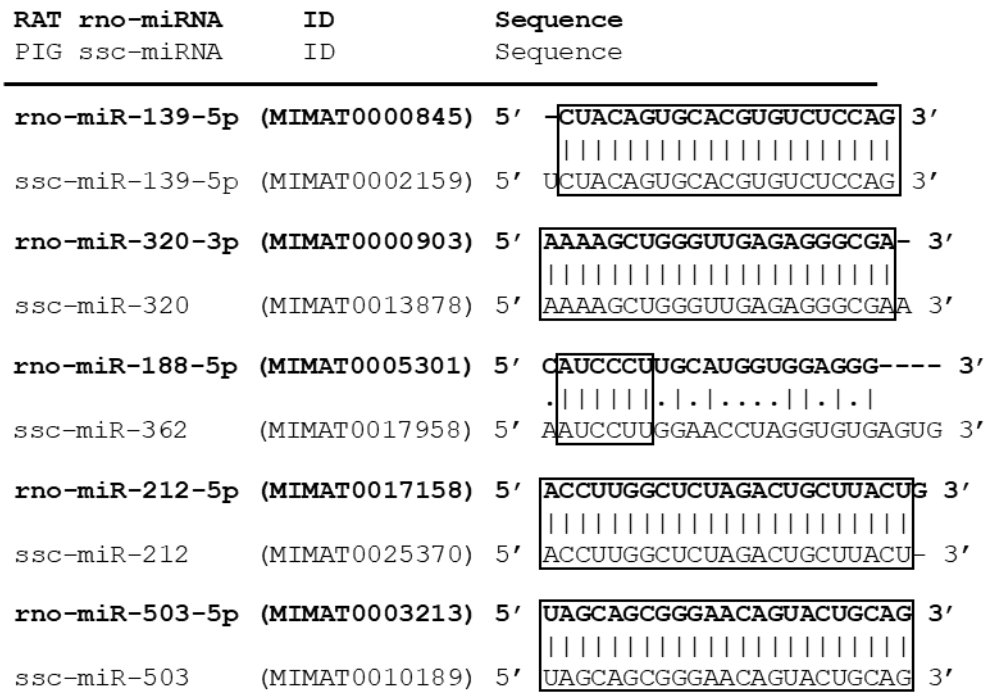

**Figure 13** Predicted interaction network of ProtectomiR microRNAs (A) showing the network neighbourhood of predicted hub genes with at least three microRNA–mRNA target interactions (node degree) (B, C). Panel (C) shows the role of the most central target hub Rictor. MicroRNAs upregulated and downregulated in pre- and/or postconditioning and target mRNAs are indicated in green, red and blue, respectively. Dark blue nodes represent mRNAs with a node degree of at least 3.

The *Rictor* gene was identified as the most central target hub with the highest node degree, interacting with five different microRNAs (miR-139-5p, miR-320, miR-212, miR-503, miR-188-5p) out of the 18 investigated protectomiRs (Figure 13 C).

### 3.3.2 *Rictor*-targeting ProtectomiRs upregulated after postconditioning in a clinically relevant porcine model

Left myocardial tissue samples were utilized from a clinically relevant porcine AMI model to validate the central role of *Rictor* in ischemic pre- and postconditioning. Therefore, first we identified the pig homologues of the *Rictor*-targeting microRNAs based on rat–pig microRNA sequence similarity. Four of the five rat microRNAs (rno-miR-139-5p, rno-miR-320, rno-miR-212, rno-miR-503) showed a total sequence match between rat and pig microRNA homologues. In the case of rno-miR-188-5p rat microRNA, we identified the scc-miR-362 with 56% homology. Ssc-miR-362 is a member of miR-188 microRNA family and it has an identical seed sequence to rno-miR-188-5p (Table 2).

**Table 2** Pig homologues of the *Rictor* targeting microRNAs were identified based on rat–pig microRNA sequence similarity.



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We found upregulation of three *Rictor* gene-targeting microRNAs out of five targeting microRNAs in the interaction network in the IPostC group. Two other microRNAs showed a tendency but not a statistically significant change (Figure 14). Interestingly, these microRNAs did not show alteration in the myocardial samples of the IPreC group.

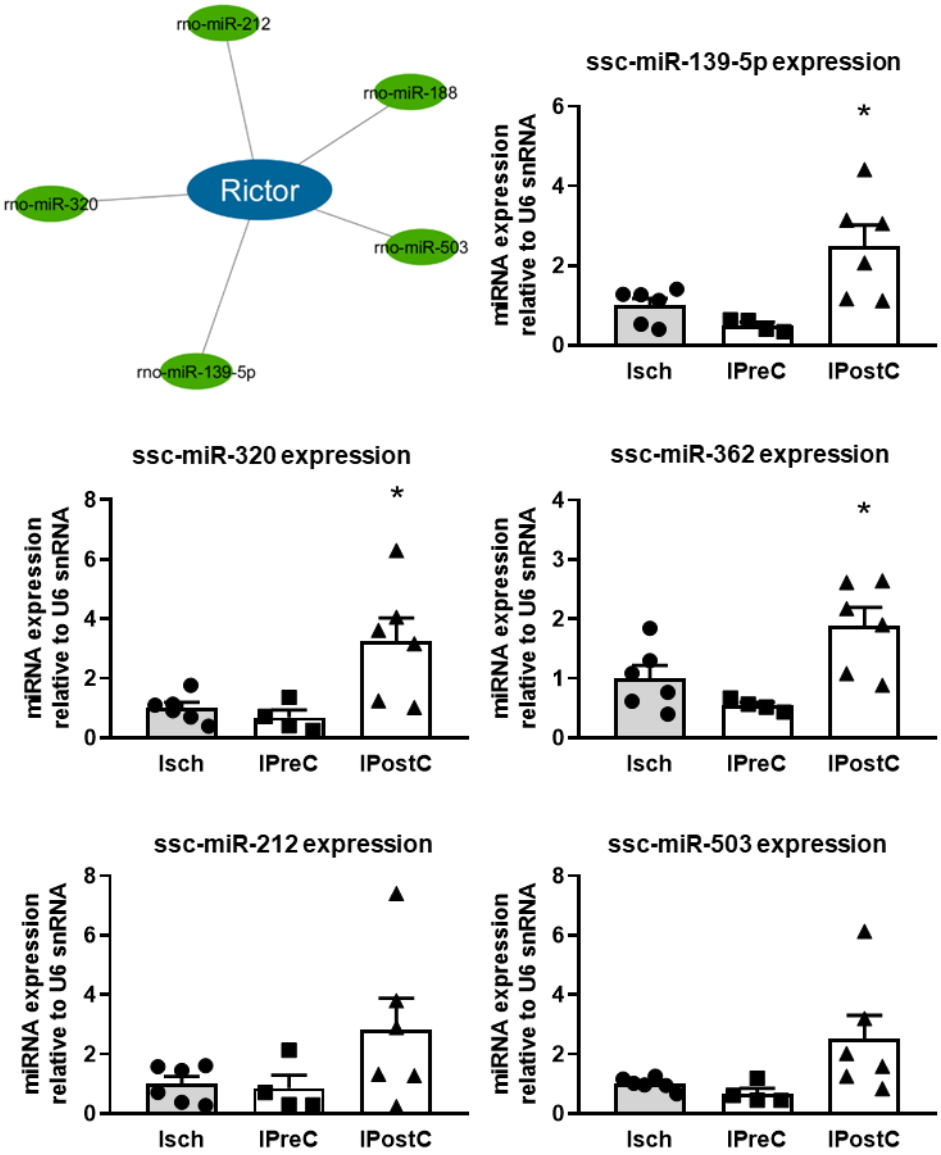


**Figure 14** Expression levels of the five *Rictor* targeting ProtectomiR microRNA homologues were tested with qPCR in ischemic preconditioning and postconditioning compared to ischemia-reperfusion (C). Black circle, square and triangle signs represent individual data points. *p < 0.05 vs. Isch group (one-way ANOVA, Dunnett post-hoc test), n=4-6.

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### 3.3.3 The central hub, *Rictor* downregulated after postconditioning in a clinically relevant porcine model

mRNA expression of the *Rictor* gene was investigated in both the ischemic and non-ischemic zones of the porcine myocardium. We observed a statistically non-significant downregulation of *Rictor* mRNA in the ischemic zone of the postconditioned group (Figure 15 A). There were no changes in *Rictor* mRNA expression in the IPreC group compared to Isch, neither in the ischemic nor in the non-ischemic (remote) myocardium zones (Figure 15 B).

Protein expression of RICTOR was in line with the mRNA level expression changes. We observed a significant downregulation of the RICTOR protein in the infarct zone (IZ) of the IPostC group as compared to the Isch group (Figure 15 C). No RICTOR protein expression changes were found in the IPreC group compared to Isch neither in the ischemic nor the non-infarcted remote zone (RZ) of the myocardium (Figure 15 D).

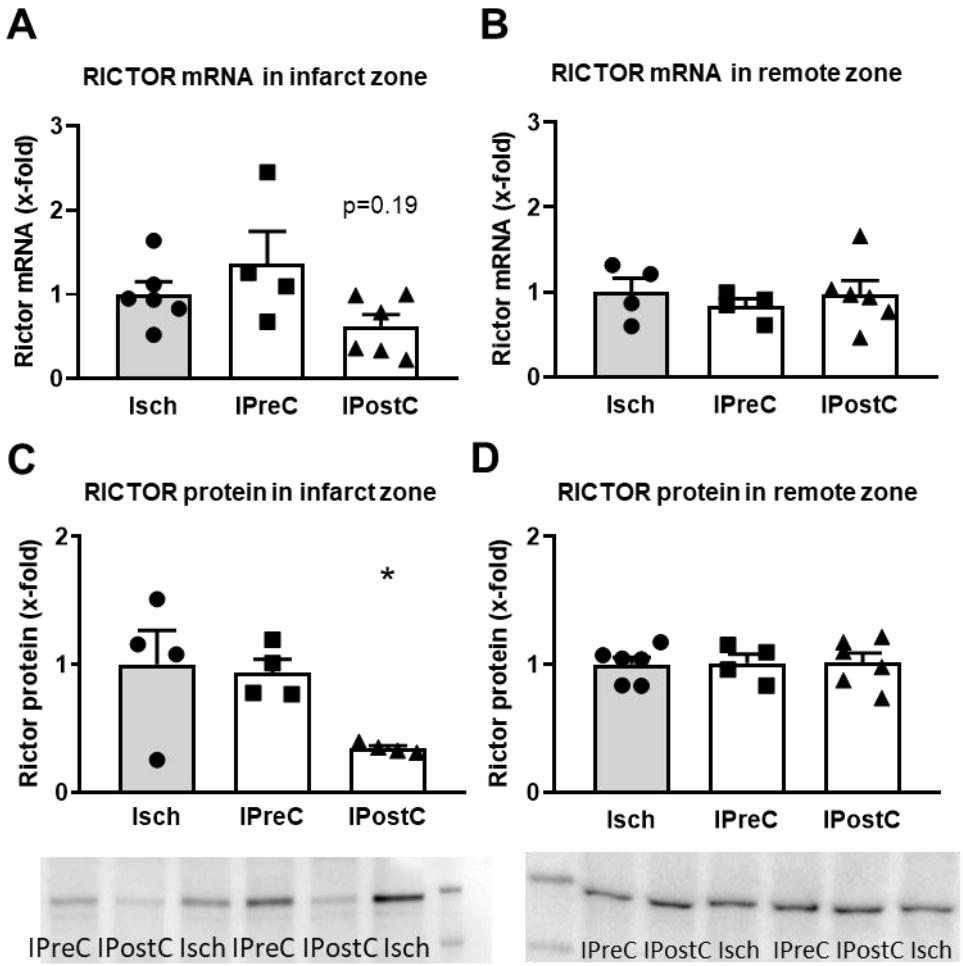


**Figure 15** Central target hub, *Rictor* mRNA expression was measured with qPCR in the ischemic zone (A) and in the non-infarcted remote zone (B) of the porcine left ventricular myocardium. RICTOR protein expression was measured with Western blot in the ischemic zone (C) and in the non-infarcted remote zone (D). Black circle, square and triangle signs represent individual data points normalized to total protein staining. *$p < 0.05$ vs. Isch group (one-way ANOVA, Dunnett post-hoc test), n=4-6.

# 4 Discussion

The present dissertation involves the results of a series of studies to investigate the three aforementioned objectives, discussion of which is detailed in the following sections.

## 4.1 Development of an *in vitro* combination model of hypercholesterolemia or metabolic disease condition with ischemia/reperfusion injury

As to our first objective, i.e. to develop an *in vitro* medium throughput test system of cardiac myocytes that mimics ischemia/reperfusion injury in presence of hypercholesterolemia and hyperglycemia, we developed an *in vitro* medium throughput cell-based test system of primary isolated rat cardiac myocytes subjected to simulated ischemia/reperfusion in presence of hypercholesterolemia using tailored hypercholesterolemic supplementation (hiChol) with or without hyperglycemia. HiChol supplementation reduced cell viability and increased oxidative stress, which effects were aggravated by SI/R and additional hyperglycemia. Moreover, HiChol supplementation blocked the protective effect of the NO-donor SNAP against SI/R injury. This is the first demonstration that the combination of the current hypercholesterolemic/metabolic disease medium and SI/R in cardiac myocytes mimics the cardiac pathology of the co-morbid heart with I/R in an *in vitro* setting.

*In vitro*, well-designed cell culture models are an increasingly important element of preclinical studies on coronary artery disease (CAD). These *in vitro* models often neglect the influence of lipids and lipid dysregulation on cell properties, however, hypercholesterolemia is a principal factor of CAD [11] and directly effects the myocardium itself [87, 152, 153].

If an external source of lipids is available, mammalian *in vitro* cells prefer to uptake lipids from the cell culture medium and enzymes related to endogenous lipid synthesis became inhibited. Thereby, the lipid composition of *in vitro* cells can be modulated by controlling their external lipid supply and a carefully planned feeding strategy grants the possibility to develop efficient *in vitro* models mimicking the real *in vivo* conditions [154, 155]. In the present model, tailored hypercholesterolemic supplementation was taken up by neonatal cardiac cells in a concentration dependent manner.

Although, hiChol-supplemented normoxic neonatal rat cardiac myocytes did not show reduced cell viability, the direct harmful effect of hypercholesterolemia on the myocardium has been shown previously. After several months of cholesterol feeding, both systolic and diastolic function impairments were detected without hypertrophy or atherosclerosis in rabbits [152, 156]. In the present model, hypercholesterolemia induced an increased level of superoxide formation in cardiac myocytes even in normoxic condition. This finding is in line with *in vivo* data, where increased superoxide formation has been observed in hypercholesterolemic rat myocardium [153], which can associate with diastolic dysfunction [87].

Simulated ischemia/reperfusion (SI/R) induced cell death aggravated harmful effect of hypercholesterolemia in neonatal cardiac myocytes, similarly to what was described in animal models previously [84]. In our model, the decreased viability of cardiac myocytes was associated with increased levels of total ROS and superoxide. Fundamental role of increased ROS in myocardial injury induced by ischemia/reperfusion is an extensively studied phenomenon [157-161]. However, ROS mediated signalling pathways were not investigated in the present study.

To further validate our model, we utilized a well-studied cardioprotective NO-donor compound, S-Nitroso-N-acetyl penicillamine (SNAP) [162-164] to test if its cardiocytoprotective effect is blocked by hypercholesterolemia. In the present model, SNAP protected against SI/R injury the normocholesterolemic, but not the hypercholesterolemic cardiac myocytes.

The presence of diabetes might interfere with the cardioprotective mechanisms, attenuating the effectiveness of these therapeutic strategies [11]. Here we found that the applied metabolic disease condition worsened the survival of cardiac myocytes even in normoxic condition. Reduction in cell viability and increase in the level of oxidative stress were further aggravated in ischemia. In a diabetic mice model, the exacerbation of heart failure after MI has been observed via increasing NAD(P)H oxidase-derived superoxide [165].

These results further prove the validity of our present *in vitro* I/R and hypercholesterolemia/metabolic disease model, and suitability for testing

cardioprotective compounds in the presence of hypercholesterolemia or metabolic disease co-morbidity.

### 4.2 Identification of microRNAs associated with cardiac adaptation and remodelling

As to our second objective, i.e. to identify miRNAs associated with cardiac adaptation and remodelling induced by acute myocardial infarction**,** we presented *Swiprosin-1* expression pattern, increased expression after I/R injury and inhibition after ischemic conditioning, associated with cardiac adaptation and remodelling induced by acute myocardial infarction in the rat heart. Moreover, in pigs the expression pattern of *Swiprosin-1* was most pronounced in the infarct zone of reperfused hearts where cardiac remodelling occurs. This expression was associated with ssc-miR-34c miRNA expression. This is the first demonstration of *Swiprosin-1* expression changes in I/R alone or in combination with ischemic conditioning are associated with expression of miRNAs, indicating a complex regulatory network in cardiac myocytes.

*Swiprosin-1* is expressed in various tissues and known to be involved in structural adaptation of cardiac myocytes. Its expression and function is required for isolated adult cardiac myocytes to adapt to culture conditions, e.g. pseudopodia formation, cell spreading. Additionally, it seems to be involved in the desensitization of β-adrenergic receptors [147]. Assuming that cardiac myocytes adapt to cardiac stress in a similar way, *Swiprosin-1* may play a key role in cardiac remodelling, de- and redifferentiation [166].

In the current study, we described that *Swiprosin-1* expression changes during cardiac repair processes in rats, and pigs. Moreover, the expression of *Swiprosin-1* is strongest in those areas of the heart where cardiac remodelling occurs, i.e. in the infarct zone of reperfused pig hearts. This finding underlines that *Swiprosin-1* plays a key role in cardiac repair and by that it may be involved in the cardiac remodelling.

The expression of *Swiprosin-1* was associated with miRNA expressions, indicating a complex regulatory network in cardiac myocytes. Two miRNAs were identified with a similar direction of regulation of expression to *Swiprosin-1*, namely, rno-miR-32-3p and rno-miR-34c-3p.

MiR-32-3p was also associated with other genes that are well known to affect cardiac remodelling (e.g. genes involved in fibrosis, inflammation, sarcomeric remodelling and fatty acid metabolism) [167-169], but showed a positive correlation with genes associated with hypertrophy and fibrosis. In contrast, miR-34c-3p was not associated with maladaptive gene reprogramming such as miR-32-3p and displayed a nearly exclusive co-regulation with *Swiprosin-1* and *Arrestin-2*. Of note, I/R-dependent regulation of miR-34c was found in rat and pig tissues as well. miR-32-3p and miR-34c have not been previously associated with myocardial remodelling and represent another new finding of this study.

The design of the current study does not allow identifying potential mechanisms by which *Swiprosin-1* may affect the remodelling process, however highlights the regulatory role of miR-34c.

### 4.3 Identification of key molecular targets of ProtectomiRs

As to our third objective, i.e. to identify key molecular targets of cardioprotective microRNAs - ProtectomiRs and confirm their association with cardioprotection in a clinically relevant porcine model, we analysed a rat protectomiR miRNA – target mRNA molecular network *in silico* and found the *Rictor* gene as centre of the network with the highest node degree. Furthermore, we experimentally validated decreased expression of *Rictor* gene and increased expression of its targeting microRNAs in the postconditioned myocardium of translational pig model of acute myocardial infarction and cardioprotection. This is the first demonstration that *Rictor* is the central molecular target of ProtectomiRs and that decreased *Rictor* expression may regulate ischemic postconditioning-, but not preconditioning-induced acute cardioprotection.

ProtectomiRs, microRNAs with cardioprotective potential, were identified previously by microRNA expression pattern analysis in acute myocardial infarction and cardioprotection induced by ischemic pre- and postconditioning in rats [116].

In the present study, we predicted the mRNA targets of 18 potential ProtectomiRs *in silico*. Here we utilized unbiased target prediction and network visualization for better understanding the mRNA targets regulated by the cardioprotection-associated ProtectomiRs according to recommendations [121]. mRNA targets with the highest

number of interactions, represent the hubs of the constructed interaction network. These mRNA hubs could be classified into two subgroups. Ten of the hubs were organized around let-7b, let-7e and let-7i microRNAs, representing a smaller isolated subnetwork (e.g., *Arih1, Cd244, Crisp1, Ctbs, Ehhadh, Hsd17b2, Mapk6, Pde12, Slc20a1* and *Yod1*). Meanwhile, the other five multiple targeted hub genes (*Rictor, E2f5, Ets1, Srsf7* and *Mier3*) are part of a larger component of the network, which contains the majority of the predicted targets.

The present microRNA-mRNA target interaction network analysis revealed the *Rictor* gene as the highest degree hub with five interactions with protectomiR microRNAs. Therefore, in the present study we focused on the highest degree of *Rictor* gene expression, and have not further studied other high-degree mRNA targets.

The protein product of *Rictor* gene is a key member of the mTORC2 protein complex, which regulates multiple cellular functions, e.g. cell survival, proliferation, migration and cytoskeletal remodelling [170]. The fundamental role of mTORC2 activation in cell survival, in cardiac adaptation and in cardioprotection by ischemic preconditioning was shown in previous publications [171, 172]. We identified here the Rictor gene with the highest node degree, interacting with 5 different protectomiRs that were upregulated in ischemic pre- and/or postconditioned rat myocardium [116]. Based on the antagonistic microRNA-target interactions, we predicted the downregulation of *Rictor* gene expression during cardioprotection by the upregulated targeting ProtectomiRs, which we measured at the mRNA and protein level.

ProtectomiRs and their target *Rictor* expression was experimentally validated in a large animal model of cardioprotection after we confirmed that rat protectomiRs can be translated to pigs based on sequence homology. In postconditioned pig myocardium three out of five mRNAs showed significant upregulation. However, none of the protectomiRs were changed in the preconditioning. Furthermore, RICTOR protein expression was downregulated in the postconditioned but not in the preconditioned group.

These results show that protectomiRs and their central target to inhibit *Rictor* expression may contribute to cardioprotection by ischemic postconditioning characterized mainly by microvascular protection as seen by decreased tissue oedema and microvascular obstruction in this pig model [150, 173]. However, it cannot be excluded that

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downregulation of the RICTOR protein in postconditioning may contribute to the observation that postconditioning does not decrease infarct size but show protection only on the microvasculature.

## 5 Conclusions and future perspectives

With the objectives of our work, we have presented both model systems that can highlight implementation of co-morbidities in early testing, we utilized a clinically relevant porcine model in our investigations and we highlighted the importance microRNAs combined with unbiased target search. These methods can help us to identify novel players in cardioprotection.

The inclusion disease conditions can improve the relevance of early stage testing to identify drug candidates with limited effects and importance of confounding factors. The presented *in vitro* test system of hypercholesterolemia and metabolic disease condition combined with ischemia/reperfusion injury mimics the *in vivo* co-morbidity condition of myocardial ischemia/reperfusion injury. Thereby, the presented test systems can be utilized as a screening platform for testing potential cardiocytoprotective drug candidates.

To progress in clinical application, proof of concept and safety evaluation in large animal models are necessary steps before the start of first-in-human trials. Therefore, miRNA and mRNA expression alterations were first identified in a well characterized rodent post-MI model and later validated on myocardial tissue samples from a clinically relevant closed-chest porcine acute myocardial infarction and cardioprotection model. We highlighted expression changes of *Swiprosin-1* and miR-34c in the post-MI heart.

Unbiased global approaches could be more capable to identify signalling networks activated in cardioprotection. These molecular networks offer to identify mediators and functional clusters that are central in the studied phenotype. Therefore, we presented that the applied network theoretic approach is suitable to identify novel mediator and common target of ProtectomiR microRNAs, *Rictor*, in ischemic postconditioning.

Approaches based on micorRNAs and on their targets demonstrated effectiveness in large animal models and several RNA-based drugs targeting cardiovascular diseases have already been approved, development of additional non-coding RNA-based therapies are also currently under way [174]. Some miRNA based approaches demonstrated infarct size sparing effect already in large animal models [123-125], and first miRNA inhibitor for the reduction of myocardial infarction caused damages is now in clinical development [126].

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## 6 Summary

**Background:** Translation of cardioprotection has been largely disappointing so far, due to the scarce application of (1) studies taking into account co-morbidities, (2) clinically relevant animal models in target discovery and validation, (3) novel targets and approaches such as miRNAs and network analysis.

**Aims:** Therefore, here we aimed (1) to develop an *in vitro* test system of cardiac myocytes that mimics I/R injury in presence of hypercholesterolemia and hyperglycemia; (2) to identify miRNA associated with cardiac adaptation and remodelling induced by MI and (3) to identify key molecular targets of cardioprotective miRNAs - ProtectomiRs and confirm their association with cardioprotection in pig model of MI.

**Methods and results:** We utilized neonatal cardiac myocytes, who took up cholesterol from the treatment media at a concentration-dependent manner. Hypercholesterolemia alone did not alter cell viability, however, in combination with SI/R the damage was aggravated. Furthermore, hypercholesterolemia inhibited the protective effect of the NO donor compound. Metabolic disease condition alone and in combination with SI/R further increased cell damage. Post-MI expression of *Swiprosin-1*, a molecule related to remodelling like events, was induced by post-MI, but not increased in IPre and IPostC rat hearts. Rno-miR-32-3p and rno-miR-34c-3p were identified, as miRNAs associated with the regulation pattern of *Swiprosin-1*. Moreover, expression pattern of *Swiprosin-1* and ssc-miR-34c was also validated in clinically relevant porcine MI and cardioprotection model. Potential molecular targets of ProtectomiR miRNAs were identified by network approach. The *Rictor* was identified as the most central hub in the protectomiR-target mRNA molecular network and further validated in a clinically relevant porcine cardioprotection model. Three *Rictor*-targeting pig homologue of rat ProtectomiRs significantly up-, while *Rictor* was downregulated in the postconditioned pig heart.

**Conclusions:** (1) We demonstrated the implementation of co-morbidities *in vitro* can be applied in early testing of potential cardioprotective compound. (2) We identified and validated in a clinically relevant porcine model *Swiprosin-1* and miR-34c in association with post-MI remodelling and cardiac repair. (3) We presented an unbiased network approach to identify *Rictor*, in ischemic postconditioning. We assume the presented methodologies and results can help to identify novel, more reliable therapeutic targets.

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