IDENTIFICATION AND VALIDATION OF CARDIOPROTECTIVE MICRORNAS IN CELLULAR MODELS OF ISCHEMIA/REPERFUSION INJURY

PhD thesis

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1. Introduction

Ischemic heart disease (IHD) is the leading cause of death worldwide, causing millions of deaths each year (Naghavi et al, 2024). The standard treatment for acute myocardial infarction is reperfusion therapy, including percutaneous coronary intervention or coronary artery bypass grafting, that restores the blood flow after an ischemic event (Byrne et al, 2023). Paradoxically, the restoration of blood flow can further increase the infarct size, a phenomenon known as ischemia/reperfusion (I/R) injury (Frantz et al, 2022). Currently, there are no effective therapies for I/R injury in clinical practice. In preclinical studies, ischemic conditioning, applying short cycles of occlusion and reperfusion cycles to the coronary artery or remote organs, showed promise, but its clinical translation has not yet been successful (Hausenloy & Yellon, 2011).

Preclinical models play a crucial role in investigating the mechanisms of myocardial I/R injury and in the development of cardioprotective treatments (Heusch, 2017). In addition to animal models, *in vitro* cell culture-based platforms are valuable tools for modeling cardiovascular diseases and conducting high-throughput

drug screening assays. Cell culture models for IHD include primary isolated cardiomyocytes, induced pluripotent stem-cell (iPSC) derived cardiomyocytes and cell lines derived from different rodent or humans sources (Lindsey et al, 2018). However, a systematic comparison of these cellular models with each other and a thorough investigation of their transcriptomic differences from their corresponding source tissue is currently lacking in the literature.

MicroRNAs (miRNAs) are short non-coding RNAs involved in post-transcriptional regulation. Since their discovery, miRNAs have attracted interest as potential therapeutic agents as they can simultaneously target multiple mRNAs, allowing the modulation of complex disease-related phenotypes, like I/R injury (Shah & Giacca, 2022). Several miRNAs, termed protectomiRs, have been shown to participate in cardioprotective pathways induced by ischemic conditioning (Varga et al, 2014). Investigating the molecular pathways underlying ischemic conditioning within a translational model of myocardial infarction might reveal novel protectomiR candidates for the development of cardioprotective therapeutics.

2. Objectives

In vitro cardiomyocyte models, such as primary cells, cell lines, and iPSC-derived cardiomyocytes have been not been systematically compared to date, though they are commonly used for testing new cardioprotective agents against I/R injury. Investigating the molecular mechanisms of ischemic conditioning in these models may identify novel therapeutic targets, including protectomiRs, which are promising candidates to treat I/R injury. This thesis aims to:

I. Systematically compare rat, mouse, and human cardiomyocyte models in the aspects of transcriptomic profile, response to hypertrophic stimuli, and tolerance to simulated I/R injury to identify their similarities and cell-specific traits.

II. Discover novel protectomiRs by:

IIa. Analysing the miRNA expression profile of a porcine model of acute myocardial infarction in combination with ischemic conditioning to identify protectomiRs.

IIb. Validating the cardioprotective effect of the protectomiR candidates *in vitro* in rat and human cardiomyocyte models of simulated I/R injury

IIc. Investigating the mechanism of action of the novel protectomiRs through bioinformatics analysis.

3. Methods

3.1. Cell line maintenance and differentiation

H9C2, AC16 and HL-1 cell lines were maintained in DMEM, DMEM-F12 and Claycomb medium, respectively, supplemented with 10% FBS in case of H9C2 and HL-1 or 12.5% FBS in case of AC16 in a standard cell culture incubator. H9C2 and AC16 cells were differentiated to a more mature cardiomyocyte phenotype with the supplementation of all-trans retinoic acid for 8-10 days.

3.2. Human induced pluripotent stem cell (hiPSC) culture and differentiation

Human iPSCs were differentiated to cardiomyocytes (hiPSC-CMs) from XCL-1 cell line. The cells were kept in RPMI 1640 medium supplemented with insulin-free B27 and 10 μM CHIR99021 for 2 days. Then, medium was changed to RPMI supplemented with insulin-free B27 for 1 day, followed by the addition of 2.5 μM C59 Wnt inhibitor on the following day. From day 5 the medium was changed every two days to RPMI supplemented with B27 containing insulin. From day 10 to 14, metabolic selection was performed by culturing cells in glucose-free RPMI supplemented with B27. All experiments were conducted between days 35-40.

3.3. Primary neonatal rat and murine cardiomyocyte isolation

Neontal rat cardiomyocytes (NRCM) and neonatal murine cardiomyocytes (NMCM) were isolated from 1-3 days old Wistar rats or C57BL/6 mice using the Pierce™ Primary Cardiomyocyte Isolation Kit. Briefly, after cervical dislocation, hearts were dissected, and placed in ice-cold Hanks' Balanced Salt Solution, minced into 1-3 mm pieces, washed, and digested for 30 minutes at 37 °C with isolation enzymes. The digested tissue was then washed, resuspended in DMEM with 10% FBS and 1% Antibiotic-Antimycotic, and seeded onto plates for culture at 37 °C with 5% CO_2. The experiments were conducted with cells on day 1 or 3 post-isolation.

3.4. Tissue collection from human, rat and murine hearts

Human left ventricular tissues were obtained from healthy organ donors (n=3) without cardiovascular disease at the Cardinal Stefan Wyszyński Institute of Cardiology. Left ventricular tissue was collected from healthy male Wistar rats (7-8 weeks, 280-330 g, n=3) and C57BL/6 mice (11-12 weeks, 25-30 g, n=3) by flash-freezing in liquid nitrogen, and storage at -80 °C until use.

3.5. RNA isolation and sequencing

Cells and tissue samples were lysed in QIAzol, and total RNA was extracted using the Direct-zol RNA MiniPrep System with DNase treatment. RNA quality and concentration were assessed with the TapeStation and Qubit systems. Libraries for gene expression profiling were prepared using the QuantSeq 3' mRNA-Seq Kit, and sequenced on the NextSeq 500 platform. RNA sequencing data were processed by trimming adapters and low-quality bases, followed by quality control and alignment to species-specific reference genomes. Differential expression and principal component analyses were performed. Functional analysis focused on cardiovascular-related Gene Ontology terms, comparing groups using signed cosine distance between their functional expression vectors and reference tissues.

3.6. Hypertrophic stimuli of cardiomyocytes

AC16 cells, differentiated AC16 cells, H9C2 cells, differentiated H9C2 cells, and NRCMs were treated with angiotensin II (100 nM-1 μM) or isoprenaline (100 nM or 1 μM) for 24 hours to assess hypertrophic or adrenergic responses (n=3-4). After treatment, cells were harvested for

RNA isolation and RT-PCR to analyze the expression of hypertrophy markers or cell surface area analysis.

3.7. MiRNA isolation and high-throughput RT-PCR

miRNA was isolated from left ventricular tissues of pigs subjected to 90 minutes of left anterior descending coronary artery occlusion (AMI) combined with ischemic preconditioning (IPreC), postconditioning (IPostC) or remote ischemic preconditioning (RIPerC) using the High Pure miRNA Isolation Kit and quantified with a NanoDrop 1000 (n=3). miRNA was reverse-transcribed to cDNA, and 4 ng of cDNA was used for RT-PCR with miRNA-specific primers targeting 220 pig miRNAs selected from miRBase database and literature. High-throughput RT-PCR was performed on the LightCycler 1536 System, and miRNA expression was analyzed using the ΔΔCt method. Differentially expressed miRNAs by all three conditioning methods were selected as protectomiR candidates for *in vitro* validation in rat and human cardiomyocytes.

3.8. Cross-species miRNA sequence similarity

NCBI RNA BLAST and the miRBase database were used to identify rat and human orthologues of selected protectomiRs. Those miRNAs which showed 100%

sequence similarity across all three species were selected for the subsequent *in vitro* screening in NRCMs.

3.9. MiRNA transfection of cardiomyocytes

NRCM and AC16 cells were transfected with 25, 50, or 100 nM of Dharmacon miRIDIAN microRNA Mimics or Hairpin Inhibitors using DharmaFECT1 for 10 hours (n=4-6). A non-targeting miRNA from *Caenorhabditis elegans*, miR-67b at 50 nM served as a negative control.

3.10. Simulated ischemia/reperfusion injury

Simulated ischemia/reperfusion (sI/R) injury was performed by replacing the medium with hypoxic or normoxic solutions and incubating cells in a hypoxic chamber (1% O_2) for 6 hours (NRCM, NMCM) or 16 hours (AC16, H9C2, HL-1, hiPSC-CMs). Cell viability was assessed using calcein-AM fluorescence or the CellTiter-Glo Luminescent Viability Assay.

3.11. MiRNA target prediction, and functional analysis

Targets of endogenous and porcine, rat, and human miR-450 were predicted using miRNAtarget™, which integrates validated and predicted interactions from miRTarBase, miRDB, and TargetScan databases. Cardiac-expressed targets were identified from rat and human RNA sequencing

data, and common targets across species were matched using Ensembl orthologs. Gene Ontology and KEGG enrichment analyses were performed on porcine, rat, and human targets using PANTHER and DAVID, identifying shared biological processes and pathways.

4. Results

4.1. Systematic comparison of cardiomyocyte models

4.1.1. Transcriptomic profile analysis

We performed deep RNA sequencing to compare gene expression profiles of common cardiomyocyte models with their source tissues by determining the number of differentially expressed genes. NRCM, NMCM, and hiPSC-CM cells showed greater similarity in their transcriptomic profile to adult cardiac tissues than cell lines, regardless of their differentiation status.

For a functional analysis of gene expression profiles, GO terms were selected for relevant cardiac functions, and we calculated signed cosine distances based on the differential expression of genes related to these GO terms. HiPSC-CMs were more similar to human cardiac tissues than AC16 cells, while AC16 differentiation did not significantly alter functional gene expression. H9C2 and NRCMs showed low similarity to rat myocardium, while HL-1 and NMCMs better matched murine tissue but differed in inflammation, endocardium, and muscle differentiation genes. Overall, primary cells and hiPSC-CMs are transcriptomically and functionally closer to adult cardiac tissues than cell lines.

4.1.2. Response to hypertrophic stimuli

Cell lines and primary NRCMs were treated with angiotensin II or isoprenaline for 24 hours to assess hypertrophic and adrenergic responses. NRCMs showed increased cell surface area, while other cell types did not show hypertrophic response. Hypertrophy marker mRNA levels (*Nppb, Ctgf, Col1a1, Col3a1*) remained unchanged across all models after treatment.

4.1.3. Response to simulated I/R injury

Primary NRCM and NMCM cells were subjected to 6 hours of simulated ischemia (sI), while cell lines and hiPSC-CMs were treated for 16 hours, followed by 2 hours of reperfusion before assessing viability. Responder ratios, showing significant viability decreases, were 75% for NRCMs and 50% for NMCMs after 6 hours sI. hiPSC-CMs had 0% response at 6 hours but 83.3% after 16 hours. Immortalized lines showed greater resistance against sI, as proliferative AC16 and H9C2 cells had responder ratios of 45.5% and 76.9%, respectively, which increased to 100% after differentiation. HL-1 cells showed only 17% response, indicating the highest resistance to hypoxia. Therefore, differentiation increases hypoxic sensitivity of cell lines, while hiPSC-CMs are the most valuable models for sI/R.

4.2. Identification of protectomiRs

4.2.1. miRNA expression profile of pigs subjected to ischemic conditioning

To identify novel cardioprotective miRNAs, we used a clinically relevant closed-chest porcine AMI model with ischemic conditioning and analyzed miRNA expression changes linked to cardioprotection. IPreC, IPostC, or RIPerC protocols were shown to reduce infarct size, microvascular obstruction, or edema. Expression of 212 miRNAs was compared between AMI and ischemic conditioning groups, revealing significant up- and downregulation: IPreC (29 up, 28 down), IPostC (19 up, 35 down), RIPerC (33 up, 35 down). Four miRNAs were consistently upregulated, and ten downregulated across all protocols, highlighting them as key cardioprotective candidates.

4.2.2. In vitro validation of the cardioprotective effect of selected protectomiR candidates

We assessed sequence similarity of the 14 protectomiR candidates across species using NCBI RNA Blast and miRBase, finding 12 had 100% rat and human orthologs. miR-105-2 showed <60% similarity and miR-424-3p lacked a rat ortholog, so both were excluded from rat cardiomyocyte validation. NRCMs were transfected with

miRNA mimics or antagomiRs in 25, 50 or 100 nM concntration and subjected to sI/R injury. The mimics of rno-miR-450a-3p and rno-miR-451-5p at 25 nM significantly improved cell survival, while other mimics or antagomiRs showed no benefit, some worsening injury at higher doses. In human AC16 cells, miR-450a mimic enhanced survival, but miR-451 mimic did not. Taken together, miR-450a was the most promising protectomiR candidate.

4.2.3. Functional analysis of miR-450a target genese

To explore miR-450a's cardioprotective mechanisms, we predicted its mRNA targets in pigs, rats, and humans. Filtering for cardiac-expressed genes resulted in 607 common targets across all species, involving key predicted targets like SMAD2, DAPK2, SOD2, RNF169, and genes involved in inflammation and cell survival. GO analysis revealed 421 shared enriched biological processes related to mRNA processing, TGFβ signaling, apoptosis, and DNA damage response. KEGG pathways analysis found 13 common enriched pathways, including PI3K, mTOR, and sphingolipid signaling. These results suggest miR-450a promotes cardioprotection by modulating signaling pathways critical to myocardial function.

5. Conclusions

This study systematically compared cardiomyocyte models, identified novel cardioprotective miRNAs, validated their effects *in vitro,* and explored their molecular mechanisms.

Cardiac cell lines like H9C2, HL-1, and AC16 were shown to be useful for models for IHD but have limitations affecting results. Primary cultures, especially hiPSC-CMs, better mimic adult cardiac tissue but are less available for high-throughput screening. Thus, appropriate model selection is crucial for *in vitro* preclinical studies.

From a porcine myocardial infarction model, we identified 14 protectomiRs regulated by ischemic pre-, post-, and remote conditioning.

Among these, miR-450a and miR-451 mimics showed direct cardioprotective effects in neonatal rat cardiomyocytes, with miR-450a also protective in human AC16 cells.

Predicted targets suggest miR-450a acts via multiple cardioprotective pathways including apoptosis, DNA damage response and signaling pathways.

In conclusion, miR-450a is a promising therapeutic candidate to fulfill the unmet need for novel effective cardioprotective treatments with a multi-target effect.

6. Bibliography of the candidate's publications

The publications involved in the current thesis:

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