NOVEL CARDIOPROTECTIVE MECHANISMS IN TRANSLATIONAL MODELS OF THE ISCHEMIC HEART

PhD thesis

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1 Introduction

Myocardial ischemia (MI) can manifest as acute occlusion of the coronary artery, which leads to lack of blood flow, oxygen and nutrient supply. Reperfusion, the first line treatment of MI, brings additional damage named "reperfusion injury". Protection against this ischemia-reperfusion (I/R) injury is an unmet clinical need.

Brief non-lethal cycles of ischemia and reperfusion, termed as "ischemic conditioning", dramatically reduce I/R damage. Ischemic preconditioning (IPreC), when these cycles are applied prior MI, provides powerful endogenous cardioprotection, but its clinical application is limited. In case of ischemic postconditioning (IPostC), when the cycles are applied at the beginning of the reperfusion, preclinical studies showed protection of the heart against lethal I/R-induced injury. However, the results of clinical studies with IPostC are controversial.

Pharmacological agents can be utilized also to induce cardioprotection against I/R injury. Many potential cardioprotective agents successful in preclinical settings failed after preliminary studies. Meanwhile, others are still under evaluation, but results are often inconsistent.

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 approaches to induce cardioprotection are also needed.

1.1 Translation of cardioprotection – role of co-morbidities

The complexity of the preclinical test systems often lacks concomitant co-morbidities, such as hypercholesterolemia or metabolic disease condition.

Hypercholesterolemia is a well-established risk factor of cardiovascular diseases. Hyperlipidemia and hypercholesterolemia are present in most patients with myocardial infarction and induce the loss of cardioprotection in preclinical studies. Additionally, diabetes mellitus and hyperglycemia are also major components of chronic metabolic disease and they can make the heart more

susceptible to I/R injury. This way inclusion of co-morbidities in early testing of a potential cardioprotective therapy can be recommended.

1.2 Translation of cardioprotection – importance of large animal studies

An additional factor to improve translation of cardioprotection to humans is the application of data obtained from large animal models with high translational value.

Domestic pigs (sus scrofa domestica) are closest to humans in terms of the anatomy of the heart, hemodynamics, and development of myocardial infarction. Critical endpoints, including infarct size and microvascular obstruction as well as oedema, have been established allowing correlation of preclinical outcomes with future clinical endpoints. Meanwhile, large animal models offer opportunity to assess molecular mechanisms parallel with clinical endpoints. This way involvement of large animals in the preclinical studies should be included.

1.3 Novel targets and approaches – microRNAs in cardioprotection

MicroRNAs are fine regulators of gene expression in cardiac physiology and pathology through incomplete base-pairing with their target mRNAs, leading to mRNA degradation or inhibited protein translation.

Dysregulation of microRNAs after MI has been described in I/R injury and in cardioprotection. MicroRNAs associated with cardioprotection, termed ProtectomiRs and can be identified with the comparative analysis of their expression in MI and in ischemic conditioning. Modulation of microRNAs can reduce infarct size or inhibit the development of post-MI heart failure.

Single microRNAs regulate protein translation from multiple mRNAs and each mRNA is regulated by several microRNAs.

This offers the application of a new omics-based approach and enables the unbiased network theoretic identification of new, possibly more relevant molecular pathways and mediators to induce cardioprotection. Considering the low number of microRNAs (only about 2000 microRNA), it is more cost-effective to measure the expression of all microRNAs. Nevertheless, by *in silico*

evaluation, the most important mRNA mediators could be identified relying solely on the microRNA expression pattern with a network theoretic approach.

This molecular network approach offers, beside the rigorous mathematical description, the possibility to visually identify mediators and functional clusters that are central in the studied phenotype.

2 Objectives

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

1) 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) The aim was to identify microRNAs associated with cardiac adaptation and remodelling induced by acute myocardial infarction in rat and porcine models.
3) 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.

3 Methods

Methodologies are detailed in the publications, on which this thesis is based.

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

Neonatal rat cardiac myocytes were cultured in normoglycaemic medium supplemented with vehicle or with hypercholesteraemic supplementation (hiChol), which was applied in three gradually increasing concentrations suitable for obtaining the responses by the cells.

Cellular cholesterol content of the treated cardiac myocytes was controlled to validate cholesterol uptake by cells (Figure 1 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 1 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 1 C).

Additionally, to mimic metabolic disease condition hiChol supplementation was completed with high glucose concentration (25 mM) and 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 1 D).

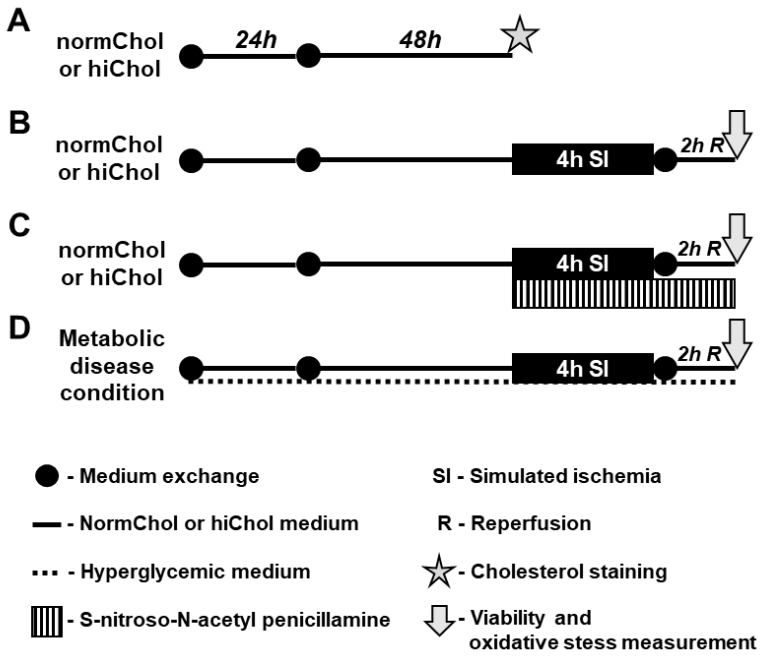

Figure 1 *In vitro combination model of hypercholesterolemia or metabolic disease condition with ischemia/reperfusion injury experimental design.*

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. 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 microRNAs, 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 microRNA in a translational porcine model.

We utilized samples from our previously described and well-characterized animal models which are detailed in the thesis.

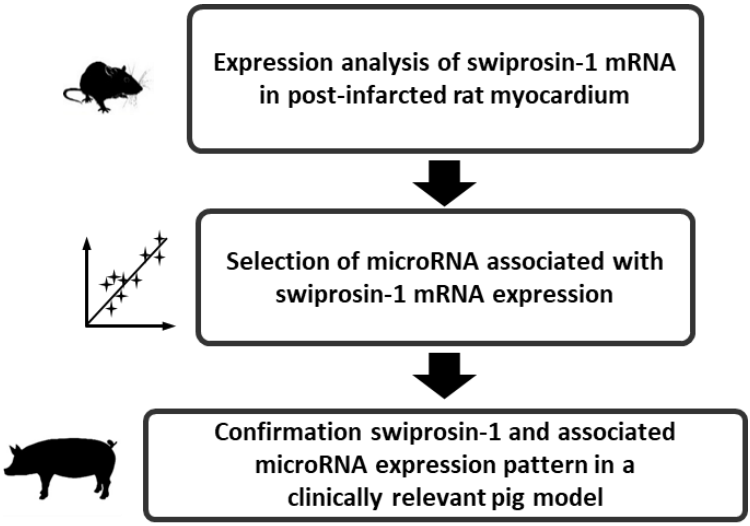

Figure 2 *Identification of microRNAs associated with cardiac adaptation and remodelling experimental flowchart*

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 were predicted by miRNAtarget software and visualised in a microRNA-target mRNA network. 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 the same previously published, well characterized, clinically relevant, closed-chest porcine model which was cited in previous chapter and further detailed in the thesis. Tissue samples were collected after 3 hour reperfusion.

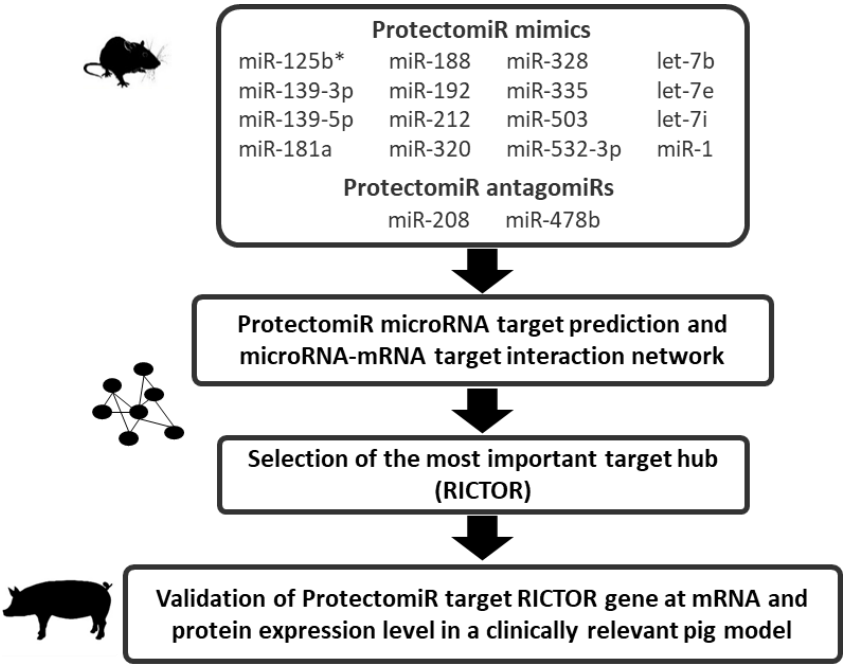

Figure 3 *Identification of key molecular targets of ProtectomiRs with an unbiased network approach experimental flowchart*

4 Results

4.1 Rat cardiac myocytes taken up cholesterol in a concentration dependent manner

Filipin staining reflected the cholesterol content of the cardiac myocytes and showed that cholesterol uptake from the hiChol supplements was efficient and cholesterol content increased in cardiac myocytes at concentration dependent manner.

4.2 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. However, superoxide levels were significantly elevated in all groups, 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. SI/R injury alone increased both total ROS and superoxide levels in normocholesterolemic (normChol) groups, which were further increased in the highest concertation hypercholesterolemic group (hiChol3).

4.3 Pharmacological cardiocytoprotection by NO donor against simulated ischemia/reperfusion injury is 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. The protective effect of SNAP was abolished in each hiChol supplemented groups.

4.4 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). In these groups, total ROS and superoxide levels increased correspondingly. Simulated ischemia-reperfusion further decreased cell viability in hiChol2 and hiChol3, while total ROS and superoxide levels increased.

4.5 *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.

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

364 microRNAs were constitutively detected in rat heart tissue samples. Among them, 27 microRNAs were either positively (n = 15) or negatively (n = 12) associated with the mRNA expression of *Swiprosin-1* in all samples. Only 12 of 27 microRNAs showed an ischemia/reperfusion-dependent regulation such as *Swiprosin-1*, and only in two cases could the induction of microRNA 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.

In the rat cardiac tissue, beyond *Swiprosin-1*, mRNA expression of 72 different genes related to cardiac biology was analysed previously. A detailed analysis of further co-regulated mRNAs with both microRNAs was performed in the current work by investigating a potential linear correlation between mRNAs and microRNAs. 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.

4.7 *Swiprosin-1* and ssc-miR-34c expression correlates 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. In the reperfused heart, ssc-miR-34c was again increased by ischemia/reperfusion, and this was again abrogated by IPreC and IPostC.

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

The eighteen different previously described 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. In this interaction network, 84 mRNAs had interactions with more than one microRNA, and 15 mRNAs interacted with at least three microRNAs.

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.

4.9 *Rictor*-targeting ProtectomiRs upregulated after postconditioning in a clinically relevant closed chest porcine cardioprotection 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.

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. Interestingly, these microRNAs did not show alteration in the myocardial samples of the IPreC group.

4.10 The central hub, *Rictor* downregulated after postconditioning in a clinically relevant porcine cardioprotection 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. 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.

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 of the IPostC group as compared to the Isch group. No RICTOR protein expression changes were found in the IPreC group compared to Isch neither in the ischemic nor the non-infarcted remote zone of the myocardium.

5 Conclusions

With the objectives of our work, we have sought to both develop model system that can implement co-morbidities in early testing, identify microRNAs as novel players in post-MI remodelling and presented and unbiased microRNA target search, a method to identify the key players involved in cardioprotection.

- The inclusion of disease conditions, can improve the relevance of early stage testing to identify drug candidates with limited effects and importance of confounding factors. The presented cell-based *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 system can be utilized as a screening platform for testing potential cardioprotective drug candidates in the presence of these co-morbidities.
- To progress in drug development evaluation in large animal models are necessary steps before the start of first-in-human trials. Therefore, in our work the analysed microRNA and mRNA expression alterations were first identified in well characterized rodent myocardial infarction model and later validated on myocardial tissue samples from a clinically relevant closed-chest porcine acute myocardial infarction and cardioprotection model. We highlighted expression of *Swiprosin-1* and miR-34c in post-infarct myocardium and their expression pattern suggests an important role in cardiac repair both in rats and pigs.
- Unbiased global microRNA-target network approach could be more capable to identify novel targets of cardioprotection. These molecular networks offer beside the rigorous mathematical description the possibility to visually 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 potential therapeutic target, *Rictor,* in ischemic conditioning induced cardioprotection. However, further studies are needed to clarify its relevance as therapeutic targets.

Approaches based on microRNAs and on their targets demonstrated effectiveness in large animal models, and the first miRNA inhibitor against post-MI remodelling in now in clinical development.

6 Bibliography of own publications

6.1 Own publications involved in the current thesis

1. **Makkos A**, Szántai Á, Pálóczi J, Pipis J, Kiss B, Poggi P, Ferdinandy P, Chatgilialoglu A, Görbe A. A Comorbidity Model of Myocardial Ischemia/Reperfusion Injury and Hypercholesterolemia in Rat Cardiac Myocyte Cultures. Front Physiol. 2020 Jan 9; 10:1564. **IF: 4.566**

2. Giricz Z, **Makkos A**, Schreckenberg R, Pöling J, Lörchner H, Kiss K, Bencsik P, Braun T, Schulz R, Ferdinandy P, Schlüter KD. Swiprosin-1/EFhD-2 Expression in Cardiac Remodeling and Post-Infarct Repair: Effect of Ischemic Conditioning. Int J Mol Sci. 2020 May 9; 21(9):3359. **IF: 5.924**

3. **Makkos A**, Ágg B, Varga ZV, Giricz Z, Gyöngyösi M, Lukovic D, Schulz R, Barteková M, Görbe A, Ferdinandy P. Molecular Network Approach Reveals Rictor as a Central Target of Cardiac ProtectomiRs. Int J Mol Sci. 2021 Sep 2; 22(17):9539. **IF: 5.924**

6.2 Own publications not involved in the current thesis

1. Baranyai T, …, **Makkos A**, …, Giricz Z. Acute hyperglycemia abolishes cardioprotection by remote ischemic perconditioning. Cardiovasc Diabetol. 2015 Nov 18; 14:151. **IF: 4.534**
2. Baranyai T, …, **Makkos A**, …, Ferdinandy P. In vivo MRI and ex vivo histological assessment of the cardioprotection induced by ischemic preconditioning, postconditioning and remote conditioning in a closed-chest porcine model of reperfused acute myocardial infarction: importance of microvasculature. J Transl Med. 2017 Apr 1; 15(1):67. **IF: 4.197**
3. Ágg B, Baranyai T, **Makkos A**, …, Ferdinandy P. MicroRNA interactome analysis predicts post-transcriptional regulation of ADRB2 and PPP3R1 in the hypercholesterolemic myocardium. Sci Rep. 2018 Jul 4; 8(1):10134. **IF: 4.011**
4. Lázár B, Brenner GB, **Makkos A**, …, Zádori ZS. Lack of Small Intestinal Dysbiosis Following Long-Term Selective Inhibition of Cyclooxygenase-2 by Rofecoxib in the Rat. Cells. 2019 Mar 15; 8(3):251. **IF: 4.366**
5. Brenner GB, **Makkos A**, …, Ferdinandy P. Hidden Cardiotoxicity of Rofecoxib Can Be Revealed in Experimental Models of Ischemia/Reperfusion. Cells. 2020 Feb 26; 9(3):551. **IF: 6.600**

6. László SB, …, **Makkos A**, …, Zádori ZS. Chronic treatment with rofecoxib but not ischemic preconditioning of the myocardium ameliorates early intestinal damage following cardiac ischemia/reperfusion injury in rats. Biochem Pharmacol. 2020 Aug; 178:114099. **IF: 5.858**
7. Szabo D, …, **Makkos A**, …, Tamas A. PACAP-38 in Acute ST-Segment Elevation Myocardial Infarction in Humans and Pigs: A Translational Study. Int J Mol Sci. 2021 Mar 12;22(6):2883. **IF: 5.924**
8. Brenner GB, …, **Makkos A**, …, Ferdinandy P. Post-Myocardial Infarction Heart Failure in Closed-chest Coronary Occlusion/Reperfusion Model in Göttingen Minipigs and Landrace Pigs. J Vis Exp. 2021 Apr 17; (170). **IF: 1.355**
9. **Makkos A**, …, Ferdinandy P. Systematic review and network analysis of microRNAs involved in cardioprotection against myocardial ischemia/reperfusion injury and infarction: Involvement of redox signalling. Free Radic Biol Med. 2021 Aug 20; 172:237-251. **IF: 7.376**
10. Onódi Z, …, **Makkos A**, …, Varga ZV. AIM2-driven inflammasome activation in heart failure. Cardiovasc Res. 2021 Jun 12: cvab202. **IF: 10.787**
11. Vörös I, …, **Makkos A**, …, Ferdinandy P. Somatostatin and Its Receptors in Myocardial Ischemia/Reperfusion Injury and Cardioprotection. Front Pharmacol. 2021 Nov 5;12:663655. **IF: 5.811**
12. Onódi Z, …, **Makkos A**, …, Varga ZV. Systematic transcriptomic and phenotypic characterization of human and murine cardiac myocyte cell lines and primary cardiomyocytes reveals serious limitations and low resemblances to adult cardiac phenotype. J Mol Cell Cardiol. 2021 Dec 24;165:19-30. **IF: 5.000**

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