Görbe Anikó PD 106001 (2013 0101-2016 0630)

The mechanisms of myocardial cell injury and cell death in response to impaired coronary perfusion continue to be a subject of intense investigations for the diagnosis and treatment of patients with ischemic heart disease. An extensive body of evidence has documented the cellular and subcellular alterations in reperfusion period in response to oxygen and substrate deprivation affecting all cell types, including cardiac myocytes. In vitro experimental investigations of different molecules in the reperfused heart tissue is an important step in the development of cardioprotective therapies. Accordingly, this project focuses on three specific aims regarding in vitro cell-based systems.

1. Testing possible cytoprotective effect of the proteoglycan decorin molecule on neonatal rat cardiac myocytes *(project for first year, continued during second and third year)*
2. Investigation of the behavior of iPS-derived cardiac myocytes after ischemic/reperfusion injury in simulated ischemia/reoxygenation (SI/R) system in vitro *(project for second year)*
3. Identification cardiac marker positive cell population of iPS-derived cardiac myocyte embryonic bodies and testing cardioprotective nitric oxide action against SI/R injury on them *(project for third year).*
4. Testing the response of an innovative engineered heart tissue system (EHT) subjected to ischemia/reperfusion in vitro, parallel with experiments on iPS-derived cardiac myocytes *(project for second and third year)*

Below I report the progress and final results of the project related to the specific aims:

1. An in vitro cell-based test system is reproducible and cost effective using neonatal rat heart for isolation of cardiac myocytes. Simulated ischemia/reoxygenation system on neonatal cardiac myocytes was tested with a non-selective MMP inhibitor Ilomastat **(Bencsik et al, 2014),** which can be applied as positive control. Exogenously administered decorin showed concentration dependent protection on cardiac myocytes. We checked the cardioprotective mechanism by exclusion of the potential proliferation effect of proteoglycans by BRDU assay and found that proteogylcans have no proliferative effects, so they exert true cytoprotective effect in the cell survival assays. Another aim was to confirm that NO signaling plays a causative role in decorin-induced cytoprotection. We proved that a NO synthase inhibitor has partially attenuated the decorin-induced protection against cell death due to simulated ischemia/reoxygenation. Additionally, we checked the involvement of the downstream PKG-mediated cytoprotective pathway and found that decorin does not activate this pathway. Because of the partial protection via NO-signalling pathway, other potential target, TLR4 signaling pathway was also investigated by application of TAK 242 inhibitor. This cascade was not involved in decorin induced cardioprotection. For further exploration of molecular mechanism of reperfusion injury, we performed western blot testing for the Safe and Risk pathways as well. Furthermore we introduced simulated ischemia/reoxygenation system on adult cardiac myocytes, where exogenously administered decorin showed concentration dependent protection. These new results were summarized and presented as oral presentation at a national and poster presentation at national and international meetings **(Gaspar et al, 2014).** The project is successfully completed and draft manuscript is under preparation: **Görbe A,** Gáspár R, Páloczi J, Varga ZV, Pipis J, Szántai Á, Csont T, Ferdinandy P, Cardiovascular Research Group, Department of Biochemistry, University of Szeged and Pharmahungary Group, Szeged, Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary. **Small leucine rich proteoglycan decorin protects cardiomyocytes against simulated ischemia/reperfusion injury.** Additionally, our research group worked on parallel project focusing on the cardiocytoprotective effect of another small leucine rich proteoglycan

biglycan, which project was finalized earlier and had priority with its publication. This MS is under revision in J Mol Cell Cardiol. Furthermore we developed a similar in vitro neonatal cardiac myocyte based model for investigation of radiation induce heart damage. That project was successfully published last week **(Kiscsatari et al, 2016).**

2. Another effective treatment of patients suffering from MI could be cell replacement therapy. Stem cell-derived cardiomyocytes are promising cell source for cardiac repair after myocardial infarction. We investigated human embryonic stem cell (ESC)-derived cardiomyocytes and applied previously tuned SI/R system and fluorescent viability assay on these cells. Here we tested well-known cardiocytoprotective molecules on this system, like an NO-donor and the B-type natriuretic peptide. These results was published this year. **(Paloczi et al, 2016).** The emerging induced-pluripotent stem cell (iPSC) technology has a great impact on the field of medicine ever since the ground-breaking discovery in 2006 that overexpression of four specific transcription factors was able to turn back the developmental clock of somatic cells into an embryonic-like state. We have cooperation with Biotalentum Ltd, and our partner provided IPS cells for our SI/R experiments: we successfully started to perform experiments on mouse iPS cell lines (3.4 and 4.1 lines). Fluorescent activated cell sorting system was introduced for separation of cardiac myocytes from embryonic bodies (containing mixed cell population after cardiogenic differentiation) with detection of intracellular cardiac troponin I and temporally expressed vascular cell adhesion protein 1 (VCAM-1). After successful application of suitable digestion protocol to get single cell suspension, now we are able test the hypoxic sensitivity of cardiac myocyte population. IPS embryonic bodies grown in 24-well format plates were already tested, and both cell lines showed similar sensitivity to simulated ischemia in comparison to embryonic stem cell bodies. There are several technical difficulties with IPS cell generation; therefore we started cooperation with an Italian partner to get IPS cells. After training by the principal investigator of Italian partner we isolated fibroblast from adult heart for IPS induction and cardiac myocyte differentiation. We released a common paper with this group about IPS-derived cardiac myocytes **(Madonna et al, 2014).**
3. We continued the project on IPS-derived cardiac myocytes. The intracellular antigen cTnI was remarkably expressed in both day-8 and -16 samples with all types of digestions. The highest cTnI expression was registered on day-8 with application of trypsin. The cell surface antigen VCAM-1 was not detectable after trypsin digestion. However, the application of collagenase type IV on day-16 resulted in the highest ratio of VCAM-1 positive cells. IPS (line 3.4) EBs and VCAM positive cells were more sensitive for SI/R injury than IPS 4.1 cell line. Interestingly, SNAP protected full EBs of HM1 cell line from SI/R, but not immunopositive cardiac myocytes isolated from the EBs. We conclude that the cardiocytoprotective NO donor protects full EBs against SI/R injury, but not the cardiac myocytes in the EBs, suggesting that iPS-derived cardiac myocytes at the current development stage are not suitable for testing cardiocytoprotective mechanisms. These results were summarized and presented as oral presentation at a national and poster presentation at national and international meetings **(Szantai et al, Görbe et al 2015).** The project was successfully completed and draft manuscript is under preparation: **A. Görbe**, J. Pálóczi, E Ruivo, Á Szántai, R Gáspár, J Kobolák, A. Dinnyés, P. Ferdinandy. **Nitric oxide mediated cytoprotection against simulated ischemia/reperfusion caused injury in induced pluripotent stem cell-derived cardiac myocytes.**

4. Because of unforeseen difficulties with the intellectual property issues of the use of IPS cells, to minimize a risk of this project, we have started a novel project on 3D engineered heart tissue in cooperation with Prof. Thomas Eschenhagen's group (Universitatklinikum Eppendorf, Hamburg) as approved by OTKA office. In vitro drug screening and disease modeling is constantly growing research field, in which three dimensional tissue engineering became a routinely applicable tool. The response of engineered heart tissue (EHT) to ischemia/reperfusion (I/R) was observed. The 24-well EHT test system is applicable to test cardioprotective compounds in vitro and monitor several functional and biochemical endpoints, which otherwise could be only measured in vivo or ex vivo heart preparations. The study was published to Plos One **(Görbe et al, 2015).**

In summary, the progress of the project was according to the plans, and finally overdelivered. We have obtained valuable results as follows in brief. Simulated ischemia/reoxygenation system on neonatal rat cardiac myocytes was validated by positive control compounds. The molecular mechanism behind of cardiocytoprotective effect of proteoglycan decorin was tested in this system. Additionally a new in vitro test system was developed for testing radiation induced heart damage. We successfully investigated the sensitivity of IPS derived cardiac myocytes to hypoxia. We have implemented a technique for their separation from other cell types. We have shown that cardiocytoprotective NO donor protects full EBs against SI/R injury, but not the cardiac myocytes in the EBs, suggesting that iPS-derived cardiac myocytes at the current development stage are not suitable for testing cardiocytoprotective mechanisms. We continued successful international cooperation in our IPS project and in a novel a project on 3D engineered heart tissue to minimize the risk of the present OTKA project due to technical and intellectual property difficulties with the IPS technology.