Heart failure is one of the major cause of death in the Western world. With this object, it is not surprising, that numerous studies have been launched to discover novel ways to reverse the pathological processes of this vital organ. Utilization of progenitor cells is promising, but still remains controversial. Continuous technical hurdles, including nature and origin of stem cells, ways of application, survival or transformation rate impede common use. Recent results suggest not the transformed stem cells, but the actual molecular factors secreted by the injected progenitors may be responsible for the potential tissue protection. In our earlier studies we discovered a small secreted 43 aa peptide Thymosin beta-4 (TB4) protects the injured heart and initiates numerous regenerative processes after cardiac hypoxia when applied systemically in adult mammals. We also presented, the C-terminal 4aa variable domain of the peptide is capable to achieve similar impacts. Because of these findings, we hypothesize understanding the potential molecular mechanisms and pathways initiated by TB4 may be instrumental towards discovering novel ways of post-hypoxic treatment of the damaged heart. Recent results suggest, small non-coding RNAs (microRNAs or miRs) may play important role in the pathogenesis of heart failure. MiRs are known to regulate the expression of genes that govern the process of adaptive and maladaptive cardiac remodeling. To put these observations together, in this grant proposal we aimed to perform a systematic analysis of TB4 initiated miRNA expression. In our studies we focused on acute cardiac hypoxia.

RESULTS:

AIM 1. *To identify miRNAs altered by systemic TB4 administration in adult mammals after hypoxia in vivo and to determine the underlying molecular pathways that are governed by their expression.*

1.1. In the first year of this grant proposal we generated cardiac infarction by ligating the left descending coronary artery in adult C57BL/6J mice and treated them with TB4 (2x15) and PBS (2x15) respectively. One half of the animals were harvested 24, second half 72 hours after ligation. Cardiac function of the animals was recorded by echocardiography before treatment and at the time of harvesting. We separated the infarcted core, remote and border areas and collected the tissue in Trizol reagent followed by prompt freezing of the samples in liquid nitrogen (Figure 1.).

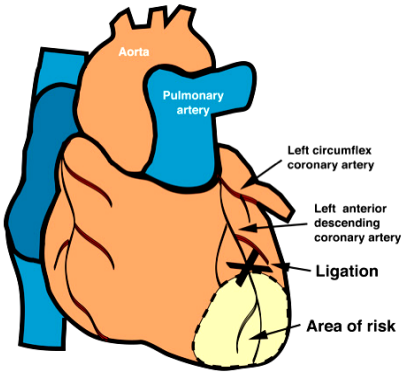

Figure 1. Generation of cardiac infarction in adult mouse hearts.

1.2. To investigate miRNA and protein expression, we isolated total RNA and protein from the same sample pool in accordance to our previously published protocols. We submitted 2µgs of total RNA to LC Sciences and to our own research team (Szentagothai Research Centre/Csaba

Fekete) for microarray analysis and NGS sequencing. Dual submission was necessary to reveal possible novel miRNAs not included in regular mouse microarray protocols. The experiments also supported establishment of proper protocol flow in our local sequencing facilities. We found, the results of our research team significantly overlapped with the microarray data generated by the Houston facilities. Later investigations were financed by independent departmental resources.

1.3. Since primary morphological alterations occur in the infarcted core, our initial analyses were executed and focused on this area of the damaged heart. The results revealed 119 potential miR targets to be altered by TB4 after systemic administration following hypoxia in adult mice. Three miR families with proven heart expression revealed particularly interesting and are in focus of our research. Currently we are performing real-time PCR analyses to confirm the alterations of the members of these families in each experimental sample. We intend to publish the outcome of these results in the close future. 25% of the remaining targets were altered at days 1 and 3 respectively, while 32% at day 1 only. As expected, analysis of the border tissue revealed inconsistent, the results require further investigation.

AIM 2. *To define the in vitro and in vivo biological and functional changes initiated by TB4 altered miRNAs.*

2.1. In our earlier publication we demonstrated TB4 affects not only the core of infarction, but also the remote healthy cells of the heart. We found TB4 initiates significant proliferation in the remote epicardium and reminds the adult heart on its embryotic state. As expected, we detected significant alterations in miR expression at day 3 in this area. Supporting our earlier observations, one of the most potential miRs is a known potent regulator of angiogenesis and progenitor activation. Analysis, confirmation and development of this study is in progress. Our ultimate plan is to publish our findings regarding this miR in the close future.

2.2. As described and presented in our earlier reports in detail, currently we are working on finalizing our publication regarding miR1196 and ROCK1 expression following TB4 treatment in the infarcted heart (Table 1. (Unpublished data)).

Probe ID	Sample A signal Experiment	Sample B signal Control	log2 (SampleA/ Sample B)
mmu-mir-1196	1,164.48	374.46	-1,66

Table 1. mmu-mir-1196 expression via miRNA microarray (Unpublished data).

We completed the *in vivo* experiments and protein confirmation studies (Figure 2. (Unpublished data)). However, to successfully publish our results in a well-respected Q1 journal it is a requirement to confirm our results in a human setting. We successfully received the necessary primary cultures and are in progress of confirming our *in vivo* results in human cardiac myocytes, human coronary endothelial cells and human coronary smooth muscle cells respectively. The outcome is promising and supports the *in vivo* findings. Unfortunately, we recently faced minor technical problems (unexpected de-attachment of some critical cell types). Once issues are cleared with the supplier, results will be ready for submission.

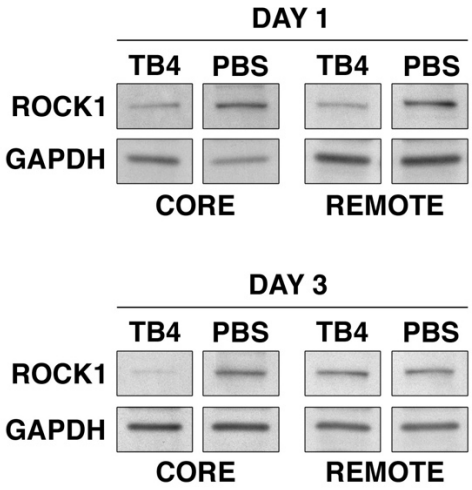

Figure 2. ROCK1 expression after TB4 treatment in infarcted adult mice hearts by Western blot (Unpublished data).

2.3. As result of a collaboration with Prof. Christian Kupatt's laboratories (porcine models) in Munich (Germany), we published our findings describing the *in vitro* and *in vivo* molecular effect of TB4's special domains in the infarcted adult mammalian rodent and porcine heart during this grant period in 2015. Hinkel R, Ball HL, DiMaio JM, Shrivastava S, Thatcher JE, Singh AN, Sun X, Faskerti G, Olson EN, Kupatt C and *Bock-Marquette I. C-terminal AGES domain of Thymosin β4 promotes post-ischemic cardiac function and repair ***JMCC.*** (2015) Aug 5;87:113-125. (*Corresponding and senior author). At this time the PI of this grant proposal was recipient, and the project was equally supported by the "European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/2-11-1-2012-0001 'National Excellence Program". As we were informed by program officials, only one of the Hungarian funding sources were allowed to be acknowledged in the submission, thus regrettably we were unable to list this present grant on our accepted publication.

2.4. Future Plans: in our original proposal we aimed to generate synthetic anti-miRs or miR mimics and test these constructs in *in vitro* and *in vivo* settings with an ultimate goal to approach clinical utilization and pharma. Lacking proper technical support and environment however, we were unable to create drug quality constructs at our facilities. Thus, execution of this part of the proposal requires collaboration with experts of the field. We intend and are in progress to establish such collaboration with the Van Rooij lab at the Hubrecht Institute in the Netherlands and with Miragen Therapeutics Inc. in Boulder Co. USA.

In summary, as projected, a global screen to identify the potential effect of TB4 on miRNA expression in the hypoxic adult mammalian heart has been successfully completed. 119 potential miR targets have been identified. At this point -with permission- we intend to withheld the full list of targets because of future patent potentials. Confirmation of numerous predicted targets has been completed. Further investigations of three miR families and additional single targets are in progress. Final experiments supporting a manuscript discussing our novel findings about the effect of TB4 on miRNA initiated ROCK1 expression is in preparation and close to submission. Additionally, a manuscript discussing the effect of TB4 and its domains on cardiac regeneration and repair has been published. Our findings were presented at three international meetings and in one dissertation.

Finally, we are in progress to establish a collaboration with two research groups to initiate potential clinical utilization of the targets we discovered in the course of our studies.

Acknowledgement
We would like to express our sincere thanks and gratitude to the Committee and the National Research, Development and Innovation Office for supporting our team and research in the last five years.