PROJECT CLOSING REPORT

Cardiac adaptation to ischemic stress in chronic kidney disease and diet-induced hypercholesterolemia: the role of microRNAs

In the present exploratory project, we proposed experimental justification of the hypothesis that metabolic diseases affect cardiac miRNAs and mRNAs and thereby influence gene expression response to adaptation to acute ischemia/reperfusion injury induced by ischemic preconditioning. Therefore, in the current proposal, we aimed to investigate 1) the effect of hypercholesterolemia and chronic kidney disease (CKD) on cardiac expression of miRNAs and mRNAs; and 2) the differential effect of hypercholesterolemia and CKD on changes in cardiac miRNA/mRNA expression induced by ischemia/reperfusion with ischemic preconditioning.

1. DETAILS OF THE RESEARCH PERFORMED

1.1. Cardiac effects of hypercholesterolemia

1.1.1. Diet-induced hypercholesterolemia model

1.1.1.1. Characterization of the model

First we have set up a rat model of diet-induced hypercholesterolemia and characterized the model. Hypercholesterolemia was induced by a diet supplemented with 2% cholesterol and 0.25% cholic acid (control animals received standard rat chow). Elevated serum total cholesterol levels were found 4, 8 and 12 weeks after the diet was started, proving the development of hypercholesterolemia. After 12 weeks, signs of steatosis were seen in the liver due to hypercholesterolemia, however, neither macroscopic morphology nor function of the heart appeared to be markedly affected as assessed by echocardiography. Based on these data, 8 weeks of cholesterol-enriched diet was used for further experiments. Detailed data are published in: *Csonka et. al, Lipids Health Dis, 2016*; and *Demján et al, Evid Based Complement Alternat Med, 2020*. We also had a chance to prepare a review article about hypercholesterolemia-induced oxidative/nitrative stress in the heart (*Csonka et al., Oxid Med Cell Longev, 2016*).

We have also performed profiling of the cardiac lipidome after 8 weeks of cholesterol-enriched diet, and found that hypercholesterolemia not only resulted in a significant increase in tissue cholesterol levels, but also induced a marked rearrangement of the cardiac lipidome (Figure 1.). These results will be used as background data in a future publication related to our hypercholesterolemia model.

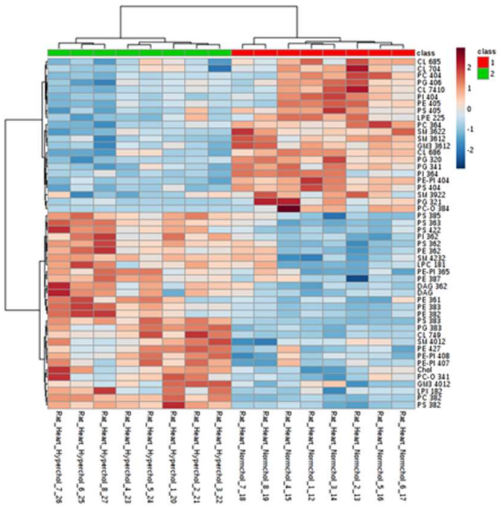

Figure 1. *Hierarchical cluster analysis of the cardiac lipidome in hypercholesterolemic and normocholesterolemic samples.*

1.1.1.2. Ischemia/reperfusion injury and the effect of ischemic preconditioning

In order to determine the effect of hypercholesterolemia on cardiac adaptation to ischemic stress, male Wistar rats were fed a cholesterol-enriched diet to induce hypercholesterolemia. Hearts were isolated from both normocholesterolemic and hypercholesterolemic animals, and were perfused ex vivo and subjected to ischemia/reperfusion with or without ischemic preconditioning. After perfusion, infarct size was determined, and we confirmed that the infarct size limiting effect of ischemic preconditioning was attenuated in the hearts of cholesterol-fed animals when compared to normocholesterolemic controls. These results are published in: *Szabó et al., Int J Mol Sci, 2020*.

1.1.1.3. The effects of hypercholesterolemia on cardiac miRNA expression

In order to determine the effects of hypercholesterolemia on cardiac miRNA expression, a new set of experiments were performed to provide tissue samples for RNA isolation and analysis of miRNA expression by next generation sequencing. Hearts of normo- or hypercholesterolemic animas were subjected to 3 different *ex vivo* perfusion protocols: i) short term basal perfusion, ii) 35 min global ischemia followed by 2h reperfusion, or iii) ischemic preconditioning + ischemia/reperfusion protocols. There were no remarkable changes in basal expression of cardiac miRNAs in the hypercholesterolemic vs. normocholesterolemic groups based on next generation sequencing data. However, since we have previously described that decrease of cardiac miR-25 in hypercholesterolemia may lead to oxidative stress, we decided to prepare a review article on the role of miR-25 in various diseases (*Sárközy et al, Oncotarget, 2018*).

More importantly, the ischemic preconditioning protocol caused significant alterations in the expression of several miRNAs compared to the ischemia/reperfusion group (Figure 2A). Interestingly, the pattern of miRNAs showing altered expression due to preconditioning has been markedly affected by hypercholesterolemia (Figure 2B). These alterations in miRNA expression levels may be responsible at least in part for the attenuated cardioprotection elicited by preconditioning in hypercholesterolemia. In line with these, we have demonstrated here that the expression of miR-125b-1-3p, a protectomiR previously shown to be associated with ischemic preconditioning, was increased due to preconditioning in normocholesterolemic hearts, however, this altered expression due to preconditioning was abolished in hypercholesterolemia. These latter results have been published in: *Szabó et al., Int J Mol Sci, 2020*. Moreover, we are currently preparing a manuscript on the overall effect of preconditioning on cardiac miRNA expression in both normo- and hypercholesterolemic animals and plan to submit the manuscript for publication in the first half of 2021.

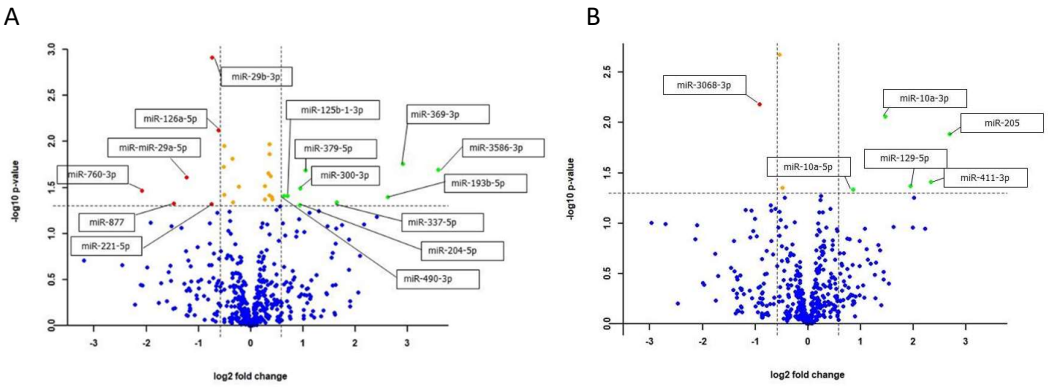

Figure 2. *Differentially expressed cardiac miRNAs after ischemic preconditioning compared to ischemia/reperfusion in hearts from normocholesterolemic (A) or hypercholesterolemic (B) rats. Each dot reperesents an identified miRNA. Red and green dots show miRs with significantly dowregulated and upregulated expression, respectively. Yellow dots are miRNAs with p < 0.05, but with fold change less than our cut-off value. Blue dots are miRNAs without significant expression change among the groups.*

1.1.1.4. The effects of hypercholesterolemia on cardiac mRNA expression

To assess cardiac mRNA expression, next generation sequencing was performed in the same experimental groups described in the previous section. Hypercholesterolemia altered the basal cardiac expression of 54 transcripts. Some of these transcripts were related to cholesterol homeostasis, cardiac contractile function, or redox homeostasis.

Transcriptomic alterations in response to ischemic preconditioning were analyzed in normo- and hypercholesterolemic hearts compared to their respective ischemia/reperfusion control groups. In our experimental setup 41 differentially expressed genes were identified due to preconditioning in normocholesterolemic hearts. Interestingly, preconditioning significantly altered the expression of 106 genes in the hypercholesterolemic myocardium compared to the respective ischemia/reperfusion group. However, the transcripts showing altered expression due to preconditioning in normocholesterolemic hearts were not changed in the same manner in preconditioned hypercholesterolemic hearts (for typical patterns and examples see Table 1.).

Gene symbol	Expression change		
	NPre vs. NIR	HPre vs. HIR	HPre vs. NPre
Ccl24	↑	↑	Ø
Tnfaip6	↑	↑	Ø
Card10	↓	↓	Ø
Mcf2l	↓	↓	Ø
Sox18	↓	↓	Ø
Reg3b	↑	↓	Ø
Reg3g	↑	↓	Ø
Myl7	↑	Ø	↓
Myl4	↑	Ø	↓
Sln	↑	Ø	↓
Chd	↓	Ø	↑
Myh11	↓	Ø	↑
Acta1	Ø	↑	↑
Csf3	Ø	↑	↑
Il1rn	Ø	↑	↑
Ptgs2	Ø	↑	↑
Serpina3n	Ø	↑	↑
RT1-T24-3	Ø	↓	↓

Table 1. *Differentially expressed genes due to preconditioning in normo- & hypercholesterolemia*

Pathway enrichment analysis was carried out with gene set enrichment analysis (GSEA) to reveal potential networks in our unfiltered, whole transcriptome data. We have found that several gene sets related to endoplasmic reticulum stress were enriched in response to preconditioning in normocholesterolemia (Figure 3). GSEA analysis failed to identify any potential networks in hypercholesterolemia. These observations may suggest the implication of ER stress in preconditioning-induced cardioprotection and therefore the loss of this network in hypercholesterolemia might have contributed to the attenuated cardioprotective effect of ischemic preconditioning.

To confirm the importance of activation of ER response pathways in cardioprotection, we have treated H9c2 cells subjected to simulated ischemia/reperfusion with pharmacological inducers of ER stress. We have found that the inducers dose-dependently decreased cell death due to simulated ischemia/reperfusion. We plan to prepare a manuscript using these results in the next three months.

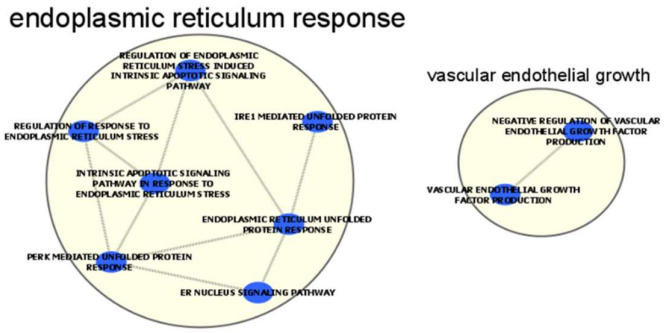

Figure 3*. Enrichment map of the major gene sets influenced by IPre in normo-cholesteorlemia. Pathway enrichment analysis was performed with gene set enrichment analysis. Blue circles represent enriched gene sets (nodes), grey lines shows the network between the gene sets (edges).*

1.1.1.5. The effects of hypercholesterolemia on the cardiac proteome

Proteomic analysis of cardiac samples was carried out by mass spectrometry. Ischemic preconditioning induced significant alterations in the myocardial level of several proteins compared to ischemia/reperfusion samples in normocholesterolemia. However, hypercholesterolemia substantially modified the influence of preconditioning on the cardiac proteome.

1.1.2. Goto-Kakizaki rat model

The Goto-Kakizaki (GK) rat is a widely used non-obese model of metabolic disease. In our study, GK rats were characterized by significantly increased cholesterol and fasting blood glucose levels, and significantly impaired glucose tolerance and insulin sensitivity as compared to controls. The cardiac gene expression profile of Goto-Kakizaki (GK) rats was determined by microarray analysis. In the hearts of GK rats, 204 genes showed significant up-regulation and 303 genes showed down-regulation as compared to controls according to microarray analysis. Detailed results have been published in Cardiovascular Diabetology (*Sárközy et al., Cardiovasc Diabetol. 2016*).

1.1.3. Fructose-enriched diet

Increased fructose intake is a characteristic component of an unhealthy diet and believed to lead to development of metabolic diseases in humans. Therefore, we have fed Wistar rats with a fructose supplemented diet for 24 weeks. We observed a mild prediabetes in this model, which was associated with cardiac dysfunction and with alterations in the myocardial proteome and lipidome including changes in cardiolipin remodeling. Detailed results are published in: *Szűcs et al., Oxid Med Cell Longev, 2019*. Since there were no significant effects on serum lipid parameters in this model, and due to limitations of financial support, we have not used this model for miRNA and/or mRNA sequencing.

1.2. Cardiac effects of chronic kidney disease

1.2.1. Chronic kidney disease model

1.2.1.1. Characterization of the model

First we have set up a rat model of chronic kidney disease and characterized the model. Chronic kidney disease was induced in male Wistar rats by partial (5/6) nephrectomy and a sham operated group served as control. After 2 months, significantly increased levels of serum urea and creatinine, urine protein, and decreased creatinine-clearance proved the development of chronic kidney disease. Transthoracic echocardiography showed the development of a mild left ventricular hypertrophy (increased wall thickness) and a mild cardiac dysfunction in CKD rats as compared to their sham operated controls. Histology confirmed left ventricular hypertrophy and fibrosis in the hearts of rats suffering from chronic kidney disease. Detailed results were published in: *Sárközy et al., Sci Rep, 2019*. In our chronic kidney disease model we have tested the effects of the angiotensin receptor blocker losartan and the beta3 agonist mirabegron on cardiac dysfunction, hypertrophy and fibrosis induced by chronic kidney disease. Losartan appeared to be more effective in exerting beneficial effects when compared to mirabegron. We have prepared a manuscript based on these data and recently submitted the paper to Disease Models & Mechanisms (*Kovacs ZZA, Szucs G, Freiwan M, Kovacs MG, Marvanykovi FM, Dinh H, Siska A, Farkas K, Kovacs F, Kriston A, Horvath P, Kovari B, Cserni BG, Cserni G, Foldesi I, Csont T, Sarkozy M. Comparison of the anti-remodeling effects of losartan and mirabegron in a rat model of uremic cardiomyopathy. Disease Models &Mechanisms. 2021. submitted*).

1.2.1.2. Ischemia/reperfusion injury and the effect of ischemic preconditioning

A set of male Wistar rats were operated in order to induce chronic kidney disease. Hearts isolated from rats with chronic kidney disease were perfused according to Langendorff to induce 35 min global ischemia and 120 min reperfusion with or without ischemic preconditioning. At the end of reperfusion the hearts were sliced and stained for determination of infarct size. Preconditioning significantly decreased infarct size in the hearts of both sham and chronic kidney disease animals. Since chronic kidney disease occurs in females at a high prevalence, we have looked at the cardioprotective effect of preconditioning in female Wistar rats suffering from chronic kidney disease. We have found that preconditioning attenuated infarct size in female rats both in the sham operated and the nefrectomized groups. Nevertheless, the extent of protection appeared to be smaller, as infarct size was significantly lower in hearts from female rats subjected to ischemia/reperfusion. While phosphorylation of STAT3 was increased in sham operated preconditioned hearts, this effect of preconditioning disappeared in chronic kidney disease. Based on these results we have prepared a manuscript and recently submitted it to Biology of Sex Differences, where it is under revision now (*Sarkozy M, Márványkövi FM, Szűcs G, Kovács ZZA, Szabó MR, Gáspár R, Siska A, Földesi I, Csont T. Ischemic preconditioning protects against ischemia-reperfusion injury in chronic kidney disease in both males and females. Biology of Sex Differences. 2021, under revision*). In addition, we have published a review article on the effect of metabolic diseases on cardiac STAT3 signaling under basal conditions or in the settings of ischemia/reperfusion with or without preconditioning (*Pipicz et al., Int J Mol Sci, 2018.*).

1.1.1.3. The effects of chronic kidney disease on cardiac miRNA expression

In order to determine the effects of chronic kidney disease on cardiac miRNA expression, RNA was isolated from left ventricular tissue and analysis of miRNA expression was performed by next generation sequencing. According to our results, 10 miRNAs were upregulated and 3 miRNAs were downregulated in the hearts due to chronic kidney disease (Figure 4.).

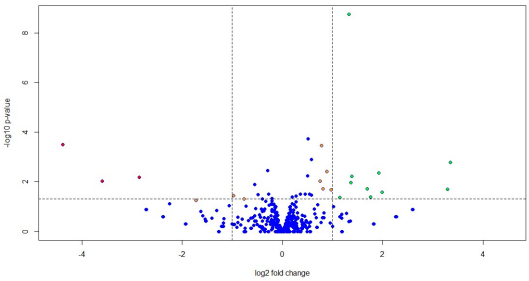

***Figure 4**. Differentially expressed cardiac miRNAs in hearts from rats with chronic kidney disease or sham operated controls. Each dot represents an identified miRNA. Red and green dots show miRs with significantly dowregulated and upregulated expression, respectively. Yellow dots are miRNAs with p < 0.05, but with fold change less than our cut-off value. Blue dots are miRNAs without significant expression change among the groups.*

Using qPCR analysis, we have also tested the expression of a few selected miRNAs and their potential targets that were suggested to be associated with myocardial hypertrophy and found that expression of miR-212 was significantly increased in the left ventricles of chronic kidney disease rats. Also western blot analysis was done for phosphorylated and total Akt, ERK, and FOXO3. Akt phosphorylation increased, while FOXO3 protein level decreased in chronic kidney disease samples. We have also investigated the role of miR-212 in CKD-induced cardiac hypertrophy, fibrosis, and cardiac dysfunction, and based on the results we have published an article in Scientific Reports (*Sarkozy et al., Sci Rep. 2019*). The same pathway has been also investigated in a side project using a model of radiation-induced heart damage where similarly to chronic kidney disease we observed cardiac hypertrophy, fibrosis, and cardiac dysfunction. These results have been published in Frontier's in Oncology (*Sarkozy et al., Front Oncol, 2019*).

The modulatory effect of ischemic preconditioning on cardiac miRNA expression in sham operated or chronic kidney disease rats was also determined and results are demonstrated on Figure 5. We are planning to prepare a manuscript using these data in the second half of 2021.

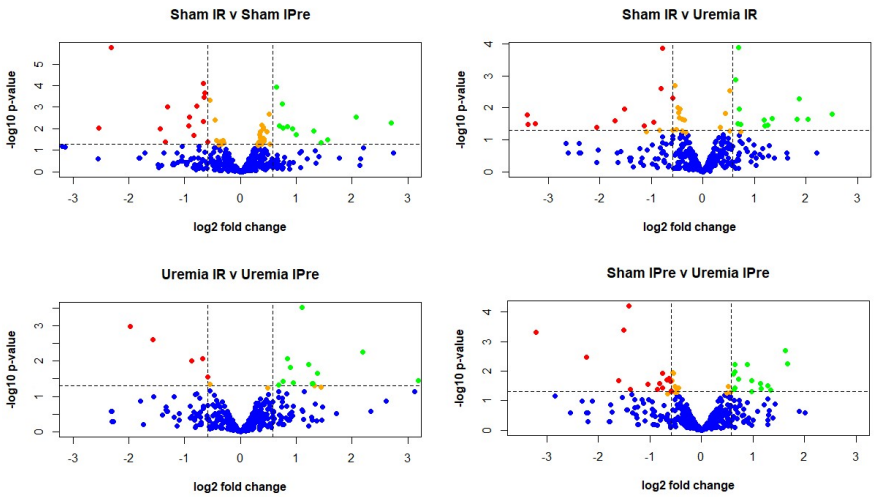

Figure 5. *Differentially expressed cardiac miRNAs after ischemic preconditioning (IPRE) in hearts from chronic kidney disease (Uremia) or sham operated rats. Each dot reperesents an identified miRNA. Red and green dots show miRs with significantly dowregulated and upregulated expression, respectively. Yellow dots are miRNAs with p < 0.05, but with fold change less than our cut-off value. Blue dots are miRNAs without significant expression change among the groups. IR: ischemia/reperfusion.*

1.1.1.4. The effects of chronic kidney disease on cardiac mRNA expression

Cardiac gene expression was assessed at the transcript level in the hearts of sham operated or nefrectomized rats. Ischemic preconditioning resulted in a differential expression of 108 cardiac mRNAs in the sham operated animals, and 261 mRNAs in the animals with chronic kidney disease compared to the respective ischemia/reperfusion controls. Moreover, expression of 245 transcripts were different between the preconditioned sham operated and chronic kidney disease groups. These results may suggest that the preconditioning-induced cardioprotection in chronic kidney disease likely involve – at least in part - different molecular mechanism as seen in control subjects. We are planning to prepare a manuscript based on these data in the last quarter of 2021.

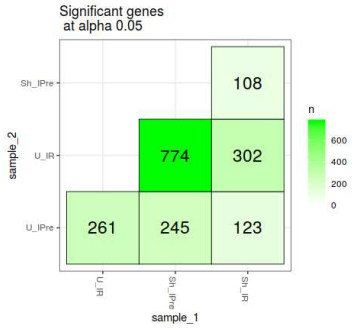

Table 1. *Differentially expressed genes due to preconditioning (IPre) and ischemia/reperfusion (IR) in sham operated and chronic kidney disease rats*

2. SUMMARY OF SCIENTIFIC OUTPUT

After completion of the research program, to date, we have published 11 *in extenso* articles related to the current project in internationally recognized scientific journals (9 of them are Q1 according to SJR). Cumulative impact factor of these publications are approximately 40, and these papers received more than 220 citations in the last 5 years according to Google Scholar. In addition, currently we do have 2 more papers submitted to scientific journals for evaluation, one of them is under revision. Moreover, we are planning to submit at least 3 new manuscripts in this year based on the findings of the present project. On the long run, several other original manuscripts or review articles related to the current project can be and will be published. Poster or oral presentations of the results on scientific conferences were also done, however, these were somewhat limited in the last year due to the COVID-19 pandemic. Besides scientific publications, these results have been and will be used for the preparation of several PhD thesis or diploma thesis. A number of undergraduate research (TDK) presentations have been delivered and several prizes were collected on the local and national students' undergraduate research conferences.

An unfortunate trend we experienced during the supporting period is that purchasing operations at our University became extremely slow due to exaggerated bureaucracy. This caused severe difficulties in planning chronic experiments (e.g. in case of animals!) and resulted in some delays in their execution. We just hope that this trend will be reversed in the future. Obviously the COVID-19 pandemic also caused some problems during the last year of the project. There were periods when PhD students and undergraduate students were not allowed to visit the buildings of the University, purchasing and administration further slowed down, and some separated incidents of COVID infections also occurred. Despite these difficulties we still think that the project is a successful one, and the financial support helped our laboratory to reach our goals.

3. RESEARCH SITES AND PARTICIPANTS

All animal experiments, heart perfusions, cell culture experiments, western blots, and some of the PCR measurements were performed in the laboratory led by the PI in the Department of Biochemistry, Faculty of Medicine, University of Szeged. The participants listed in the funding contract (Csaba Csonka, Márta Sárközy, Renáta Gáspár, Márton Pipicz, Kamilla Gömöri) were involved in these research activities with some minor changes. Kamilla Gömöri changed workplace and left the project after 18 months, and she was replaced by new researchers temporarily joining to the project (László Gruber-Nagy, Erzsébet Benkő, Bella Bruszel). Also there were 5 PhD students showing substantial or partial contribution to this project (Márton Szabó, Virág Demján, Andrea Sója, Zsuzsanna Kovács, Petra Diószegi), and it should be noted that at the time of application it was not possible to involve PhD students in the project as participants with FTE values. However, in reality their contribution is very significant and important. In addition to researchers and PhD students, 5 undergraduate medical students were also involved in the project. Unfortunately, it is evident by now that we will have no chance to motivate these students to carry on a scientific track in the future. This is mainly due to the humiliating salaries in the research career especially when compared to the recently updated salaries of doctors working in patient care. Immediate actions should be taken in order to prevent the loss of the most promising talents from the scientific field and make the research career more attractive in Hungary.

Next generation sequencing was performed in the Department of Biochemistry and Molecular Biology, Faculty of Science and Informatics, University of Szeged with the help of Nóra Zsindely and László Bodai. Renáta Gáspár and Márton Szabó, members of the PI's laboratory, also contributed to these measurements.

Some other measurements were done in collaborations. Some of the serum laboratory parameters were determined in the Department of Laboratory Medicine, Faculty of Medicine, University of Szeged with the help of Andrea Siska and Imre Földesi. Lipidomic profiling was carried out in the Biological Research Center with the help of Gábor Balogh, Mária Péter, and László Vígh. Proteomic analysis was performed in the Department of Medicinal Chemistry, Faculty of Medicine, University of Szeged with the help of Zoltán Szabó, Tamás Janáky, and Bella Bruszel who was temporarily hired part-time with the support of the current project. Histology was performed with the help of Bence Kővári and Gábor Cserni. Microarray analysis and some PCR measurements were performed in the Biological Research Center by Ágnes Zvara and László Puskás.