Author's response to reviews

Title: Transcriptional profiling of left ventricle and peripheral blood mononuclear cells in a rat model of postinfarction heart failure

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Author's response to reviews: see over
Dear Dr. Ng,

Thank you very much for considering a revision of our submitted manuscript. Following your suggestion we have now included a molecular interaction network analysis to elucidate tetraspanin 12 association with myocardial infarction and cardiovascular system dysfunction. The following revisions have been made to the manuscript and all the Reviewers’ comments have been addressed (using the Reviewers’ numbering system):

Reviewer 1

We would like to thank the Reviewer for helpful comments which greatly improved and clarified the manuscript.

1. The explanation how the altered tetraspanin 12 expression in PBMCs is associated with myocardial infarction has been added in Discussion, pages 12 and 13. An additional analysis of molecular networks by Ingenuity Pathway Analysis software was performed and Figure 5 has been added in Discussion. We analyzed functional interactions between differentially expressed genes in PBMCs and showed that tetraspanin 12 is involved in a network classified as “Cardiovascular System Development and Function, Cellular Function and Maintenance, Cellular Growth and Proliferation”. Since expression levels of many molecules present in this network have actually been found to be changed, it is likely that they are connected with each
other and that their changes may represent a response to the myocardial infarction and heart failure. We discuss also other published findings that directly connect Tspan12 and functions of membrane metalloproteinases, which could explain their involvement in cardiac hypertrophy. We also propose that Tspan12 may be downregulated in the peripheral blood mononuclear cells due to neurohumoral activation associated with heart remodeling after large-size myocardial infarction. Increased wall stress and increased plasma norepinephrine level result in activation of the renin-angiotensin-aldosteron system (RAAS) followed by increase of renin and angiotensin II plasma levels. This activation is associated with increased plasma levels of vasopressin and endothelin. It is possible that these neurohormones circulating in the plasma might influence expression of the various genes in PMBCs, through activation of the receptors present on the surface of these cells. Moreover, activated mononuclear cells are recruited to the infarcted myocardium where altered expression of Tspan12 may affect processes related to left ventricular remodelling and heart failure. This may explain why Tspan12 is downregulated only in the peripheral blood mononuclear cells, but not in the heart.

2. To address the Reviewer’s criticism on the ceruloplasmin upregulation in PBMCs the 7th paragraph in Discussion, pages 11 and 12, has been reconstructed. Although ceruloplasmin is synthesized primarily by hepatocytes, its production by activated mononuclear cells has also been reported. Here we showed that both PBMCs and failing myocardium may be a major source of ceruloplasmin that is subsequently found in serum of patients with heart failure. After completion of our work several studies have been published showing a correlations between serum ceruloplasmin levels and the extent of heart failure. Although the direct mechanism connecting ceruloplasmin and pathogenesis of heart failure remains unclear, upregulation of CP may be important in the progression of heart failure due to its property of an acute phase protein, antioxidant function and regulation of nitric oxide homeostasis.
Reviewer 2

We would like to thank the Reviewer for the insightful comments and the assessment of the analysis.

1. A comment on the selection of reference genes used in RT-qPCR reactions has been added in Materials and Methods, page 6. For evaluation of stable reference genes we performed a comparison of four candidate genes recommended in the literature, hypoxanthine-guanine phosphoribosyltransferase (\textit{Hprt1}), glyceraldehyde-3-phosphate dehydrogenase (\textit{Gapdh}), TATA box binding protein (\textit{Tbp}) and polymerase (RNA) II (DNA directed) polypeptide A, transcript variant 2 (\textit{Polr2a}). A GeNorm algorithm-based selection has revealed the following gene stability values (M-value; the highest M-value means the least stable transcript): \textit{Hprt} and \textit{Gapdh} –0.464, \textit{Tbp} – 0.615, \textit{Polr2a} – 0.692. For this reason, the expression data were normalized to the \textit{Gapdh} and \textit{Hprt1} genes.

2. The RT-qPCR reactions were done in triplicates from a single RT reaction. We agree that duplicate cDNA synthesis is recommended when mRNA quantification is the major aim by itself, but please notice that our RT-qPCR was only performed for validation of the results of the microarrays analysis, on which results we build the whole paper. We obtained an excellent correlation between the microarray and RT-qPCR results for the selected gene set. Nevertheless, following the Referees’ suggestion to better support our main results we performed a second cDNA synthesis with the same primers, reaction conditions and total amount of RNA. The most important results are presented in the table below:
### Results from RT-qPCR

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>GenBank ID</th>
<th>Fold Change I cDNA synthesis</th>
<th>P-value</th>
<th>Significance</th>
<th>Fold Change II cDNA synthesis</th>
<th>P-value</th>
<th>Significance</th>
<th>Fold Change I cDNA synthesis</th>
<th>P-value</th>
<th>Significance</th>
<th>Fold Change II cDNA synthesis</th>
<th>P-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp</td>
<td>NM_012532</td>
<td>2.5</td>
<td>0.034</td>
<td>*</td>
<td>2.3</td>
<td>0.039</td>
<td>*</td>
<td>6.2</td>
<td>0.001</td>
<td>**</td>
<td>3.3</td>
<td>0.001</td>
<td>**</td>
</tr>
<tr>
<td>Tspan12</td>
<td>NM_001015026</td>
<td>-2.7</td>
<td>0.022</td>
<td>*</td>
<td>-3.22</td>
<td>0.019</td>
<td>*</td>
<td>1</td>
<td>0.992</td>
<td>ns</td>
<td>1.1</td>
<td>0.734</td>
<td>ns</td>
</tr>
</tbody>
</table>

As can clearly be seen, the overall agreement between the present and previous results is satisfactory.

3. The explanation how our RT-qPCR procedure adheres to the MIQE guidelines has been added in Materials and Methods, page 6. We made sure that the main points [design of the experiment, sample collection, RNA preparation (DNA digestion), quality and storage, and also the reverse transcription (primers, removing DNA contamination, -RT control), primer design (localization, *in silico* specificity screen using BLAST) and qPCR normalization (no-template control, melting curve, gel electrophoresis, dilution curve, PCR efficiency), indication of the statistical methods for analysis of the results’ significance] followed the MIQE-guidelines (Taylor S; Methods; 2010;50(4):S1-5). We have also adjusted the nomenclature used to the MIQE guidelines (e.g., qRT-PCR has been changed to RT-qPCR, housekeeping gene to reference gene).

4. We agree with the Referee’s opinion that at least a duplicate cDNA synthesis should be done to evaluate the expression levels of genes with small (<2) fold-changes. The main goal of our RT-qPCR analysis, however, was the validation of results from the global profiling and confirming similarities between tissues (myocardium, PBMCs). That global analysis indicated only five candidate genes as possible heart failure markers, two of which were also
significantly changed in the PBMCs, both more than two-fold (Cp, Tspan12). The additional cDNA synthesis and subsequent qPCR strengthened that conclusion.

Minor Essential Revisions:

The spelling of the word “hybridization” on page 5 has been corrected.

The last sentence in Conclusions, page 13, has been removed.

Discretionary Revisions:

We have included a PCA plot of the gene expression data from PBMCs as Figure 4 in Results.

In addition, 11 new references have been added and references 21-42 and 45 renumbered accordingly.

We hope that with these changes the manuscript will be acceptable for publication in your Journal. We look forward to hearing from you,

yours sincerely,

Beata Burzynska

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