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Reviewer A

The authors describe new cellular signaling pathways involved in right heart failure using a translational study design by performing transcriptome analysis of right ventricular tissue from adults with complex congenital heart disease and clinical signs of right ventricular stress.

Because of the lack of healthy myocardial tissue, the authors are comparing RNA sequencing results from affected patients with data obtained from a cohort of infants with similar underlying structural heart disease but without long-standing right ventricular hemodynamic stressors.

While this causes bias in interpreting data correctly, I still believe that the authors findings serve as basis for further and focused investigations of the signaling pathways described and contribute to the field of congenital heart disease.

Writing and language are excellent. However, following revisions are recommended prior to publication:

Comment 1:

Can the authors explain more in detail why comparison with myocardial tissue from age-matched healthy patients cannot be performed by using post-mortem samples obtained from the department of pathology?

Reply 1:

We thank the reviewer for pointing this out and clarified this in the text by adding “The major limitation of the current study is the lack of whole transcriptome profile of RV tissue gained from healthy patients without structural heart disease and without myocardial disease state as a control group. The use of a donor heart, which is discarded for organ transplantation, would have provided an opportunity to remove healthy tissue from the right ventricle and use it as a reference material. Nevertheless, it must be noted, that not-transplantable donor hearts are often not completely healthy with exposition to abundant factors, typically changing gene expression. Besides, the rejection of a donor heart is a rarity. Also, the falsification of the results of gene sequencing by temporal issue and processing complications should also not be underestimated. Because of altered myocardial gene expression after organ ischemia and organ processing outside the body it

is not ideal to use not-transplantable donor hearts as healthy reference tissue (40).” to the Discussion on page 14, line 335.

Comment 2:

The authors are mentioning rhythm disturbances as sequelae of right ventricular remodeling. However, clinical data regarding arrhythmias are not reported in the tables.

Reply 2:

We appreciate the reviewer´s comment and agree that it is of importance to mention rhythm disturbances as sequelae of right ventricular remodeling. The electrocardiogram signs of right ventricular impairment in patient with complex congenital heart disease were taken from the scientific statement of the American Heart Association, including right-axis deviation, presence of incomplete or complete right bundle branch block, length of QRS interval, abnormal repolarization, atrial and/or ventricular tachyarrhythmias and implantation of implantable cardioverter-defibrillator (1). We modified Table 2 “Clinical parameters, cardiorenal and cardiohepatic serum markers, electrocardiogram as well as imaging parameters and preoperative medication.” and added selected parameters.

Comment 3:

In addition, it is unclear at what age corrective or palliative surgery was performed in the adult patient cohort and how long right ventricular hemodynamic stressors have been going on clinically. Authors shall add this information.

Reply 3:

We appreciate the reviewer´s suggestion and added the age corrective or palliative surgery was performed in the adult patient cohort in Table 1 “Patients characteristics of the adult and infant patient group.” Furthermore, in order to reflect the duration of hemodynamic right ventricular stressors, we added the sentence “Follow up time since corrective surgery was 34 years in median (range: 27 to 50 years) in adult patients with CCHD and RVI.” to the Results on page 8, line 180.

Comment 4:

Also, an exemplary figure depicting imaging criteria for right ventricular impairment would add value to the manuscript.

Reply 4:

We agree with the reviewer that an exemplary figure depicting criteria for right ventricular impairment would add value to the manuscript. Therefore, we added Figure 3 to the Results on page 8, line 192.

Comment 5:

Authors mention in their limitation, that genes associated with cardiac development excluded for further interpretation. Since reactivation of fetal gene programs is described in pathologic heart conditions, I want to ask the authors to show this list of genes and to discuss these results comparing to data in the literature.

Reply 5:

We thank the reviewer for pointing this out. Most fetal genes involved in cardiac development and heart maturation are abundantly expressed in fetal ventricles and become silent after birth (2). Reactivation of fetal gene expression in heart failure has been described. Thus, reactivation of fetal gene programs is potentially linked to adaptive and maladaptive changes in molecular signaling pathways during pathological cardiac remodeling. Studies have shown that members of the myocyte enhancer factor 2 (MEF2) family are expressed in both embryonic cardiac tissue and postnatal myocardium during hypertrophic remodeling (3). MEF2 proteins function as transcription factors by binding specific DNA sequences and thereby enhancing expression of a number of fetal and cardiac genes, including natriuretic peptide A (*NPPA*), skeletal alpha actin (*ACTA1*), desmin (*DES*) and dystrophin (*DMD*). Beside other *MEF2* genes, *MEF2C* has been detected during early cardiac development, simultaneously with the beginning of myocyte differentiation. In murine embryogenesis, targeted disruption of *MEF2C* causes inhibition of cardiac looping and right ventricular formation, leading to early death. In postnatal hearts, *MEF2C* depletion attenuated hypertrophic cardiac growth as well as the upregulation of *NPPA* as a consequence of pressure overload. Concomitant to previous observations the expression of *MEF2C* (p-value 0.043), *NPPA* (p-value 0.0062), *ACTA1* (p-value 0.0000002), *DES* (p-value 6.67E-12) and *DMD* (p-value 0.035) were found to be upregulated in our study, reinforcing the suggestion of reactivation of fetal gene programs in pathological heart conditions. Remarkably, improvement of ventricular function in heart failing patients was shown to be accompanied by decreased expression of fetal gene programs, confirming suggestions of fetal reprogramming. Reactivation of fetal gene programs in the adult failing heart remains a complex process of transcriptional, posttranscriptional and epigenetic regulatory mechanism.

We modified the manuscript as advised and added the following summarizing paragraph to the Discussion on page 15, line 360: “Studies have shown that members of the myocyte enhancer factor 2 (*MEF2*) family are expressed in both embryonic cardiac tissue and postnatal hypertrophic cardiac remodeling (43). MEF2 proteins such as MEF2C function as transcription factors, increasing expression of certain fetal and cardiac genes, including natriuretic peptide A (*NPPA*), skeletal alpha actin (*ACTA1*), desmin (*DES*) and dystrophin (*DMD*). Concomitant to previous observations expression of *MEF2C*, *NPPA*, *ACTA1*, *DES* and *DMD* were found to be upregulated in our study, reinforcing the suggestion of reactivation of fetal gene programs in pathological heart conditions (Online Table 10).”.
Furthermore, we added Online Table 10, which presents a list of reactivated fetal genes, including p value, adjusted p value and expression.

Reviewer B

The authors Pollmann et al. describe new molecular signaling pathways in right ventricular impairment of adult patients after tetralogy of Fallot (TOF) repair. The authors collected cardiac tissue of the right ventricle during cardiac surgery from adult patients and infants with TOF, which they used as a control group. They performed RNA Seq and identified 3.010 differentially expressed genes in the adult group compared to the infant group. Significantly enriched genes were involved in cellular metabolism, cell-cell communication, cell cycling and cellular contractility. Different genes were picked out and examined more closely using Western blot analysis. The authors suggest that the results can be useful for the discovery of biomarkers for disease progression and of novel therapeutic targets.
The manuscript is well written. The authors are aware of the limitation of the study due to the lack of appropriate healthy control tissue. But they were able to show that the individual groups were homogeneous and clearly distinguished from the other group based on the parameters collected.

Comment 1:

Since drugs can also influence gene expression, it would be helpful to list the medication of each patient before the heart tissue is removed and discuss any differences regarding the gene expression between the two groups.

Reply 1:

We agree with the reviewer that medication might also influence cardiac gene expression. We reviewed medical charts for preoperative medication and added this information to Table 2 “Clinical parameters, cardiorenal and cardiohepatic serum markers,

electrocardiogram as well as imaging parameters and preoperative medication.”. However, after discussing with bioinformatical experts, we deemed statistical evaluation of RNA sequencing data comparing two versus four patients not sufficient to reach efficient power and therefore did not include this analysis in our study. We added this limitation to the Discussion on page 16, line 379: “Two of the six adult patients were on cardiac medication at the time of study. The influence of this on gene expression in unknown and due to small sample size, we were not able to focus on this in the current analysis.”. Unfortunately, we were unable to find relevant data in the literature regarding the influence of cardiac medication on myocardial gene expression. However, this field is highly interesting and definitely warrants further investigations.

Comment 2:

Furthermore, it should be noted that the adult group consists almost exclusively of women (6/7), while the infant group is dominated by males. To what extent this has an influence on gene expression is questionable, but it should at least be discussed.

Reply 2:

We very much agree with the reviewer that the distribution of female and male patients in both groups should be noted. Unfortunately, in our cohort gender-matched samples of patients with CCHD and RVI for gene expression analysis was not possible due to limited availability of right ventricular tissue samples from patient meeting the inclusion criteria. Therefore, we added the following sentences to the Discussion on page 15, line 371: “Another limitation is the unequal distribution of female and male patients in the infant and adult patient group. While the infant patient group is dominated by males (6/7), the adult patient group is composed of more females (5/6). The gender disparity might have an impact on gender specific alterations in gene expression, which makes transferability more difficult. However, whereas there are contradictory data regarding gender differences in gene expression of the left ventricular myocardium in human (44) and animal (45) studies, to our knowledge the impact of gender on mRNA expression in human RV heart tissue has not been described so far in the literature.”.

Comment 3:

The conclusion that genes regarding mitotic cell cycle, cell division and DNA replication play an important role in the disease mechanism is very daring. Since the control group consists of samples from children at the age of around 5 months, it can be assumed that the growth phase of the heart is not yet completed there and therefore the genes for

mitotic cell cycle, cell division and DNA replication are upregulated. It is therefore not surprising that these genes are downregulated compared to the adult samples. For this reason, it is questionable whether an association with the disease can really be deduced from this. It would therefore be good to compare the data once again with gene expression data from cardiac tissue of adults and infants in the literature and then to discuss the issue once again under this aspect.

Reply 3:

We thank the reviewer for pointing this out. As described in literature, differentiated cells such as cardiomyocytes typically become post-mitotic and exit the cell cycle postnatal (4). Since we compared adult with infant patients, downregulation of cell cycle in adult patients might not be surprising due to the fact, that cardiac myocytes rapidly proliferate during fetal period and remain in permanent cell cycle arrest as terminally differentiated adult cardiomyocytes (5). However, in our study we analyzed differentially expressed genes in RV tissue samples of adult patient with CCHD and RVI, facing long-term hemodynamic stressors. Study results could show that the stressed myocardium retains the incapability to re-enter cell cycle as a potential adaptive strategy under pathological conditions. These findings are in line with recent data from the literature describing the very limited potential for myocardial regeneration after cardiac injuries (5). The identification of potentially capable cardiac stem cells for differentiation into cardiomyocytes and small cardiomyocytes reentering cell cycle has questioned the incapability of reentering cell cycle as adaptive response in order to restore function in stressed cardiac tissue. Because of only minimal effects, the lack of adequate models and techniques to detect cell cycle reentry, experts still belief that proliferative potential of cardiomyocytes and cardiac stem cells remain insufficient for myocardial regeneration after injury. However, the underlying mechanisms of mitotic cell cycle and differentiation potential in patients with CCHD facing adverse cardiac remodeling is poorly understood and requires further research. Probably our initial wording might be misleading not having clearly pointed out the topic precisely. Therefore, we clarified this by modifying the Discussion on page 13, line 311 accordingly:

“During the development of RV remodeling and RVI, RNA sequencing revealed a marked adjustment of gene expression, regarding mitotic cell cycle, cell division and DNA replication. Downregulation of differentially expressed genes, associated with DNA binding transcription factor activity, transcription regulator activity as well as RNA polymerase 2 transcription factor activity, support the assumption of decreased cell cycling in stressed RV myocardium of adult patients with signs of RVI. In line with above findings, reduction of gene expression with respect to nucleus, chromosome, protein-DNA complex, spindle and

replication fork strengthen suspicion of declined cell proliferation and cell maintenance in cardiac remodeling. Downregulation of cell cycle leads to a fatal lack of myocardial regeneration, due to failing proliferation of normal functioning cardiomyocytes (35). Experimental research determined cardiac loss of ability to reentry cell cycle, since differentiated cells such as adult cardiomyocytes typically become post-mitotic and exit the cell cycle postnatal (36) (37). The present study indicates that stressed myocardium retains the incapability to re-enter cell cycle in patients with CCHD and RVI, facing adverse cardiac remodeling. Cardiac tissue consequently encounters difficulties in adaption to pathologic conditions such as pressure and volume overload, which results in impaired cardiac injury repair and hypertrophy, leading to increased risk of cardiac morbidity and mortality (38) (39). The incapacity to enter cell cycle, guaranteeing cell proliferation, injury regeneration and preservation of ventricular function, aggravate pathophysiologic conditions and encourage development of RV remodeling and RVI. The prevention of cardiomyocytes to exit cell cycle and become post-mitotic could be a promising approach for innovative therapeutic strategies, in order to improve cardiac regeneration.”.