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Integrated Analysis of miRNA-mRNA Interaction in Pediatric Dilated Cardiomyopathy

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Abstract

Background—microRNAs (miRNAs) are short single stranded nucleotides that can regulate gene expression. Although we previously evaluated expression of miRNAs in pediatric dilated cardiomyopathy (DCM) by miRNA array, pathway prediction based on changes in mRNA expression has not been previous analyzed in this population. The current study aimed to determine regulation of miRNA expression by miRNA-Seq and, through mRNA-Seq, analyze their putative target genes and altered pathways in pediatric DCM hearts.

Methods—miRNA expression was determined by miRNA-Seq [n=10 non-failing (NF), n=20 DCM]. Expression of a subset of miRNAs was evaluated in adult DCM patients (n=11 NF, n=13 DCM). miRNA-mRNA prediction analysis was performed using mRNA-Seq data (n=7 NF, n=7 DCM) from matched samples.

Results—Expression of 393 miRNAs was significantly different ($p<0.05$) in pediatric DCM patients compared to NF controls. TargetScan-based miRNA-mRNA analysis revealed 808 significantly inversely expressed genes. Functional analysis suggests up-regulated pathways related regulation of stem cell differentiation and cardiac muscle contraction, and down-regulated pathways related to regulation of protein phosphorylation, signal transduction and cell communication.

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Conclusion—Our results demonstrated a unique age-dependent regulation of miRNAs and their putative target genes which may contribute to distinctive phenotypic characteristics of DCM in children.

Introduction

Dilated cardiomyopathy (DCM) is the most common type of cardiomyopathy and is characterized by ventricular chamber enlargement and contractile dysfunction (1, 2). DCM often progresses to heart failure (HF) in children as well as in adults (3). End-stage HF due to DCM is the most common indication for cardiac transplantation in children over the age of 1 (4, 5). Although DCM affects both adults and children, the etiologies are varied, with ischemic heart disease being the most frequent cause of HF in adults, whereas in children the most common cause of DCM is idiopathic (6). The outcomes of children with DCM remain poor, with 50% of pediatric patients dying or needing cardiac transplantation within 5 years of diagnosis (6). Current therapies have resulted in a significant improvement in morbidity and mortality in adults with DCM (7). Therapeutic practices in children with DCM are based on adult patient guidelines (8). However, medical therapies such as β-blockers that are proven effective in adult DCM patients, showed no significant improvement in clinical outcomes in children (9), and transplant-free survival has only minimally improved in children with DCM (10).

Our studies have demonstrated unique myocellular characteristics of the pediatric DCM heart (4, 11–13), including age-specific differences in microRNA (miRNA, miR) expression (14). miRNAs are regulatory molecules consisting of ~22 noncoding nucleotides that regulate gene expression by targeting messenger RNAs (mRNAs) resulting in mRNA degradation or translational suppression of targeted transcripts (15). Recent evidence suggests that miRNAs can modulate the expression of multiple target genes and networks related to physiological (16) and pathological processes in the heart (17), making them an important contributor to cardiovascular diseases (9). In the current study, we investigated the expression profile of miRNAs in the left ventricular tissue (LV) of pediatric DCM patients and age-matched non-failing (NF) controls by RNA-Seq. Using our previously published mRNA-Seq data (13), we performed stringent statistical analyses and machine learning tools to identify potential targets of differentially expressed miRNAs. Using Ingenuity Pathway Analysis (IPA) we identified signaling pathways related to these putative target genes including, signal transduce and activator of transcription 3 (STAT3) signaling, cardiac hypertrophy signaling, extracellular signal-regulated kinases or mitogen-activated protein kinases (ERK/MAPK) signaling, extracellular signal-regulated kinases 5 (ERK5) signaling, C-C chemokine receptor type 3 (CCR3) signaling, C-X-C chemokine receptor type 4 (CXCR4) signaling, and Hippo-signaling.

Materials and Methods

Human Tissue Samples

All subjects gave informed consent and donated their hearts to the institutional review board-approved Investigations of Pediatric or Adult heart tissue bank at the University of Colorado, Denver. NF LV tissue was from organ donors with normal heart structure

and function, whose hearts could not be placed for technical reasons (size or blood type mismatch). DCM LV tissue was from patients transplanted due to DCM of LV morphology who were transplanted secondary to HF. Inclusion criteria for pediatric DCM heart disease patients were age <18 years, presence of non-ischemic DCM without any definitive contributing comorbidity and ejection fraction < 50%. Characteristics of pediatric patients (n=13 NF and n=36 DCM, <18 years of age) and adult patients (n=13 NF and n=11 DCM and, ≥40 years of age) are listed in supplemental Tables S1 and S2 respectively. At the time of cardiac transplantation or donation, the LV was rapidly dissected in the operating room, flash-frozen, and stored at −80°C until further use.

miRNA Isolation

miRNA was extracted as per the manufacturer's protocol. Human LV samples were homogenized in QIAzol, and miRNA was extracted from pediatric LV tissue using mirVana Kit (Ambion) (n=13 NF and 28 DCM samples) or miRNeasy plus mini kit (Qiagen) (n=8 DCM samples). mirVana was used for extracting miRNA from adult LV tissue (n=13 NF and n=11 DCM). RNA quality and quantity were measured using a nanodrop spectrophotometer (ND-2000, Thermo Scientific) and the Agilent Bioanalyzer Nano RNA chip. Only samples with RNA integrity number (RIN) > 8 were used for RNA-Seq.

miRNA-Seq

Read quality was confirmed using FastQC (http://www.bioinformatics). All known mature miRNAs and hairpins were downloaded from miRBase (http://www.mirbase.org/). Currently, there are 2,588 mature miRNAs (human) in the most recent miRBase release 21. All adapter sequences were trimmed from raw sequencing files and the reads were mapped to the mature miRNAs using Bowtie (18). Counts estimates were calculated using SAM tools (http://samtools.sourceforge.) and normalized using edgeR (19) (n = 20 DCM and 10 NF pediatric hearts).

miRNA RT-qPCR

Expression of selected miRNAs was evaluated by RT-qPCR in pediatric (n=12 NF, n=30 DCM) and adult DCM patients (n=13 NF and n=11 DCM) (see Supplementary Tables S1 and S2). miRNA expression was measured by RT-qPCR using SYBR Green (miScript SYBR Green PCR kit Qiagen, Inc) and the miScript Universal Primer along with miRNA-specific primers. cDNA was synthesized using the Qiagen miScript II cDNA synthesis (Qiagen) according to manufacturer's instructions and as previously described (20). The PCR reactions were carried out in a final volume of 10 μl consisting of 1.25 ng cDNA in a Quant Studio 7 Flex. miRNA expression was normalized to 18s. miRNA primers are listed in Table S3.

Data analysis

The top 100 miRNAs by count values from DCM samples were selected for further analysis. t-test was used to compare the log2 normalized miRNA counts between DCM and NF patients. Pairwise Spearman correlation analysis between the significant 45 miRNAs and 6,273 mRNAs was calculated. miRNA targets were downloaded from TargetScan

(http://www.targetscan.org/) release 7.2, March 2018. Only targets that were significantly differentially regulated by mRNA-Seq (13) were selected for further analysis.

For each of the 45 significant miRNAs, predicted targets were filtered out as more reliable due to a significant negative correlation ($p < 0.05$). The miRNA-Seq data used for correlation analysis was from 7 NF and 7 pediatric DCM patients that matched the mRNA-Seq data. One proportion test was performed for each of the miRNAs, and only miRNAs whose predicted targets were significant by one proportion test (significantly inversely correlated targets subtracted from total significantly correlated targets over the total significantly correlated mRNA targets) were selected for further analysis (https://www.medcalc.org/calc/test_one_proportion.php). Significant miRNAs and targets were further evaluated by performing functional analysis. All analyses were performed in R.

Gene Ontology (GO) categorization was performed using PANTHER to identify the biological process associated with DCM and NF control LV tissue. Genes which were predicted targets of significantly dysregulated miRNAs and filtered by Spearman correlation analysis were used. Up-regulated and down-regulated genes were uploaded to PANTHER separately and analyzed with Fisher's exact test using the 6,273 genes we previously identified in the heart of pediatric DCM patients and NF controls with FPKM>1 as our reference list (13).

Ingenuity Pathway Analysis (IPA) was performed to investigate molecular pathways and toxicity functions associated with DCM by comparing RNA-seq-generated transcriptomes of pediatric DCM and NF control LV tissue. Genes which were predicted targets of the significantly dysregulated miRNAs and filtered by Spearman correlation analysis, were uploaded to IPA. Similarly, the 45 significantly differentially regulated miRNAs in pediatric DCM patients were analyzed using IPA to compare miRNA-Seq data to miRNA-Seq-mRNA-Seq predicted targets.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism software. Welch's t-test was used when comparing NF and DCM groups. One-way Analysis of Variance (ANOVA) and multiple group comparisons were performed when analyzing the effect of sex. Consistent with American Physiological Society recommendations on studies that include higher-level mammals and the goal of identifying putative mechanisms that could lead to further studies, statistical significance was set a priori at $P<0.1$ when evaluating sex differences, and all data are presented as mean ± SEM in the figures (21).

Results

Patient Characteristics

Characteristics of pediatric and adult patients are listed in Supplementary Tables S1 and S2 respectively. The median age for pediatric NF donors was 8.6 years with an interquartile range (IQR) of 9.4 years, and a median age of 4.3 years with an IQR of 12 years for pediatric DCM patients. 54% of the pediatric NF donors and 53% of pediatric DCM patients were females. The median age for adult NF donors was 52 years with an IQR of 11 years

and 48 years with IQR of 23 years for DCM patients. 39% of the adult NF donors and 9% of adult DCM patients were females. Angiotensin converting enzyme inhibitor, beta blockers, and diuretic treatments were more commonly used in DCM patients compared to NF donors. There were no differences in the use of inotropes between NF controls and DCM patients in the pediatric or adult cohort.

Identification of differentially expressed miRNAs

We compared the expression profile of miRNAs in the LV tissue of pediatric DCM patients versus non-failing pediatric controls using miRNA seq. 2,588 miRNAs were identified. After normalization by edgeR, 393 miRNAs showed significant differential expression ($p<0.05$) (Supplementary Table S4) and www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE99321. Of the 393 differentially expressed miRNAs, 227 miRNAs were up-regulated and 166 miRNAs were down-regulated (Figure 1A). Based on the counts of the DCM samples, the top 100 miRNAs were selected. Of these, 45 miRNAs showed significant differential expression ($p<0.05$) in the heart of pediatric DCM patients compared to NF pediatric controls (Figure 1B). Based on average counts miRNAs were ranked high to low both in DCM and NF and the top 25 most highly expressed miRNAs are listed in (Figure 1C and 1D) for NF and DCM respectively.

Validation of sequencing data by RT-qPCR

To confirm the results obtained by miRNA-Seq, a subset of miRNAs significantly changed in pediatric DCM regardless of their count levels (see Supplementary Table S4) was randomly selected (miR-301a-3p, −301b-3p, −495–3p, −17–5p, −208a-5p, −193a-5p, −107, −133a-5p, −30c-5p, −1–3p and −1261), and their expression was verified by RT-qPCR from 42 pediatric LV DCM tissue (n= 12 NF and 30 DCM – see Supplementary Table S1). Consistent with miRNA-Seq, results, RT-qPCR confirmed up-regulation of four miRNAs (miR-301a-3p, miR-301b-3p, miR-495–3p, miR-107) and down-regulation of two miRNAs (miR-17–5p, miR-208a-5p) in pediatric DCM patients. Although expression of miR-193a-5p, miR-133a-5p, miR-30c-5p, miR-1 and miR-1261 was unchanged by RT-qPCR, directionality of expression was consistent with results from miRNA-Seq, (Figure 2). Sequence variation in miRNAs can happen. These so-called isomiRs may be detected by RNA-Seq but not by RT-PCR due to primer sequence mismatch. To determine if the primers used for RT-qPCR detect all isomiRs, we evaluated the contribution of isomiRs to miRNAs that did not significantly change by RT-PCR. The percentage of isomiRs predicted to be undetected by RT-PCR based on primer sequence for miR-1, miR-30c-5p and miR-133a-5p, is likely too low to explain the differences between RT-PCR and RNA-seq. However, the percentage of isomiRs likely undetected by RT-PCR for miR-193a-5p and miR-1261 was >50%, which may explain the differences in RNA-Seq and RT-PCR results (Table 4).

Additionally, we evaluated the effect of sex on miRNA expression. Interestingly, miR-495–3p and −17–5p showed differentially significant expression only in female pediatric DCM patients compared to NF. Whereas, the expression of miR-107 and −181c-5p significantly changed only in male pediatric DCM (Figure 3A). The effect of sex differences on miRNA expression in the LV tissue of pediatric DCM was confirmed by RT-qPCR. Consistent with miRNA-Seq results, RT-qPCR revealed up-regulation of miR-495–3p and down-regulation

of miR-17–5p only in female pediatric DCM patients compared to NF, and the expression of miR-107 and 181c-5p were significantly up-regulated only in male pediatric DCM (Figure 3B).

To evaluate differences in miRNA expression profile between adults and children with DCM, RT-qPCR was also performed in the LV tissue sample from adult DCM patients only for the miRNAs differentially expressed in pediatric DCM. miR-301a-3p, miR-301b-3p, miR-495–3p and miR-107 showed similar expression profiles in pediatric and adult DCM hearts. Interestingly, although miR-208a-5p was significantly different in pediatric DCM compared to NF control and adult DCM compared to NF control the directionality of expression was opposite. Moreover, the expression profile of miR-17–5p was unchanged in adult DCM patients compared to NF control..

miRNA-Seq-based target analysis

To evaluate the putative miRNA targets, we used our previously published mRNA-Seq dataset (13). 6,273 mRNAs, FPKM>1 were filtered as putative targets of the 45 significantly different miRNAs. Following Spearman correlation analysis and one proportion test, 36 significant miRNAs and 808 (163 up-regulated and 645 down-regulated) inversely significantly dysregulated target mRNAs were used for functional analysis (Supplementary Tables S5 and S6).

Functional enrichment analysis

PANTHER was used to further categorize the 808 (645 down-regulated and 163 up-regulated) putative target genes of differentially expressed miRNAs (36 miRNAs). Several biological processes based on up-regulated and down-regulated putative target genes of altered miRNAs were significantly enriched ($p<0.05$) (Table S7 and Table S8 respectively). Specifically, modulation of chemical synaptic transmission, positive regulation of stem cell differentiation, regulation of cytoplasmic translation and cardiac muscle contraction are among the top 15 significantly enriched biological processes associated with the up-regulated putative target genes (Figure 5A and B). Cellular process, regulation of protein phosphorylation, signal transduction and cell communication are also among the top 15 significantly enriched biological processes associated with the down-regulated putative target genes (Figure 5C and D). Genes in the top ten significantly enriched biological processes are listed in supplementary Table S9.

Pathway analysis using IPA identified several significantly enriched canonical pathways ($p<0.05$) associated with putative target genes of dysregulated miRNAs. Most importantly, signaling pathways such as STAT3 pathway, UVB-induced MAPK signaling pathway, ErbB signaling, cardiac hypertrophy signaling (Enhanced), ERK5 signaling, PI3K/AKT signaling, integrin signaling, HGF signaling, FAK signaling and ErbB2-ErbB3 signaling were highly significantly enriched ($p<0.001$) in the heart of pediatric DCM patients compared to NF control (Table 1). Moreover, IPA analysis also revealed multiple significantly dysregulated ($p<0.05$) cardiac-specific pathways related to cardiac function, including cardiac enlargement, cardiac inflammation, cardiac dilation, cardiac dysfunction,

arteriopathy, heart failure, congestive cardiac failure, cardiac stenosis, hypoplasia and infarction (Table 2).

In order to evaluate if pathway analysis based solely on miRNA-Seq, data would produce results similar to predicted targets from the mRNA-seq data, IPA of the 45 significantly differentially regulated miRNAs was performed. Unlike the results using putative target genes based on mRNA-seq data, IPA does not evaluate canonical pathways based solely on miRNA expression. Moreover, most of the top significantly dysregulated pathways related to cardiotoxicity function, including cardiac enlargement, cardiac dilation, cardiac proliferation, arteriopathy and heart failure were common between the two analyses. However, top cardiotoxicity functions including cardiac inflammation, cardiac hypoplasia, congestive cardiac failure, cardiac stenosis and cardiac arrhythmia were enriched only when putative target genes were used. Furthermore, cardiac fibrosis and cardiac regeneration were enriched as top cardiotoxicity functions in IPA analysis based solely on miRNA-Seq data (Table 3).

Discussion

Despite the fact that etiologies and biological factors involved in pediatric DCM are different from that of adults, treatment of pediatric patients have highly relied on adult guidelines (6). Our previous studies have clearly shown unique myocellular characteristics of pediatric DCM patients that are different from adults (4, 11, 12), including an age-specific miRNA expression profile (14). Moreover, we have recently identified a unique cardiac gene expression profile in pediatric DCM patients (13). Since miRNAs can target expression of mRNAs from more than 60% of protein-coding genes (22, 23), we evaluated miRNA expression by miRNA-Seq, and the relationship between miRNAs and mRNAs in pediatric DCM hearts. miRNAs play an important role in the cardiovascular system by regulating cardiomyocyte growth and contractility together with the development and maintenance of cardiac rhythm (24). Moreover, several studies have implicated altered miRNA expression in cardiac hypertrophy, dysfunction as well as heart failure (25, 26). In this study, we identified, by miRNA-Seq, 393 miRNAs significantly dysregulated in pediatric DCM hearts when compared to NF controls. Furthermore, by using mRNA-Seq data, we investigated biological pathways potentially regulated by these miRNAs.

In our previous study, we investigated miRNA expression by miRNA array (14). In this study, using miRNA-Seq, we limited our functional analysis to the top 100 expressed miRNAs (by count). Previous studies using high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) showed that only the most expressed miRNAs are likely to interact with the RISC complex, suggesting that only a subset of miRNAs has a biological role (27). Therefore, although the array studies provide important information with respect to dysregulated miRNAs, miRNA-Seq studies can more reliably be used to identify putative mRNA targets.

Upregulation of miR-301a, −301b, −107 and −495 and downregulation of miR-17–5p and −208a-5p were confirmed by RT-qPCR. In addition, although not significant, directionality of changes was the same when comparing miRNA-Seq and RT-qPCR of tested miRNAs. We

and others have previously observed differences in the significance of miRNA expression by RT-qPCR when compared to arrays (14, 20, 28–30). We investigated if isomiR expression could explain the lack of significance in the RT-PCR data. The contribution of isomiRs to miRs-1, −30c-5p and −133a-5p is not likely to alter RT-PCR results. However, miRs-193a-5p and −1261 had high levels of isomiR expression. In fact, all miR-1261 sequences were different than predicted by miRbase. It is unclear if this is an organ-specific or an age-dependent response. As the field advances, it will be important to define isomiR expression by age and organ, and the effect these isomiRs may have on gene expression. Although RT-PCR results were not significant for a subset of miRs, directionality of expression is similar, suggesting that significance may be lost due to added samples. Therefore, miRNA-Seq results were used for pathway analysis. Moreover, in support of our previous study (14), age-specific dysregulation of miR-208a-5p and −17–5p was observed when comparing changes in pediatric DCM patients vs NF controls and adult DCM patients vs NF controls. Expression of miR-208a-5p in adult DCM patients was increased, but down-regulated in pediatric DCM patients compared to NF controls. Moreover, miR-17–5p was down-regulated in pediatric DCM patients, whereas no change was observed in adults, which suggest a unique miRNA regulation in pediatric DCM patients.

Several studies have shown a strong association of miR-301 with multiple human cancers including prostate cancer, pancreatic cancer and breast cancer (31–33). However, studies on the role of miR-301 in the heart are limited. High expression of miR-301a was reported in mouse hearts from late embryonic to neonatal stages when compared to adult animals, suggesting the potential involvement of miR-301a in cardiac development, differentiation and proliferation (34). A recent study on a mouse model of DCM showed a link between downregulation of miR-301a and elevated fetal gene expression as well as contractile dysfunction without hypertrophy. Furthermore, the authors also showed neither overexpression nor inhibition of miR-301a is related to cellular hypertrophy in neonatal rat cardiomyocytes (35). Interestingly, we previously showed that hypertrophy is not observed in pediatric DCM hearts (13). In addition, increased expression of miR-301b has been reported in non-valvular atrial fibrillation patients compared to healthy controls (36), whereas inhibition of miR-301b expression in neonatal rats has been implicated in reduced ATP production and contractile dysfunction (37).

Additionally, we found increased expression of miR-107 in adult DCM hearts. Consistent with our findings, increased expression of miR-107 was observed in the arterial and coronary sinus blood samples of adult ischemic heart disease and non-ischemic dilated cardiomyopathy patients (38). Moreover, miR-107 was overexpressed in endothelial progenitor cells (EPCs) and inhibits their differentiation under hypoxic conditions via targeting HIF-1β. Similarly, upregulation of miR-495 has been implicated in various cardiac injury models (39). Moreover, upregulation of miR-495 suppressed high glucose-induced extracellular matrix accumulation of cardiac fibroblasts via targeting NOD1 (40).

Previous studies have reported association of over-expression of miR-208a-3p with increased cardiomyocyte hypertrophy and fibrosis (41, 42). However, the role of miR-208a-5p has not been evaluated in the heart. Future studies will be important to investigate the role of miR-208a-5p in the heart. Previous studies have reported a strong

association of miR-17–5p with myocardial infarction (43, 44). Increased expression of miR-17–5p was observed in the plasma of acute myocardial infarction (AMI) patients suggesting that miR-17–5p can be used as a novel AMI biomarker (43). Furthermore, downregulation of miR-17–5p increased cardiac function and reduced apoptosis in an *in vivo* acute myocardial infarction rat model (44). It is interesting that expression of miR-17–5p is decreased in the pediatric DCM heart. This could be a compensatory mechanism to cardiac dysfunction or it could be related to yet undefined roles of this miRNA in end-stage HF.

Sexual dimorphism can affect gene expression profile in heart failure patients (45, 46). However, though miRNAs have been recognized as important players in cardiac function and disease (47), studies on the relationship between miRNA expression and sex are limited. A recent study evaluated the effect of sex on the expression of some miRNAs in normal as well as ischemic cardiomyopathic human hearts (48). Similarly, in the current study, expression of several miRNAs including miR-17–5p, −107, −495–3p, and −181c-5p in pediatric DCM were sex-based. Evaluating the role of these miRNAs in a sex-specific manner will be important to understand sex-specific differences in mRNA expression. Unfortunately, we were not powered to investigate these differences since mRNA-seq had only been performed in seven NF and seven DCM samples.

Though not specifically in DCM patients, the role of miR-181c-5p in the heart has been evaluated previously (49, 50). A recent study showed hypoxia/re-oxygenation stimulated expression of miR-181c-5p in H9C2 cardiomyocytes and in an *in vivo* rat cardiac ischemia/reperfusion injury model (49). Furthermore, the increased expression of miR-181c-5p aggravates NFκB-mediated inflammation *in vivo* as well as *in vitro* (50).

Functional analysis of the predicted target genes of altered miRNAs in pediatric DCM patients indicated their involvement in various biological processes and cardiac abnormalities.. Additionally, pathway analysis of putative target genes revealed several pathways predicted to be inhibited including STAT3 signaling, ERK/MAPK signaling, ERK5 signaling, CCR3 signaling and CXCR4 signaling, and only Hippo-signaling was predicted to be activated (Supplementary Table S7). Most of these dysregulated canonical pathways have important roles in various cardiac functions and abnormalities. For instance, STAT3 has been implicated in cardiac protection mechanisms both during acute and chronic stress (51, 52). However, reduced activity of STAT3 has been linked to cardiac inflammation, remodeling, as well as end-stage heart failure (53). Moreover, complete loss of STAT3 in mitochondria shows significant inhibition of complex I and II activities together with decreased membrane potential, reduced ATP production and increased ROS generation (54, 55). Interestingly, consistent with our finding of inhibited STAT3 signaling, we have previously reported mitochondrial dysfunction in pediatric DCM patients (56) suggesting that STAT3 may be implicated in the progression of pediatric DCM.

Additionally, several studies have implicated MAPK pathways, including ERK5, in cardiac development, function and pathology (57). MAPK pathways can be activated in response to hypertrophic stimuli or stressors such as oxidative stress, hyperosmosis and radiation (58). Moreover, a recent study reported the association of dysregulated miRNA expression with

ERK5-dependent initiation of an inflammatory response in cardiac tissue leading to cardiac hypertrophy (59).

A recent report showed increased expression of chemokines and their receptors, such as CXCR4, in patients with heart failure (60). CXCR4 negatively modulates the contractile function of cardiac myocytes in response to the stimulation of the β-adrenergic receptor (61). A subsequent study also showed that complete loss of CXCR4 in a mouse model results in contractile dysfunction as a result of over-activated adrenergic pathways (62). In addition, CCR3 signaling plays a significant role in eosinophil-mediated heart damage (eosinophilic myocarditis) via the eotaxin-CCR3 pathway (63).

Lastly, Hippo signaling regulates cardiomyocyte proliferation during heart development to maintain a normal heart size in mammals (65). Several studies have reported involvement of Hippo signaling in different cardiac abnormalities including, hypertrophy, heart failure, arrhythmogenic cardiomyopathy and dilated cardiomyopathy (66). It has also been reported that the Hippo signaling pathway promotes cardiac regeneration during cardiac injury through its effector Yap (67).

Our functional analysis results revealed that miRNAs significantly altered in the heart of pediatric DCM patients target genes that are associated with pathways known to be involved in mitochondrial function, hypertrophy, inflammatory response and stem cell differentiation. More specifically, up-regulated putative target genes are involved in biological processes such as positive regulation of stem cell differentiation. Interestingly, ours and others' previous evaluation of mRNA-Seq data from pediatric DCM patients suggested a gene expression profile pattern consistent with incomplete cell differentiation, absence of cardiac hypertrophy (13), absence of an inflammatory response (64) and mitochondrial dysfunction (56). Similarly, a study by Wehman et al. showed increased number of cardiac stem cells in end-stage pediatric heart failure patients (68). Pathway prediction suggests that modulating the miRNAs that are significantly dysregulated in the heart of pediatric DCM patients may provide a potential therapeutic benefit for this population.

Finally, we compared IPA analysis of miRNA-mRNA target prediction (39 miRNAs) to miRNA-Seq data only (45 miRNAs significantly differentially regulated in the heart of pediatric DCM patients, regardless of changes in mRNA expression). Interestingly, when using all 45, but not the 39 miRNAs, IPA predicted enrichment of fibrosis. We previously showed minimal interstitial fibrosis in pediatric DCM hearts (12). This suggests that results from miRNA-Seq and mRNA-Seq interaction data are more reliable predictors of pathophysiological changes than results from miRNA-Seq only.

Conclusion

In this study, our findings revealed alteration of several miRNAs in pediatric DCM patients compared to NF control. We also showed age- and sex-specific regulation of miRNAs in pediatric DCM patients. The putative target genes of dysregulated miRNAs were involved in pathways related to cardiac toxicity. Therefore, further investigations of the implication of

dysregulated miRNAs in the hearts of children with DCM may help lead to identification of potential age-specific miRNA-based therapy.

Limitations

There are important limitations to the study. The observed changes in pediatric DCM hearts are tissue bank-based studies and these studies are cross-sectional. Moreover, we have not evaluated if the changes observed are physiological or pathological. Although we have shown several miRNAs are differentially expressed in pediatric DCM and analyzed their putative target genes predicted by TargetScan, we acknowledge that these target genes need to be confirmed. miRNA-Seq, as well as RT-qPCR were performed using heart tissue which contains not only cardiomyocytes but also endothelial cells and fibroblasts. Therefore, the observed changes in miRNA expression may not be specific to cardiomyocytes. Furthermore, since we were limited by the number of patients that had mRNA-Seq performed, we were not able to evaluate the putative miRNA targets based on sex. Further evaluation of the role of these miRNAs in a sex-specific manner will be important to understand sex-specific differences in mRNA expression. Lastly, we recognize these studies do not define phenotypic characteristics of the pediatric heart. We are currently investigating aspects of contractile dysfunction and cellular composition of these hearts, but these studies are beyond the scope of this work.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Maron BJ et al. Contemporary definitions and classification of the cardiomyopathies: An American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functio. Circ. 113, 1807–1816 (2006).

2. Pinto YM et al. Proposal for a revised definition of dilated cardiomyopathy, hypokinetic non-dilated cardiomyopathy, and its implications for clinical practice: A position statement of the ESC working group on myocardial and pericardial diseases. Eur. Heart J 37, 1850–1858 (2016). [PubMed: 26792875]

3. Kirk R. et al. The Registry of the International Society for Heart and Lung Transplantation: Thirteenth official pediatric heart transplantation report 2010. J. Heart Lung Transplant 29, 1119–1128 (2010). [PubMed: 20870166]

4. Miyamoto SD et al. Beta-adrenergic adaptation in paediatric idiopathic dilated cardiomyopathy. Eur. Heart J 35, 33–41 (2014). [PubMed: 22843448]

5. Veltmann C, Bauersachs J. & Berliner D. Dilated cardiomyopathies and non-compaction cardiomyopathy. Herz. 45, 212–220 (2020). [PubMed: 32107565]

6. Towbin JA et al. Incidence, causes, and outcomes of dilated cardiomyopathy in children. JAMA. 296, 1867–1876 (2006). [PubMed: 17047217]

7. Jayaprasad N. Heart failure in children. Heart Views.17,92–99 (2016). [PubMed: 27867456]

8. Kirk R. et al. The International Society for Heart and Lung Transplantation Guidelines for the management of pediatric heart failure: Executive summary. J. Heart Lung Transplant 33, 888–909 (2014). [PubMed: 25110323]

9. Shaddy RE et al. Carvedilol for children and adolescents with heart failure: A randomized controlled trial. JAMA. 298, 1171–1179 (2007). [PubMed: 17848651]

10. Singh RK et al. Survival Without Cardiac Transplantation Among Children With Dilated Cardiomyopathy. J. Am. Coll. Cardiol 70, 2663–2673 (2017). [PubMed: 29169474]

11. Nakano SJ et al. Age-related differences in phosphodiesterase activity and effects of chronic phosphodiesterase inhibition in idiopathic dilated cardiomyopathy. Circ. Heart Fail 8, 57–63 (2015). [PubMed: 25278000]

12. Woulfe KC et al. Fibrosis and Fibrotic Gene Expression in Pediatric and Adult Patients With Idiopathic Dilated Cardiomyopathy. J. Card. Fail 23, 314–324 (2017). [PubMed: 27890770]

13. Tatman PD et al. Pediatric dilated cardiomyopathy hearts display a unique gene expression profile. JCI insight. 2, e94249 (2017).

14. Stauffer BL, Russell G, Nunley K, Miyamoto SD & Sucharov CC MiRNA expression in pediatric failing human heart. J. Mol. Cell. Cardiol 57, 43–46 (2013). [PubMed: 23333438]

15. Ambros V. microRNAs: tiny regulators with great potential. Cell. 107, 823–826 (2001). [PubMed: 11779458]

16. Cordes KR & Srivastava D. MicroRNA regulation of cardiovascular development. Circ. Res 104, 724–732 (2009). [PubMed: 19325160]

17. Vegter EL, van der Meer P, de Windt LJ, Pinto YM & Voors AA MicroRNAs in heart failure: from biomarker to target for therapy. Eur. J. Heart Fail 18, 457–468 (2016). [PubMed: 26869172]

18. Langmead B. & Salzberg SL Fast gapped-read alignment with Bowtie 2. Nat.Methods 9, 357–359 (2012). [PubMed: 22388286]

19. Robinson Mark D, McCarthy Davis J. & Smyth Gordon K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26, 139–140 (2010). [PubMed: 19910308]

20. Sucharov CC et al. Micro-RNA expression in hypoplastic left heart syndrome. J. Card. Fail 21, 83–88 (2015). [PubMed: 25291457]

21. Curran-Everett D. & Benos DJ Guidelines for reporting statistics in journals published by the American Physiological Society. Am. J. Physiol. Regul. Integ.r Comp. Physiol 287, R247–249 (2004).

22. Agarwal V, Bell GW, Nam JW & Bartel DP Predicting effective microRNA target sites in mammalian mRNAs. eLife. 4, e05005 (2015).

23. Friedman RC, Farh KKH, Burge CB & Bartel DP Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 19, 92–105 (2009). [PubMed: 18955434]

24. Hata A. Functions of MicroRNAs in Cardiovascular Biology and Disease. Annu. Rev. Physiol 75, 69–93 (2013). [PubMed: 23157557]

25. Condorelli G, Latronico MV & Cavarretta E. microRNAs in cardiovascular diseases: current knowledge and the road ahead. J. Am. Coll. Cardiol 63, 2177–2187 (2014). [PubMed: 24583309]
26. Wojciechowska A, Braniewska A. & Kozar-Kamińska K. MicroRNA in cardiovascular biology and disease. Adv. Clin. Exp. Med 26, 865–874 (2017). [PubMed: 29068585]
27. Spengler RM et al. Elucidation of transcriptome-wide microRNA binding sites in human cardiac tissues by Ago2 HITS-CLIP. Nucleic Acids Res. 44, 7120–7131 (2016). [PubMed: 27418678]
28. Callari M. et al. Comparison of Microarray Platforms for Measuring Differential MicroRNA Expression in Paired Normal/Cancer Colon Tissues. PLoS ONE. 7, e45105 (2012).
29. Git A. et al. Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA. 16, 991–1006 (2010). [PubMed: 20360395]
30. Sucharov CC et al. Myocardial microRNAs associated with reverse remodeling in human heart failure. JCI Insight. 2, e89169 (2017).
31. Wang W, Liu M, Guan Y. & Wu Q. Hypoxia-responsive Mir-301a and Mir-301b promote radioresistance of prostate cancer cells via downregulating NDRG2. Med. Sci. Monit 22, 2126–2132 (2016). [PubMed: 27327120]
32. Xia X. et al. Downregulation of miR-301a-3p sensitizes pancreatic cancer cells to gemcitabine treatment via PTEN. Am. J. Transl. Res 9, 1886–1895 (2017). [PubMed: 28469793]
33. Zheng JZ et al. Elevated miR-301a expression indicates a poor prognosis for breast cancer patients. Sci. Rep 8, 2225 (2018). [PubMed: 29396508]
34. Zhen LX et al. MiR-301a promotes embryonic stem cell differentiation to cardiomyocytes. World J. Stem Cells 11, 1130–1141 (2019). [PubMed: 31875873]
35. Rangrez AY et al. MicroRNA miR-301a is a novel cardiac regulator of Cofilin-2. PLoS ONE. 12, e0183901 (2017).
36. Wang JG et al. Differential expressions of miRNAs in patients with nonvalvular atrial fibrillation. National Medical Journal of China. 92, 1816–1819 (2012). [PubMed: 22944230]
37. Tatekoshi Y. et al. Translational regulation by miR-301b upregulates AMP deaminase in diabetic hearts. J. Mol. Cell. Cardiol 119, 138–146 (2018). [PubMed: 29733818]
38. Marques FZ, Vizi D, Khammy O, Mariani JA & Kaye DM The transcardiac gradient of cardio-microRNAs in the failing heart. Eur. J. Heart Fail 18, 1000–1008 (2016). [PubMed: 27072074]
39. Clark AL et al. miR-410 and miR-495 Are Dynamically Regulated in Diverse Cardiomyopathies and Their Inhibition Attenuates Pathological Hypertrophy. PLoS One. 11, e0151515 (2016).
40. Wang X, Jin H, Jiang S. & Xu Y. MicroRNA-495 inhibits the high glucose-induced inflammation, differentiation and extracellular matrix accumulation of cardiac fibroblasts through downregulation of NOD1. Cell. Mol. Biol. Lett 23, 23 (2018). [PubMed: 29760746]
41. Callis TE et al. MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. J. Clin. Invest 119, 2772–2786 (2009). [PubMed: 19726871]
42. van Rooij E. et al. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science. 316, 575–579 (2007). [PubMed: 17379774]
43. Xue S. et al. Circulating MiR-17–5p, MiR-126–5p and MiR-145–3p Are Novel Biomarkers for Diagnosis of Acute Myocardial Infarction. Front Physiol. 10, 123 (2019). [PubMed: 30833907]
44. Yang S. et al. Downregulation of microRNA-17–5p improves cardiac function after myocardial infarction via attenuation of apoptosis in endothelial cells. Mol. Genet. Genomics 293, 883–894 (2018). [PubMed: 29536180]
45. Haddad GE et al. Human cardiac-specific cDNA array for idiopathic dilated cardiomyopathy: Sex-related differences. Physiol. Genomics 33, 267–277 (2008). [PubMed: 18303083]
46. Molina-Navarro MM et al. Differential gene expression of cardiac ion channels in human dilated cardiomyopathy. PLoS ONE. 8, e79792 (2013).
47. Joladarashi D, Thandavarayan RA, Babu SS & Krishnamurthy P. Small engine, big power: microRNAs as regulators of cardiac diseases and regeneration. Int. J. Mol. Sci 15, 15891–15911 (2014).
48. Tsuji M. et al. Sexual dimorphisms of mRNA and miRNA in human/murine heart disease. PLoS ONE. 12, e0177988 (2017).

49. Ge L. et al. miR-181c-5p Exacerbates Hypoxia/Reoxygenation-Induced Cardiomyocyte Apoptosis via Targeting PTPN4. Oxid. Med. Cell. Longev 2019, 1957920, (2019).
50. Wang S. et al. MiR-181c-5p Promotes Inflammatory Response during Hypoxia/Reoxygenation Injury by Downregulating Protein Tyrosine Phosphatase Nonreceptor Type 4 in H9C2 Cardiomyocytes. Oxid. Med. Cell. Longev 2020, 7913418, (2020).
51. Zouein FA, Kurdi M. & Booz GW Dancing rhinos in stilettos: The amazing saga of the genomic and nongenomic actions of STAT3 in the heart. JAKSTAT. 2, e24352 (2013).
52. Zouein FA et al. Pivotal Importance of STAT3 in Protecting the Heart from Acute and Chronic Stress: New Advancement and Unresolved Issues. Front Cardiovasc. Med 2, 36, (2015). [PubMed: 26664907]
53. Hilfiker-Kleiner D, Hilfiker A. & Drexler H. Many good reasons to have STAT3 in the heart. Pharmacol. Ther 107, 131–137 (2005). [PubMed: 15963355]
54. Sarafian TA et al. Disruption of astrocyte STAT3 signaling decreases mitochondrial function and increases oxidative stress in vitro. PLoS ONE. 5, e9532 (2010).
55. Wegrzyn J. et al. Function of mitochondrial Stat3 in cellular respiration. Science. 323,793–797 (2009). [PubMed: 19131594]
56. Chatfield KC et al. Dysregulation of cardiolipin biosynthesis in pediatric heart failure. J. Mol. Cell. Cardiol 74, 251–259 (2014). [PubMed: 24937604]
57. Rose BA, Force T. & Wang Y. Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale. Physiol. Rev 90, 1507–1546 (2010). [PubMed: 20959622]
58. Sugden PH & Clerk A. Cellular mechanisms of cardiac hypertrophy. J. Mol. Med. (Berl) 76, 725–746 (1998). [PubMed: 9826118]
59. Yu B. et al. Inhibition of microRNA-143–3p attenuates myocardial hypertrophy by inhibiting inflammatory response. Cell Biol. Int 42, 1584–1593 (2018). [PubMed: 30203887]
60. Damås JK et al. Myocardial expression of CC- and CXC-chemokines and their receptors in human end-stage heart failure. Cardiovasc. Res 47, 778–787 (2000). [PubMed: 10974226]
61. Pyo RT et al. CXCR4 modulates contractility in adult cardiac myocytes. J. Mol. Cell.Cardiol 41, 834–844 (2006). [PubMed: 17010372]
62. LaRocca TJ et al. CXCR4 cardiac specific knockout mice develop a progressive cardiomyopathy. Int. J. Mol. Sci 20, 2269 (2019).
63. Diny NL et al. Macrophages and cardiac fibroblasts are the main producers of eotaxins and regulate eosinophil trafficking to the heart. Eur. J. Immunol 46, 2749–2760 (2016). [PubMed: 27621211]
64. Patel MD et al. Pediatric and adult dilated cardiomyopathy represent distinct pathological entities. JCI insight. 2, e94382 (2017).
65. Heallen T. et al. Hippo pathway inhibits wnt signaling to restrain cardiomyocyte proliferation and heart size. Science. 332, 458–461 (2011). [PubMed: 21512031]
66. Mia MM & Singh MK The Hippo Signaling Pathway in Cardiac Development and Diseases. Front Cell Dev. Biol 7, 211 (2019). [PubMed: 31632964]
67. Xin M. et al. Hippo pathway effector Yap promotes cardiac regeneration. Proc.Natl.Acad. Sci. U S A 110, 13839–1383944 (2013).
68. Wehman B. et al. Pediatric End-Stage Failing Hearts Demonstrate Increased Cardiac Stem Cells. Ann Thorac Surg. 100, 615–622 (2015). [PubMed: 26138767]

Impact:

- This is the first study to compare miRNA expression in the heart of pediatric dilated cardiomyopathy patients to age-matched healthy controls by RNA-sequencing
- Expression of a subset of miRNAs is uniquely dysregulated in children
- Using mRNA-seq and miRNA-seq from matched samples, target prediction was performed.
- This study underscore the importance of pediatric-focused studies.

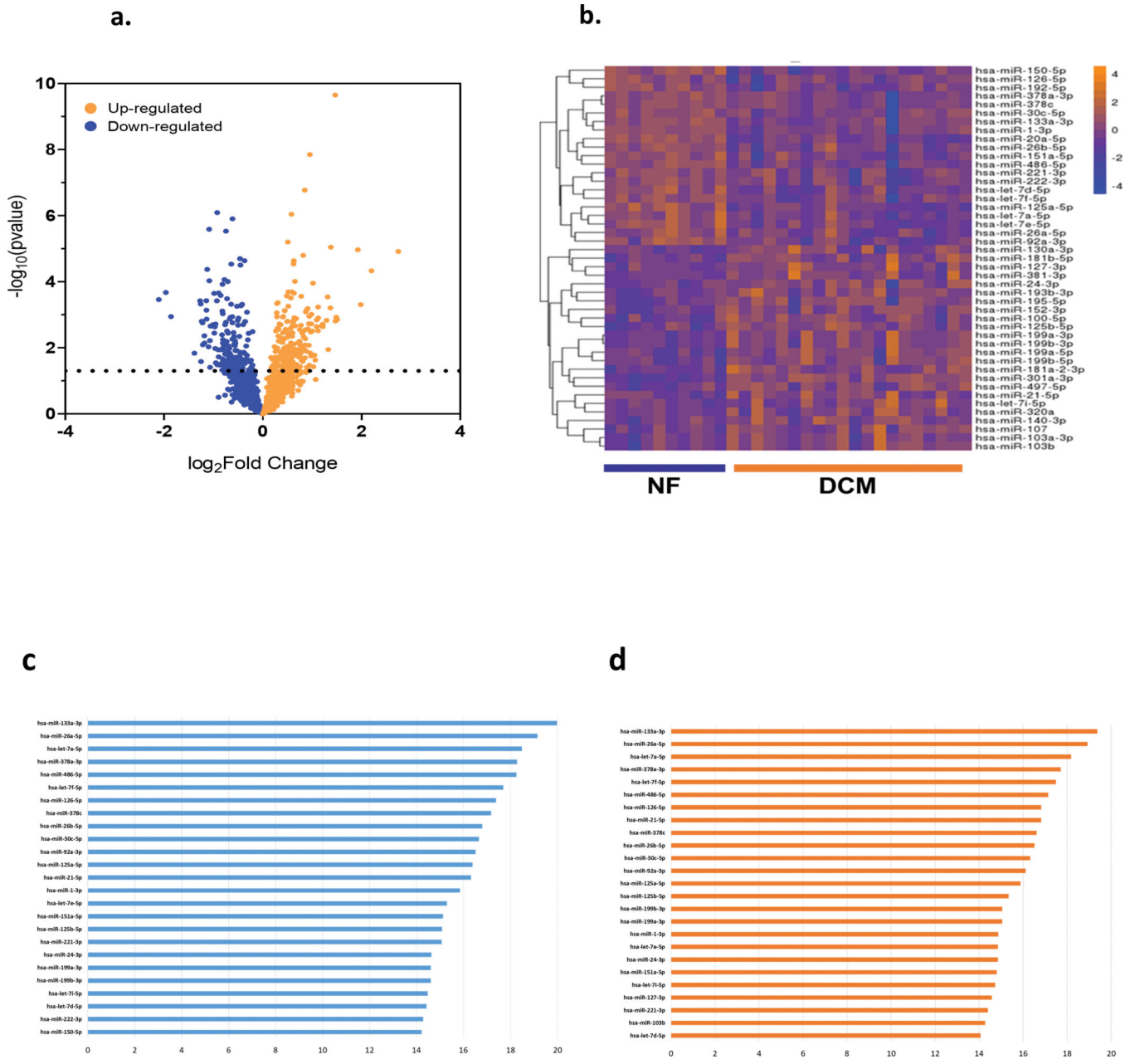

Figure 1. miRNA expression analysis in pediatric DCM patients and NF controls

(A) Volcano plot representing 2134 miRNAs detected by miRNA-Seq described as the log2 fold-change in expression (x-axis) and the log odds of miRNAs being differentially expressed (y-axis). The 393 miRNAs that are significantly differentially expressed between NF pediatric versus DCM are represented by dots above dotted line [-log10 (p-value)>1.3 or p<0.05]. **(B)** Heat map representing the 45 top abundant miRNAs (counts) that are significantly differentially expressed in pediatric DCM patients. Hierarchical clustering separated NF (n=10) and DCM (n=20) samples. **(C)** The top 25 most highly expressed miRNAs ranked based on average counts in NF controls **(D)** The top 25 most highly expressed miRNAs ranked based on average counts in DCM patients.

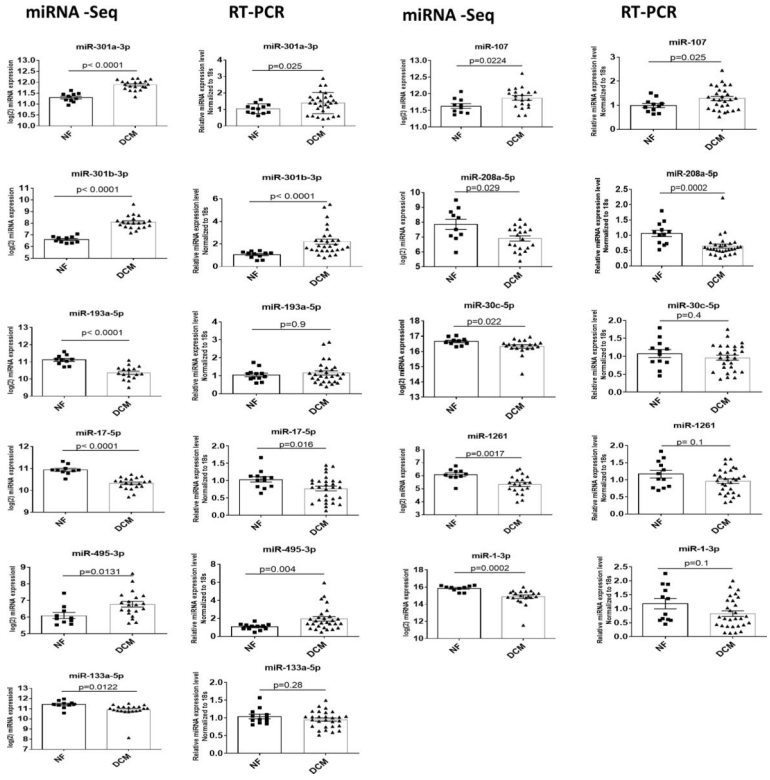

Figure 2. miRNA expression analysis in pediatric DCM patients.

miRNA expression analysis was performed by RT-qPCR (n=12 NF and n=30 DCM LV tissue). Expression was normalized to 18s. Non- failing (NF), dilated cardiomyopathy (DCM).

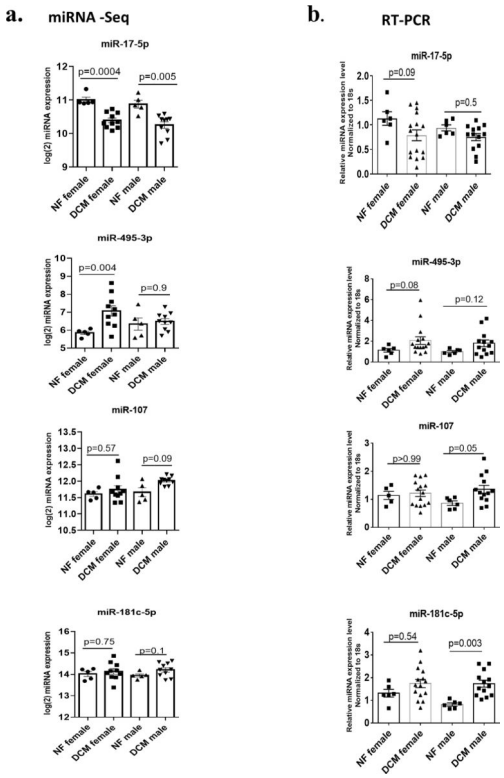

Figure 3: miRNA expression affected by sex differences in pediatric DCM patients.

(A) miRNA-Seq analysis of miRNA expression in DCM hearts (n=10 female, n=10 male) compared to NF controls (n=5 female, n=5 male). **(B)** RT-qPCR analysis of miRNA expression in DCM hearts (n=17 female, n=13 male) compared to NF controls (n=6 female, n=6 male). Only miRNAs regulated based on sex are presented.

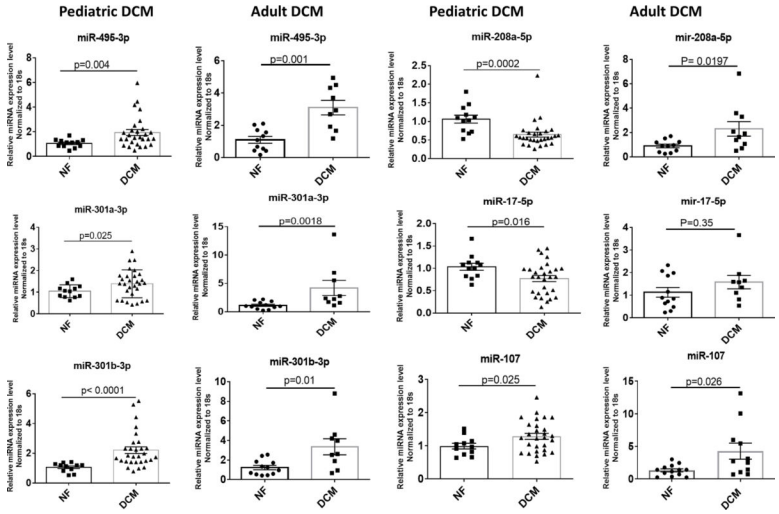

Figure 4. miRNA expression analysis in adult DCM patients.

miRNA expression analysis was performed by RT-qPCR (n=13 NF and n=11 DCM LV tissue). Expression was normalized to 18s. Non failing (NF), dilated cardiomyopathy (DCM).

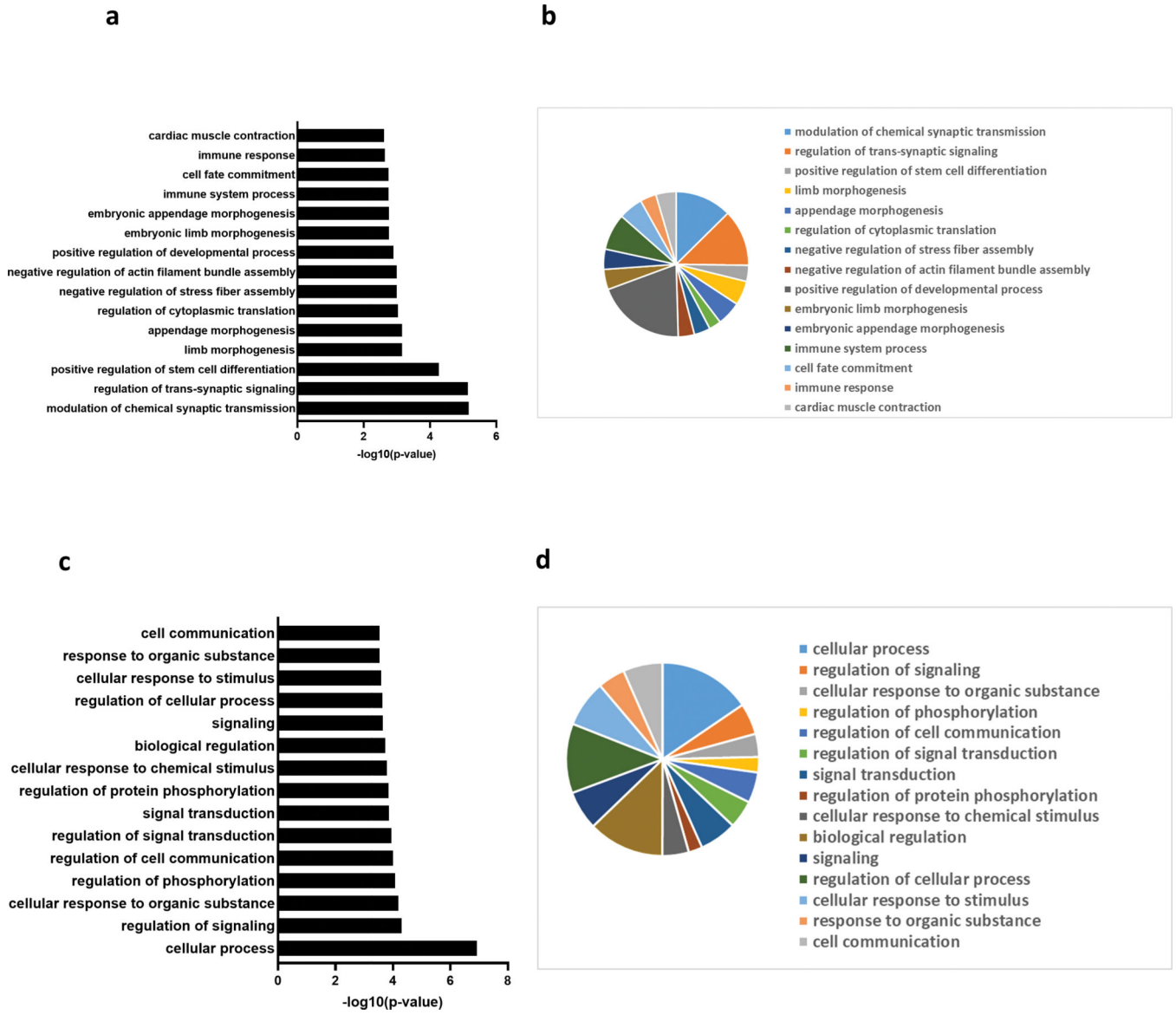

Figure 5. Gene Ontology analysis of the top biological processes predicted to be affected by changes in miRNA and mRNA expression.

(A) Gene Ontology (GO) analysis results for the top 15 significantly enriched biological process related to 163 up-regulated putative target genes in pediatric DCM patients versus NF controls identified using PANTHER [-log10 (p-value)>1.3 or p<0.05]. **(B)** Gene Ontology (GO) annotations for the top 15 significantly enriched biological processes related to 163 up-regulated putative target genes in pediatric DCM patients versus NF controls showing the number of genes involved in each biological processes represented by a pie chart. **(C)** Gene Ontology (GO) analysis results for the top 15 significantly enriched biological process related to 645 down-regulated putative target genes in pediatric DCM patients versus NF controls identified using PANTHER [-log10 (p-value)>1.3 or p<0.05]. **(D)** Gene Ontology (GO) annotations for the top 15 significantly enriched biological processes related to 645 down-regulated putative target genes in pediatric DCM patients

versus NF controls showing the number of genes involved in each biological processes represented by a pie chart.

Table 1.

IPA enriched top canonical pathways

Canonical Pathways	−log(p-value)
STAT3 Pathway	6.66
Synaptogenesis Signaling Pathway	6.6
ErbB Signaling	6.3
Cardiac Hypertrophy Signaling (Enhanced)	6.29
ERK5 Signaling	5.86
PI3K/AKT Signaling	5.8
Acute Myeloid Leukemia Signaling	5.74
HGF Signaling	5.47
FAK Signaling	5.44
ErbB2-ErbB3 Signaling	5.41
Neuregulin Signaling	5.39
UVB-Induced MAPK Signaling	5.36
Prolactin Signaling	5.35
ErbB4 Signaling	5.29
Germ Cell-Sertoli Cell Junction Signaling	5.29
Epithelial Adherens Junction Signaling	5.28
Integrin Signaling	5.24
Endometrial Cancer Signaling	4.84
1D-myo-inositol Hexakisphosphate Biosynthesis V (from Ins(1,3,4)P3)	4.83
Glioma Signaling	4.78
Reelin Signaling in Neurons	4.75
Thrombopoietin Signaling	4.66
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	4.63
FLT3 Signaling in Hematopoietic Progenitor Cells	4.59
Melanoma Signaling	4.57
UVC-Induced MAPK Signaling	4.5
Molecular Mechanisms of Cancer	4.39
Senescence Pathway	4.27
Growth Hormone Signaling	4.24

Canonical Pathways	−log(p-value)
Glioblastoma Multiforme Signaling	4.23
CNTF Signaling	4.15
NRF2-mediated Oxidative Stress Response	4.15
14-3-3-mediated Signaling	4.15
Oncostatin M Signaling	4.11
Pancreatic Adenocarcinoma Signaling	4.11
p70S6K Signaling	4.09
ERK/MAPK Signaling	4.05

Table 2.

IPA enriched top tox function related to putative target genes of miRNAs significantly altered in pediatric DCM

Categories	−log(p-value)
Cardiac Necrosis/Cell Death	5.28
Cardiac Enlargement	4.60
Cardiac Proliferation	4.22
Cardiac Dilation	4.14
Cardiac Arrythmia	3.43
Congenital Heart Anomaly	2.75
Cardiac Dysfunction	2.74
Cardiac Arteriopathy	2.27
Cardiac Stenosis	2.19
Heart Failure	1.83
Congestive Cardiac Failure	1.76
Cardiac Damage	1.75
Cardiac Hypoplasia	1.61
Cardiac Inflammation	1.61
Cardiac Infarction	1.34

Table 3.

IPA enriched top tox function related to the 45 miRNAs significantly altered in pediatric DCM

Categories	−log (p-value)
Cardiac Enlargement	13.3
Cardiac Dilation	11.1
Cardiac Fibrosis	7.3
Cardiac Necrosis/Cell Death	3.5
Cardiac Proliferation	3.4
Cardiac Arteriopathy	2.2
Cardiac Infarction	2.0
Heart Failure	1.8
Cardiac Damage	1.8
Cardiac Regeneration	1.8
Congenital Heart Anomaly	1.4

Table 4.

List of miRNAs and percentage of isomiRs not predicted to be detected by RT-qPCR primers

miRNAs	% of isomiRs not detected by RT-qPCR
miR-30c-5p	4.8
miR-133a-5p	14.7
miR-193a-5p	57.6
miR-1261	100
miR-1	0.96