Pediatric RESEARCH

BASIC SCIENCE ARTICLE

Integrated analysis of miRNA–mRNA interaction in pediatric dilated cardiomyopathy

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BACKGROUND: MicroRNAs (miRNAs) are short single-stranded nucleotides that can regulate gene expression. Although we previously evaluated the expression of miRNAs in pediatric dilated cardiomyopathy (DCM) by miRNA array, pathway prediction based on changes in mRNA expression has not been previously analyzed in this population. The current study aimed to determine the regulation of miRNA expression by miRNA-sequencing (miRNA-seq) and, through miRNA-sequencing (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 upregulated pathways related to the regulation of stem cell differentiation and cardiac muscle contraction, and downregulated pathways related to the regulation of protein phosphorylation, signal transduction, and cell communication.
CONCLUSIONS: 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.

Pediatric Research (2022) 92:98–108; https://doi.org/10.1038/s41390-021-01548-w

IMPACT:

- This is the first study to compare miRNA expression in the heart of pediatric DCM 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 underscores the importance of pediatric-focused studies.

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 year.[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

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Received: 20 November 2020 Revised: 10 March 2021 Accepted: 8 April 2021
Published online: 19 May 2021

diseases.[9] In the current study, we investigated the expression profile of miRNAs in the left ventricular (LV) tissue of pediatric DCM patients and age-matched non-failing (NF) controls by RNA sequencing (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 transducer and activator of transcription 3 (STAT3) signaling, cardiac hypertrophy signaling, extracellular signal-regulated kinase or mitogen-activated protein kinase (ERK/MAPK) signaling, extracellular signal-regulated kinase 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 nonischemic 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, ≥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 > 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 2588 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] Count 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 quantitative reverse transcription-PCR (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. Complementary DNA (cDNA) was synthesized using the Qiagen miScript II cDNA synthesis (Qiagen) according to the 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 log 2 normalized miRNA counts between DCM and NF patients. Pairwise Spearman's 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 seven NF and seven 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 that were predicted targets of significantly dysregulated miRNAs and filtered by Spearman's correlation analysis were used. Upregulated and downregulated genes were uploaded to PANTHER separately and analyzed with Fisher's exact test using the 6273 genes we previously identified in the heart of pediatric DCM patients and NF controls with FPKM (Fragments Per Kilobase of transcript per Million mapped reads) > 1 as our reference list.[13]

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 that were predicted targets of the significantly dysregulated miRNAs and filtered by Spearman's correlation analysis was 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 GraphPad Prism software. Welch's *t* test was used when comparing NF and DCM groups. One-way analysis of variance 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. Fifty-four percent of the pediatric

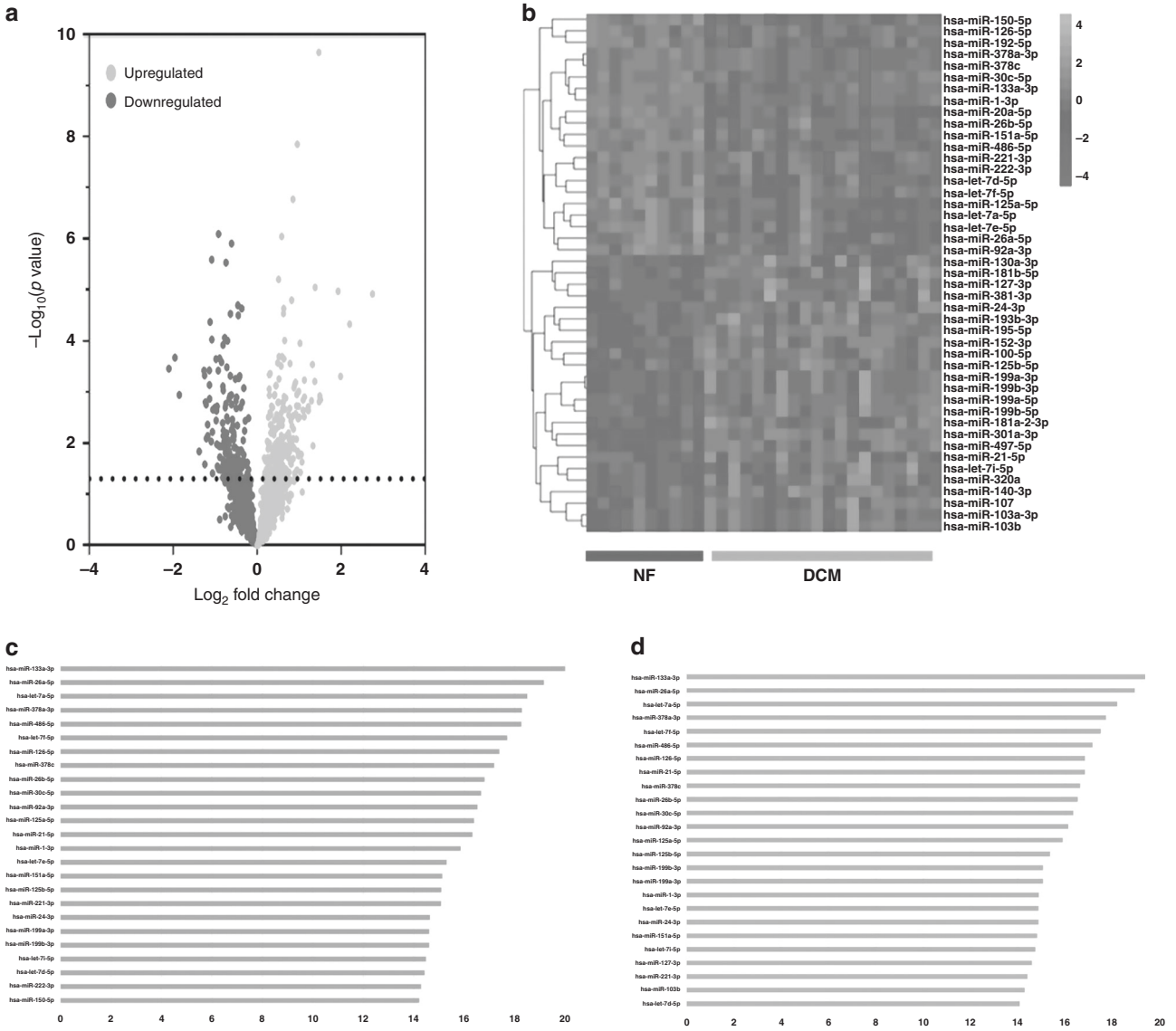

Fig. 1 miRNA expression analysis in pediatric DCM patients and NF controls. a Volcano plot representing 2134 miRNAs detected by miRNA-seq described as the log 2 fold change in expression (*x*-axis) and the log odds of miRNAs being differentially expressed (*y*-axis). The 393 miRNAs that are significantly differentially expressed between NF pediatric vs DCM are represented by dots above the dotted line [−log 10 (*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.

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. Thirty-nine percent 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 vs non-failing pediatric controls using miRNA-seq. Two thousand five hundred and eighty-eight 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 upregulated and 166 miRNAs were downregulated (Fig. 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 (Fig. 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 (Fig. 1c, d) 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, miR-301b-3p, miR-495-3p, miR-17-5p, miR-208a-5p,

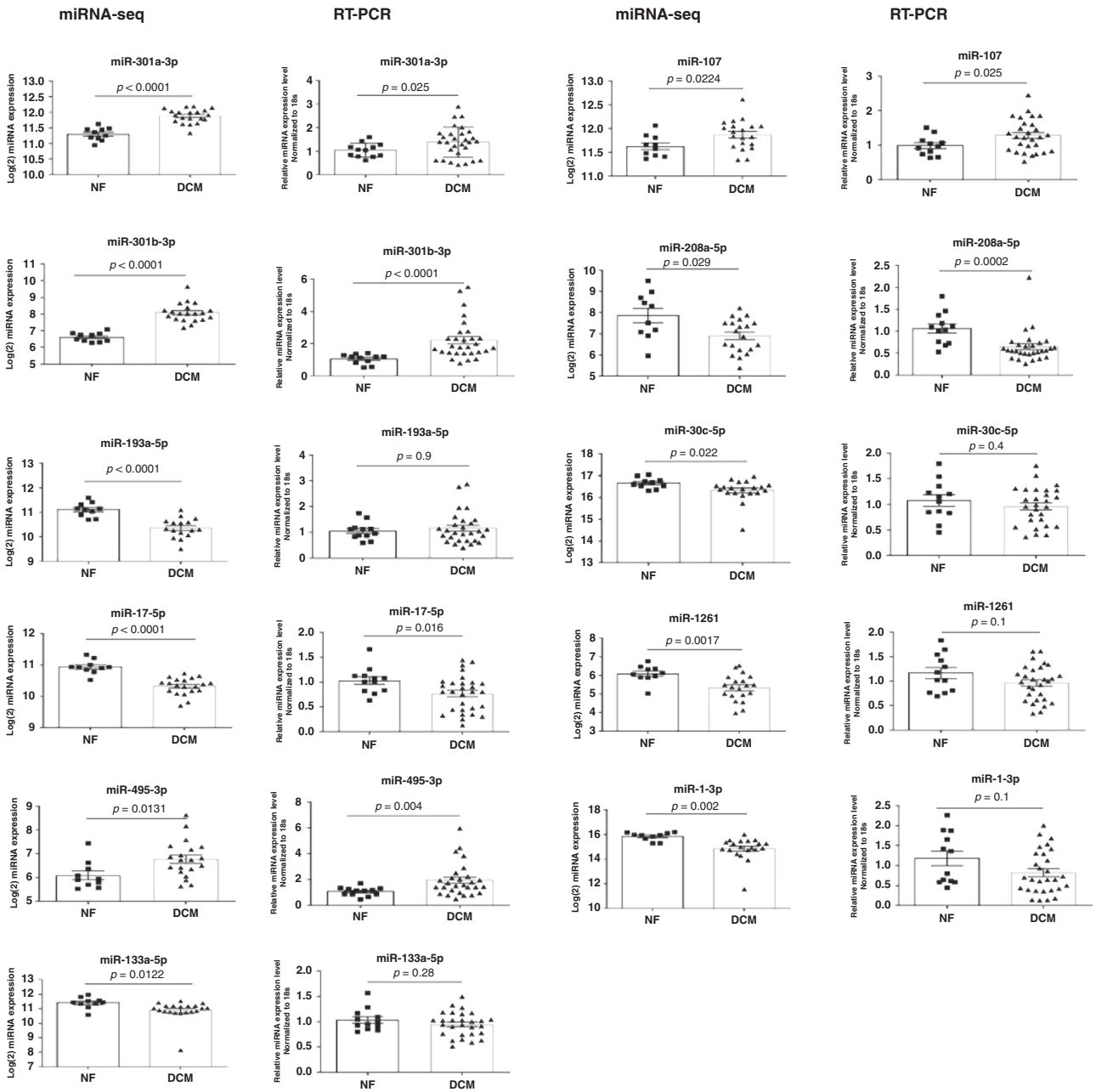

Fig. 2 miRNA expression analysis in pediatric DCM patients. miRNA expression analysis was performed by RT-qPCR ($n = 12$ NF and $n = 30$ DCM LV tissue). Expression was normalized to 18s. NF non-failing, DCM dilated cardiomyopathy.

miR-193a-5p, miR-107, miR-133a-5p, miR-30c-5p, miR-1-3p, and miR-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 upregulation of four miRNAs (miR-301a-3p, miR-301b-3p, miR-495-3p, and miR-107) and downregulation of two miRNAs (miR-17-5p and miR-208a-5p) in pediatric DCM patients. Although expression of miR-193a-5p, miR-133a-5p, miR-30c-5p, miR-1-3p, and miR-1261 was unchanged by RT-qPCR, the directionality of expression was consistent with results from miRNA-seq (Fig. 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-1-3p, 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 1).

In addition, we evaluated the effect of sex on miRNA expression. Interestingly, miR-495-3p and miR-17-5p showed differentially significant expression only in female pediatric DCM patients compared to NF. Whereas the expression of miR-107 and miR-181c-5p significantly changed only in male pediatric DCM (Fig. 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 upregulation of miR-495-3p

Table 1. 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-1-3p	0.96

and downregulation of miR-17-5p only in female pediatric DCM patients compared to NF, and the expression of miR-107 and miR-181c-5p were significantly upregulated only in male pediatric DCM (Fig. 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 the opposite. Moreover, the expression profile of miR-17-5p was unchanged in adult DCM patients compared to NF control (Fig. 4).

miRNA-seq-based target analysis

To evaluate the putative miRNA targets, we used our previously published mRNA-seq dataset.[13] Six thousand two hundred and seventy-three mRNAs, FPKM > 1 were filtered as putative targets of the 45 significantly different miRNAs. Following Spearman's correlation analysis and one proportion test, 36 significant miRNAs and 808 (163 upregulated and 645 downregulated) 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 downregulated and 163 upregulated) putative target genes of differentially expressed miRNAs (36 miRNAs). Several biological processes based on upregulated and downregulated putative target genes of altered miRNAs were significantly enriched ($p < 0.05$) (Tables S7 and 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 upregulated putative target genes (Fig. 5a, 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 downregulated putative target genes (Fig. 5c, 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, ultraviolet B-induced MAPK signaling pathway, ErbB signaling, cardiac hypertrophy signaling (Enhanced), ERK5 signaling, PI3K/AKT signaling, integrin signaling, hepatocyte growth factor signaling, focal adhesion kinase signaling, and ErbB2–ErbB3 signaling were highly significantly enriched ($p < 0.001$) in the heart of pediatric DCM patients compared to NF control (Table 2). Moreover, IPA analysis also revealed multiple significantly dysregulated ($p < 0.05$) cardiac-specific pathways related to cardiac function, including cardiac enlargement, cardiac

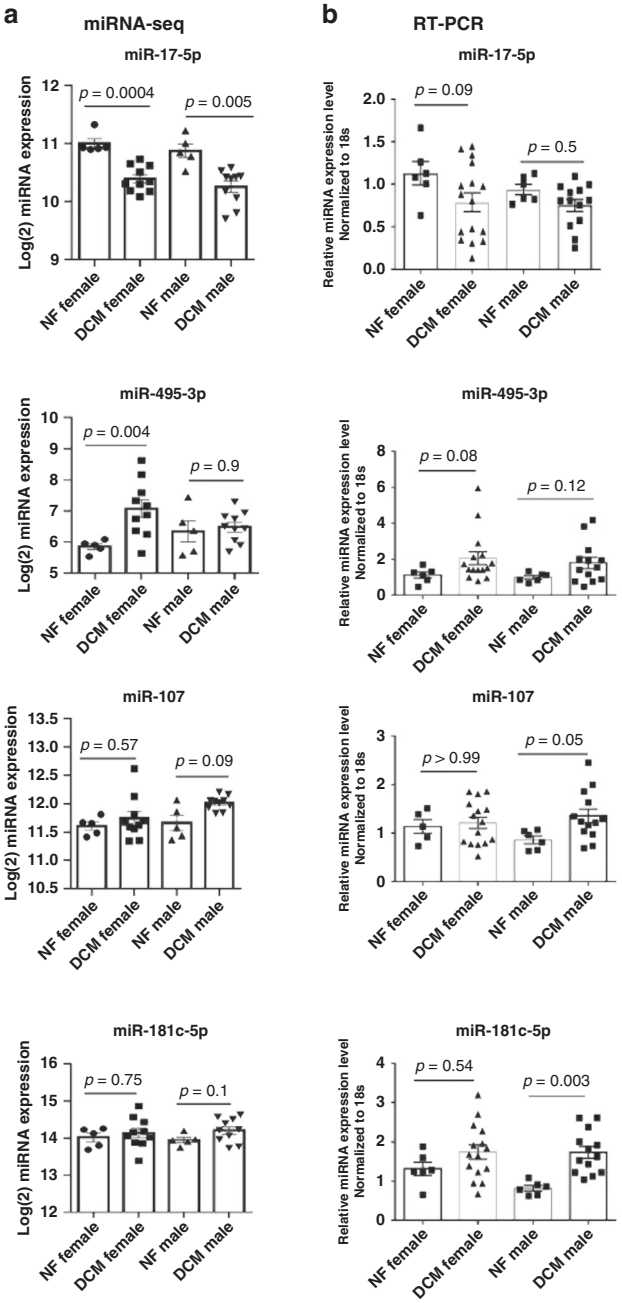

Fig. 3 miRNA expression affected by sex differences in pediatric DCM patients. a miRNA-seq analysis of miRNA expression in DCM hearts ($n = 10$ female, $n = 10$ male) compared to NF controls ($n = 5$ female, $n = 5$ male). **b** RT-qPCR analysis of miRNA expression in DCM hearts ($n = 17$ female, $n = 13$ male) compared to NF controls ($n = 6$ female, $n = 6$ male). Only miRNAs regulated based on sex are presented.

inflammation, cardiac dilation, cardiac dysfunction, arteriopathy, HF, congestive cardiac failure, cardiac stenosis, hypoplasia, and infarction (Table 3).

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

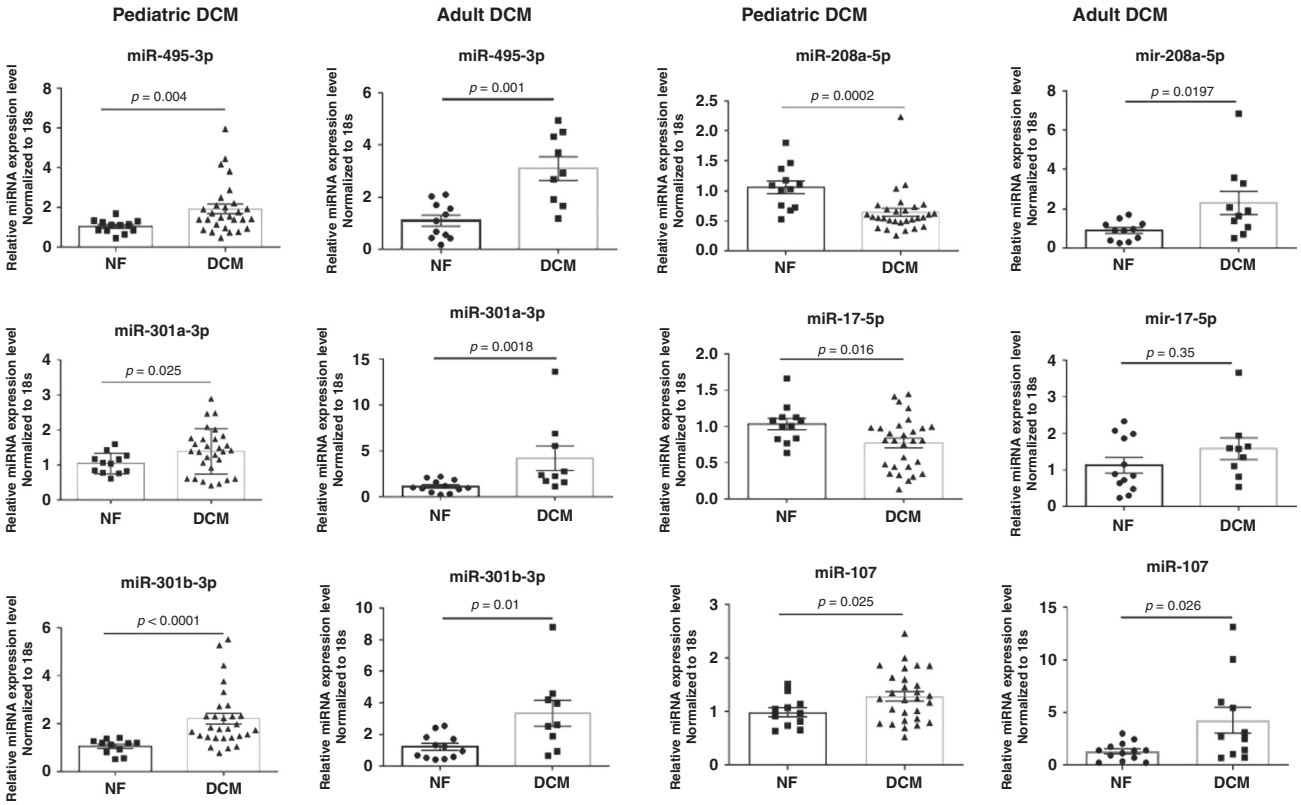

Fig. 4 miRNA expression analysis in adult DCM patients. miRNA expression analysis was performed by RT-qPCR ($n = 13$ NF and $n = 11$ DCM LV tissue). Expression was normalized to 18s. NF non-failing, DCM dilated cardiomyopathy.

related to cardiotoxicity function, including cardiac enlargement, cardiac dilation, cardiac proliferation, arteriopathy, and HF 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 4).

DISCUSSION

Despite the fact that etiologies and biological factors involved in pediatric DCM are different from that of adults, treatment of pediatric patients has 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 the expression of mRNAs from >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 and dysfunction as well as HF.[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 showed that only the most expressed miRNAs are likely to interact with the RNA-induced silencing 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-3p, miR-301b-3p, miR-107, and miR-495-3p and downregulation of miR-17-5p and miR-208a-5p were confirmed by RT-qPCR. In addition, although not significant, the 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 miR-1-3p, miR-30c-5p, and miR-133a-5p is not likely to alter RT-PCR results. However, miR-193a-5p and miR-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, the 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 miR-17-5p was observed when comparing changes in pediatric DCM patients vs

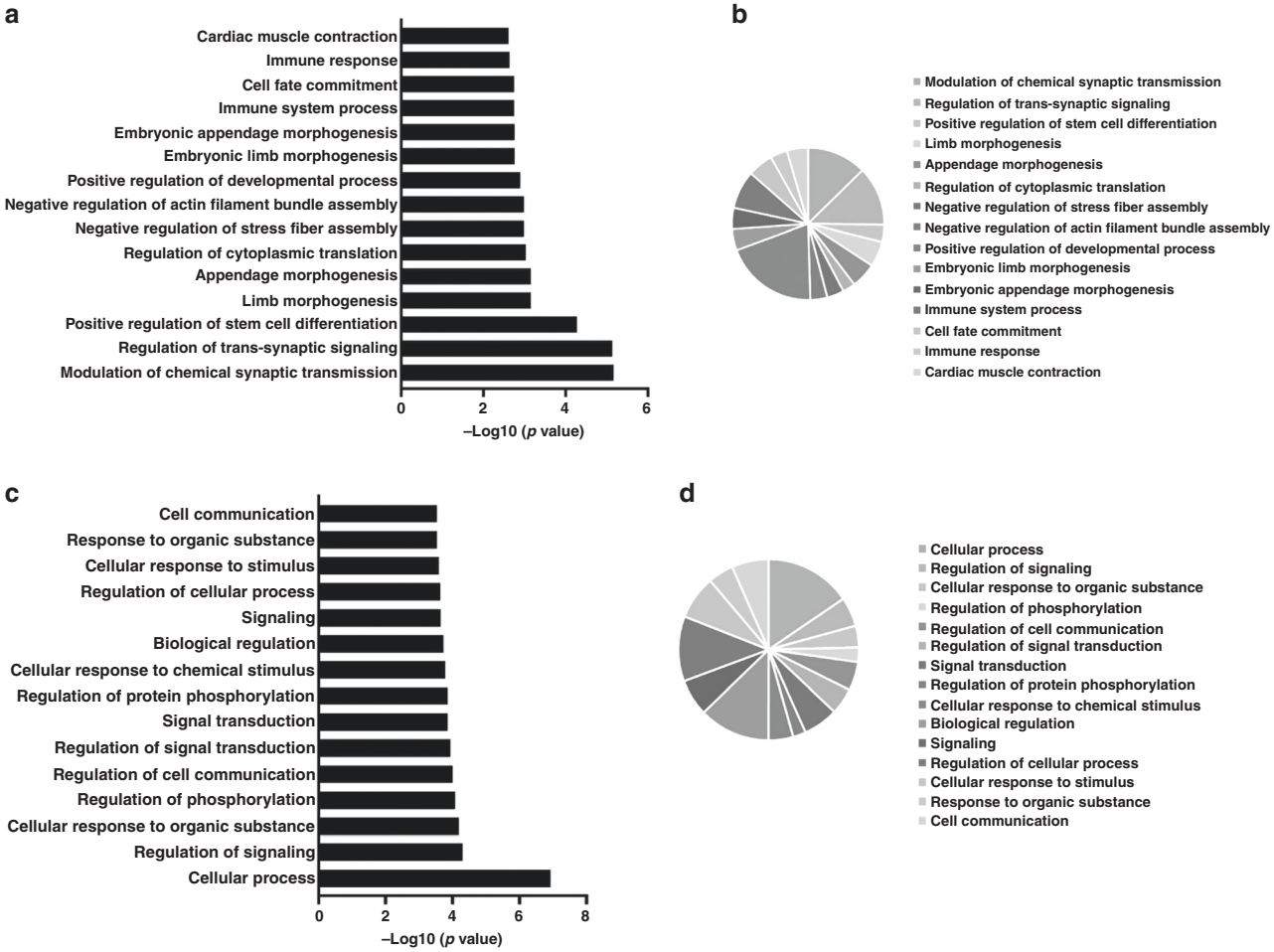

Fig. 5 Gene ontology analysis of the top biological processes predicted to be affected by changes in miRNA and mRNA expression. **a** Gene ontology (GO) analysis results for the top 15 significantly enriched biological processes related to 163 upregulated putative target genes in pediatric DCM patients vs NF controls identified using PANTHER [−log 10 (p value) > 1.3 or $p < 0.05$]. **b** GO annotations for the top 15 significantly enriched biological processes related to 163 upregulated putative target genes in pediatric DCM patients vs NF controls showing the number of genes involved in each biological processes represented by a pie chart. **c** GO analysis results for the top 15 significantly enriched biological process related to 645 downregulated putative target genes in pediatric DCM patients vs NF controls identified using PANTHER [−log 10 (p value) > 1.3 or $p < 0.05$]. **d** GO annotations for the top 15 significantly enriched biological processes related to 645 downregulated putative target genes in pediatric DCM patients vs NF controls showing the number of genes involved in each biological processes represented by a pie chart.

NF controls and adult DCM patients vs NF controls. Expression of miR-208a-5p in adult DCM patients was increased, but downregulated in pediatric DCM patients compared to NF controls. Moreover, miR-17-5p was downregulated in pediatric DCM patients, whereas no change was observed in adults, which suggests 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]

In addition, 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 nonischemic DCM patients.[38] Moreover, miR-107 was overexpressed in endothelial progenitor cells (EPCs) and inhibits their differentiation under hypoxic conditions via targeting hypoxia-inducible factor-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 an association of overexpression 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,

Table 2. 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
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 3. 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 4. 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

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 AMI rat model.[44] It is interesting that the 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 HF patients.[45,46] However, although 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, the expression of several miRNAs including miR-17-5p, miR-107, miR-495-3p, and miR-181c-5p in pediatric DCM was 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.

Although 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 nuclear factor-κB-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. In addition, 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 (Table 2). 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 and remodeling, as well as end-stage HF.[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.

In addition, 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 HF.[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, HF, arrhythmogenic cardiomyopathy, and DCM.[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, upregulated 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 an increased number of cardiac stem cells in end-stage pediatric HF 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 the 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 that 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 was 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 who 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 that 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.

ACKNOWLEDGEMENTS

We would like to acknowledge the Children's Hospital Colorado (CHCO) and University of Colorado Hospital (UCH) Cardiothoracic surgical teams including Drs. David Campbell, M.D., Max Mitchell, M.D., James Jaggers, M.D., and Matthew Stone, M.D. (CHCO), and David Fullerton, M.D., and Bret Reece, M.D. (UCH) for the procurement of heart tissue. We would also like to acknowledge Drs. Peter Buttrick, M.D. and Amrut Ambardekar, M.D. for maintenance of the adult tissue bank, and Jacqueline Holstein (CHCO) for patient recruitment and assistance with collection of historical clinical data of pediatric patients. This work was supported by the National Institutes of Health Grants (3R01 HL139968-01S1 to F.T.H., K24 HL150630 to C.C.S., R01 HL1399683 to C.C.S. and S.D.M., R01 HL107715-01A1S1 and R01 HL107715 to B.L.S.), the Jack Cooper Millisor Chair in Pediatric Heart Disease, the Rose Community Foundation, and the Colorado CTSA Grant [UL1 TR002535].

AUTHOR CONTRIBUTIONS

Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data: F.T.H., A.K.-F., L.S.T., B.L.S., and C.C.S. Drafting the article or revising it critically for important intellectual content: F.T.H., A.K.F., L.S.T., M.R.B., S.D.M., B.L.S., and C.C.S. Final approval of the version to be published: F.T.H., A.K.-F., L.S.T., M.R.B., S.D.M., B.L.S., and C.C.S.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41390-021-01548-w.

Competing interests: C.C.S. and M.R.B.: scientific founder and shareholder at miRagen Inc. C.C.S., S.D.M., and B.L.S.: scientific founders and shareholders at CoramiR Inc. The authors declare no competing interests.

Statement of consent: 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.

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REFERENCES

1. Maron, B. J. 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. *Circulation* **113**, 1807–1816 (2006).
2. Pinto, Y. M. 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).
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).
4. Miyamoto, S. D. et al. Beta-adrenergic adaptation in paediatric idiopathic dilated cardiomyopathy. *Eur. Heart J.* **35**, 33–41 (2014).
5. Veltmann, C., Bauersachs, J. & Berliner, D. Dilated cardiomyopathies and non-compaction cardiomyopathy. *Herz* **45**, 212–220 (2020).
6. Towbin, J. A. et al. Incidence, causes, and outcomes of dilated cardiomyopathy in children. *JAMA* **296**, 1867–1876 (2006).
7. Jayaprasad, N. Heart failure in children. *Heart Views* **17**, 92–99 (2016).
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).
9. Shaddy, R. E. et al. Carvedilol for children and adolescents with heart failure: a randomized controlled trial. *JAMA* **298**, 1171–1179 (2007).
10. Singh, R. K. et al. Survival without cardiac transplantation among children with dilated cardiomyopathy. *J. Am. Coll. Cardiol.* **70**, 2663–2673 (2017).
11. Nakano, S. J. 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).
12. Woulfe, K. C. et al. Fibrosis and fibrotic gene expression in pediatric and adult patients with idiopathic dilated cardiomyopathy. *J. Card. Fail.* **23**, 314–324 (2017).
13. Tatman, P. D. et al. Pediatric dilated cardiomyopathy hearts display a unique gene expression profile. *JCI Insight* **2**, e94249 (2017).
14. Stauffer, B. L., Russell, G., Nunley, K., Miyamoto, S. D. & Sucharov, C. C. MiRNA expression in pediatric failing human heart. *J. Mol. Cell. Cardiol.* **57**, 43–46 (2013).
15. Ambros, V. microRNAs: tiny regulators with great potential. *Cell* **107**, 823–826 (2001).
16. Cordes, K. R. & Srivastava, D. MicroRNA regulation of cardiovascular development. *Circ. Res.* **104**, 724–732 (2009).
17. Vegter, E. L., van der Meer, P., de Windt, L. J., Pinto, Y. M. & Voors, A. A. MicroRNAs in heart failure: from biomarker to target for therapy. *Eur. J. Heart Fail.* **18**, 457–468 (2016).
18. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357–359 (2012).
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).
20. Sucharov, C. C. et al. Micro-RNA expression in hypoplastic left heart syndrome. *J. Card. Fail.* **21**, 83–88 (2015).
21. Curran-Everett, D. & Benos, D. J. Guidelines for reporting statistics in journals published by the American Physiological Society. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **287**, R247–R249 (2004).
22. Agarwal, V., Bell, G. W., Nam, J. W. & Bartel, D. P. Predicting effective microRNA target sites in mammalian mRNAs. *eLife* **4**, e05005 (2015).
23. Friedman, R. C., Farh, K. K. H., Burge, C. B. & Bartel, D. P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **19**, 92–105 (2009).
24. Hata, A. Functions of microRNAs in cardiovascular biology and disease. *Annu. Rev. Physiol.* **75**, 69–93 (2013).
25. Condorelli, G., Latronico, M. V. & Cavarretta, E. microRNAs in cardiovascular diseases: current knowledge and the road ahead. *J. Am. Coll. Cardiol.* **63**, 2177–2187 (2014).
26. Wojciechowska, A., Braniewska, A. & Kozar-Kamińska, K. MicroRNA in cardiovascular biology and disease. *Adv. Clin. Exp. Med.* **26**, 865–874 (2017).
27. Spengler, R. M. et al. Elucidation of transcriptome-wide microRNA binding sites in human cardiac tissues by Ago2 HITS-CLIP. *Nucleic Acids Res.* **44**, 7120–7131 (2016).
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).
30. Sucharov, C. C. 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).
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).
33. Zheng, J. Z. et al. Elevated miR-301a expression indicates a poor prognosis for breast cancer patients. *Sci. Rep.* **8**, 2225 (2018).
34. Zhen, L. X. et al. MiR-301a promotes embryonic stem cell differentiation to cardiomyocytes. *World J. Stem Cells* **11**, 1130–1141 (2019).
35. Rangrez, A. Y. et al. MicroRNA miR-301a is a novel cardiac regulator of Cofilin-2. *PLoS ONE* **12**, e0183901 (2017).
36. Wang, J. G. et al. Differential expressions of miRNAs in patients with nonvalvular atrial fibrillation. *Natl Med. J. China* **92**, 1816–1819 (2012).
37. Tatekoshi, Y. et al. Translational regulation by miR-301b upregulates AMP deaminase in diabetic hearts. *J. Mol. Cell. Cardiol.* **119**, 138–146 (2018).
38. Marques, F. Z., Vizi, D., Khammy, O., Mariani, J. A. & Kaye, D. M. The transcardiac gradient of cardio-microRNAs in the failing heart. *Eur. J. Heart Fail.* **18**, 1000–1008 (2016).
39. Clark, A. L. 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).
41. Callis, T. E. et al. MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. *J. Clin. Invest.* **119**, 2772–2786 (2009).
42. van Rooij, E. et al. Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* **316**, 575–579 (2007).
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).
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).
45. Haddad, G. E. et al. Human cardiac-specific cDNA array for idiopathic dilated cardiomyopathy: sex-related differences. *Physiol. Genomics* **33**, 267–277 (2008).
46. Molina-Navarro, M. M. et al. Differential gene expression of cardiac ion channels in human dilated cardiomyopathy. *PLoS ONE* **8**, e79792 (2013).
47. Joladarashi, D., Thandavarayan, R. A., Babu, S. S. & 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.* https://doi.org/10.1155/2019/1957920 (2019).
50. Wang, S. et al. MiR-181c-5p promotes inflammatory response during hypoxia/reoxygenation injury by downregulating protein tyrosine phosphatase non-receptor type 4 in H9C2 cardiomyocytes. *Oxid. Med. Cell. Longev.* **2020**, 7913418 (2020).
51. Zouein, F. A., Kurdi, M. & Booz, G. W. Dancing rhinos in stilettos: the amazing saga of the genomic and nongenomic actions of STAT3 in the heart. *JAKSTAT* **2**, e24352 (2013).
52. Zouein, F. A. 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).
53. Hilfiker-Kleiner, D., Hilfiker, A. & Drexler, H. Many good reasons to have STAT3 in the heart. *Pharmacol. Ther.* **107**, 131–137 (2005).
54. Sarafian, T. A. 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).
56. Chatfield, K. C. et al. Dysregulation of cardiolipin biosynthesis in pediatric heart failure. *J. Mol. Cell. Cardiol.* **74**, 251–259 (2014).
57. Rose, B. A., 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).
58. Sugden, P. H. & Clerk, A. Cellular mechanisms of cardiac hypertrophy. *J. Mol. Med.* **76**, 725–746 (1998).
59. Yu, B. et al. Inhibition of microRNA-143-3p attenuates myocardial hypertrophy by inhibiting inflammatory response. *Cell Biol. Int.* **42**, 1584–1593 (2018).
60. Damås, J. K. et al. Myocardial expression of CC- and CXC-chemokines and their receptors in human end-stage heart failure. *Cardiovasc. Res.* **47**, 778–787 (2000).
61. Pyo, R. T. et al. CXCR4 modulates contractility in adult cardiac myocytes. *J. Mol. Cell. Cardiol.* **41**, 834–844 (2006).

62. LaRocca, T. J. et al. CXCR4 cardiac specific knockout mice develop a progressive cardiomyopathy. *Int. J. Mol. Sci.* **20**, 2269 (2019).
63. Diny, N. L. 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).
64. Patel, M. D. 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).
66. Mia, M. M. & Singh, M. K. The Hippo signaling pathway in cardiac development and diseases. *Front. Cell Dev. Biol.* **7**, 211 (2019).
67. Xin, M. et al. Hippo pathway effector Yap promotes cardiac regeneration. *Proc. Natl Acad. Sci. USA* **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).