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ORIGINAL RESEARCH

microRNA Expression Levels Change in Neonatal Patients During and After Exposure to Cardiopulmonary Bypass

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BACKGROUND: The systemic inflammation that occurs after exposure to cardiopulmonary bypass (CPB), which is especially severe in neonatal patients, is associated with poorer outcomes and is not well understood. In order to gain deeper insight into how exposure to bypass activates inflammatory responses in circulating leukocytes, we studied changes in microRNA (miRNA) expression during and after exposure to bypass. miRNAs are small noncoding RNAs that have important roles in modulating protein levels and function of cells.

METHODS AND RESULTS: We performed miRNA-sequencing on leukocytes isolated from neonatal patients with CPB (n=5) at 7 time points during the process of CPB, including before the initiation of bypass, during bypass, and at 3 time points during the first 24 hours after weaning from bypass. We identified significant differentially expressed miRNAs using generalized linear regression models, and miRNAs were defined as statistically significant using a false discovery rate–adjusted $P<0.05$. We identified gene targets of these miRNAs using the TargetScan database and identified significantly enriched biological pathways for these gene targets. We identified 54 miRNAs with differential expression during and after CPB. These miRNAs clustered into 3 groups, including miRNAs that were increased during and after CPB (3 miRNAs), miRNAs that decreased during and after CPB (10 miRNAs), and miRNAs that decreased during CPB but then increased 8 to 24 hours after CPB. A total of 38.9% of the target genes of these miRNAs were significantly differentially expressed in our previous study. miRNAs with altered expression levels are predicted to significantly modulate pathways related to inflammation and signal transduction.

CONCLUSIONS: The unbiased profiling of the miRNA changes that occur in the circulating leukocytes of patients with bypass provides deeper insight into the mechanisms that underpin the systemic inflammatory response that occurs in patients after exposure to CPB. These data will help the development of novel treatments and biomarkers for bypass-associated inflammation.

Key Words: cardiopulmonary bypass ■ inflammation ■ microRNA ■ neonate

Surgical palliation/repair of congenital heart defects is associated with significant morbidity, mortality, and cost. Neonatal patients are particularly at risk, having a 10% mortality and a 30% complication rate.[1,2] The majority of pediatric open-heart surgeries require the patient to be supported by cardiopulmonary bypass (CPB) to give the surgeon a bloodless field to operate in, while also minimizing ischemic damage to the body. However, patients with CPB experience significant post-CPB inflammation—which includes increased cytokine levels, inflammatory cell infiltration, vascular leak, and multiorgan dysfunction. In neonates recovering from complicated cardiac surgeries, increased cytokine levels are associated with high

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CLINICAL PERSPECTIVE

What Is New?

- We address a critical gap in knowledge by systematically mapping how microRNAs, small noncoding RNAs that play an important role in regulating genes, change in the circulating leukocytes of neonatal patients during and after cardiopulmonary bypass.
- The data presented in this publication will advance the understanding of the molecular mechanisms that drive cardiopulmonary bypass–associated inflammation and facilitate efforts to reduce cardiopulmonary bypass–associated morbidity and mortality.

What Are the Clinical Implications?

- Our findings have translational implications for patients with cardiopulmonary bypass and for other conditions that involve blood being exposed to increased shear stresses, such as ventricular assist devices, extracorporeal live support, and hemodialysis.

Nonstandard Abbreviations and Acronyms

CPB	cardiopulmonary bypass
DEG	differentially expressed gene
FDR	false discovery rate
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAPK	mitogen-activated protein kinase
miRNA	microRNA

mortality and extended intensive care stays[3]; however, the molecular mechanisms that underlie this inflammatory response are not fully understood.

In an effort to understand how CPB instigates inflammation, we have quantified functional changes in circulating leukocytes using unbiased and comprehensive 'omics approaches. Recently, we have measured global gene expression changes that occur in neonatal patients during and after CPB exposure using RNA sequencing.[4] In this report, we examine the microRNA (miRNA) changes that occur in neonatal patients with CPB. miRNAs are small noncoding RNAs that generally bind to the 3' untranslated regions of messenger RNA (mRNA) based on complimentary base pairing to target sites. miRNAs suppress gene expression through inhibition of protein translation and/or promoting degradation of the target mRNAs.[5] miRNAs modulate the protein levels of their target genes by either attenuating mRNA translation and/or promoting degradation of the target mRNAs.[6] Through disruption of these target genes, miRNAs play a vital role in numerous cellular and biological processes, including inflammatory signaling, and have been demonstrated to be differentially expressed in many human diseases, such as cardiovascular diseases.[7]

There has been great interest in identifying miRNA biomarkers for post-CPB complications. For example, miRNAs have been studied as biomarkers of myocardial injury.[8,9] Currently, a limited amount is known about the miRNA changes that occur in leukocytes in response to CPB. This knowledge gap is significant since leukocytes are crucial regulators of inflammation. Understanding global changes of miRNA in leukocytes is a critical step to better understand the mechanisms of CPB-induced inflammation.

We performed a comprehensive unbiased profiling of miRNA expression from pediatric patient leukocytes. Blood samples were collected pre-bypass, during bypass, and post-bypass surgery at 7 different time points from 5 infants with congenital heart diseases undergoing CPB surgery. Leukocytes were isolated from whole blood from each set of patient samples and miRNA levels were subsequently quantified by high-throughput small RNA sequencing. This study aimed to characterize CPB-affected leukocyte miRNA profiles and to holistically analyze how differential expression of these miRNAs may impact target gene expression and corresponding changes in biological pathways.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Human Blood Samples

Pediatric patients aged <1 month with different congenital heart defects requiring repair utilizing CPB were enrolled in our study at Mercy's Children Hospital (Kansas City, MO) (institutional review board protocol number 14110493). Written informed consent was received from participants' parents or legal guardians before inclusion into the study. A standard CPB protocol was used in all of the patients. The approach in all cases was via median sternotomy. Aortic cannulation was used for the arterial access, with either single venous cannula being placed in the right atrium or bicaval cannulation, depending on the type of the defect requiring repair. The CPB circuit was blood primed, and standard additives included methylprednisolone, sodium bicarbonate, cefazolin, and tranexamic acid. After initiation of CPB, the patients were cooled to 18 to 30 °C. Mean perfusion pressure was maintained at 25

to 35 mm Hg, and the minimum hematocrit was kept at 24%. On completion of repair, the patients were rewarmed and weaned from CPB. Modified ultrafiltration was performed in all of the patients after weaning from CPB. Blood samples of 2.5 mL were collected from an indwelling patient line or from the CPB pump, dependent on the time of the blood draw (for blood draws performed while the patient was on CPB, these samples were pulled from the CPB pump). These samples were collected in EDTA tubes at 7 time points: before CPB, 1 hour into CPB, at the end of CPB, at the end of modified ultrafiltration, and 1 hour, 8 hours, and 24 hours during postoperative recovery. Blood was centrifuged at 1000*g* for 20 minutes at room temperature, and plasma was removed. Red blood cells were lysed in RNase-free red blood cell lysis solution (PerfectPure RNA blood kit, 5Prime) for 5 minutes at room temperature. The tubes were centrifuged at 2000*g* for 5 minutes at room temperature. The pellets of nucleated cells were lysed in the cell lysis buffer and immediately stored at −80 °C until RNA extraction. The design and time course of the study are illustrated in Figure 1A. Patient demographics are shown in Table 1.

miRNA Sequencing

Total RNA from the nucleated cells was extracted using a MirVana RNA extraction kit (Invitrogen). The concentration and quality of total RNA for each sample were quantified and evaluated by spectrophotometry (Epoch; Thermo Scientific) and automated chip electrophoresis analyses (Experion, BioRad), respectively. Each sample met the quality standards of a 260/280 ratio >2.0 and an RNA integrity number >9. Illumina TruSeq RNA libraries were prepared from 1 µg of total RNA and sequenced to generate 50 base pair single-end reads using a HiSeq 1500 (Illumina) at the Children's Mercy OMICs Research Core Lab (Kansas City, MO).

Bioinformatics

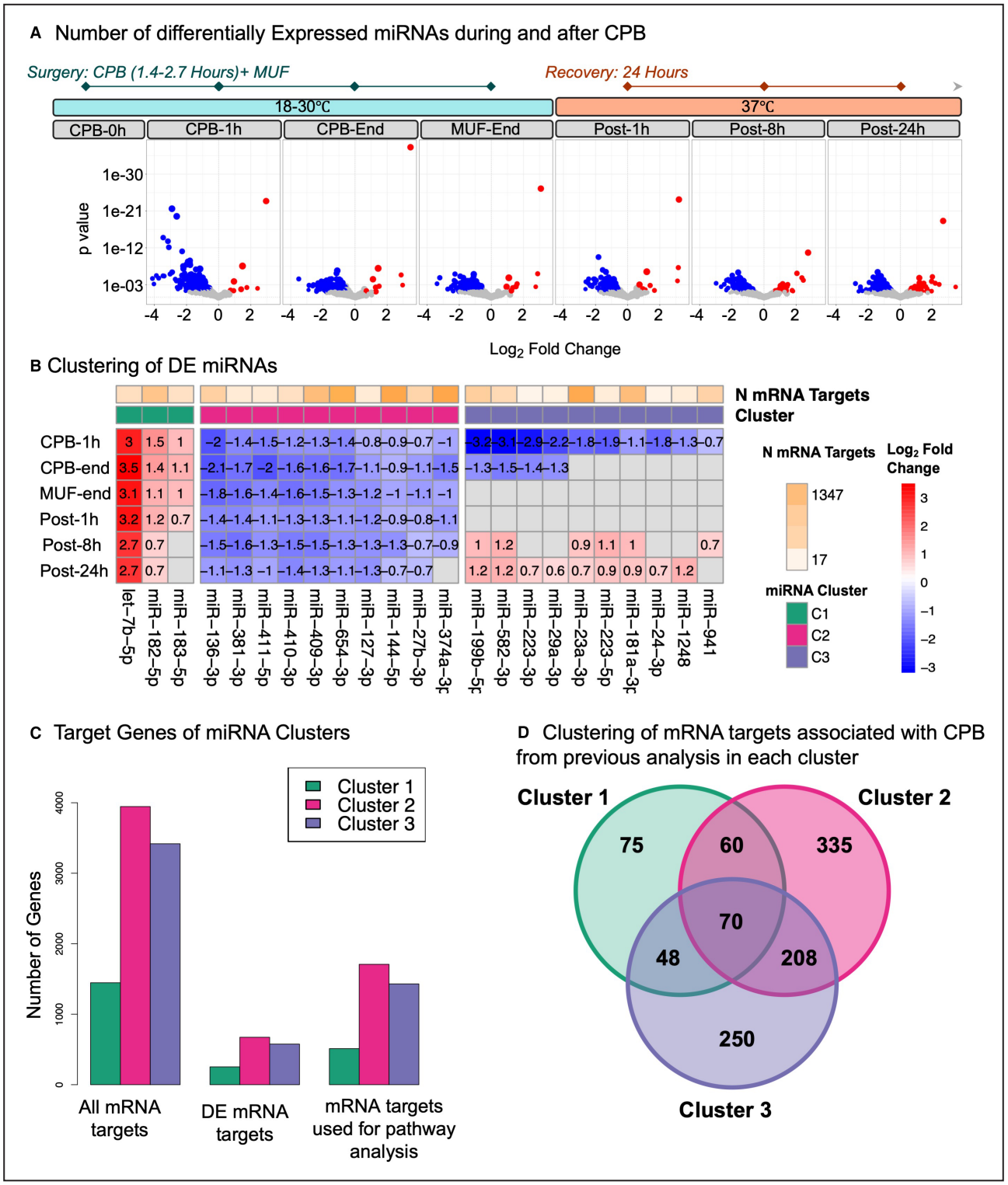
Small RNA sequencing data sets were analyzed using the TIGER pipeline.[10] Briefly, raw reads were preprocessed for quality control using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc). Cutadapt (v2.9) was used to trim 3′ adapters and reads <16 nucleotides in length were removed.[11] Preprocessed small RNA reads were collapsed into nonredundant identical files and aligned to the human genome using bowtie (v1.2.3) allowing 1 mismatch.[12] For miRNAs, nontemplated additions were clipped from the 3′ terminal end, and small RNA reads overlapping mature miRNA coordinates were counted allowing for offset positions −2, −1, 0, +1, and +2. Results were reported as raw read count and reads per million total reads. Our RNA sequencing results were processed and analyzed using DEseq2 (v1.24.0), which is a standardized approach to RNA sequencing analysis[13] with >36 000 citations to date. We performed adjustment for multiple comparisons using the Benjamini-Hotchberg approach.[14] miRNAs were considered statistically significant at a false discovery rate (FDR)–adjusted $P<0.05$. miRNAs were considered to be significantly altered during either surgery or recovery if there were significant differences for at least one time point compared with baseline within each respective stage of CPB (surgery or recovery). A cutoff of 25 reads per million was used to filter out any miRNAs with low expression. Differentially expressed miRNAs were manually clustered based on the directionality of the log fold change and visualized using the R package "pheatmap" (version 1.0.12, R Foundation for Statistical Computing) (https://www.rdocumentation.org/packages/pheatmap/versions/1.0.12/topics/pheatmap). Volcano plots and other miRNA data visualization were generated by the TIGER pipeline.[10]

Identification of Putative miRNA Targets

Putative mRNA targets for miRNAs within each cluster were assessed using TargetScan software (v7.2, targetscan.org), which predicts canonical targeting of miRNAs based on 14 features.[15] For cluster 3, which contained >10 miRNAs, we included the top 10 miRNAs based on log fold change. All mRNA targets that were not present in the paired total (mRNA) RNA sequencing data (n=12 709 genes) were removed. We performed additional filtering using the absolute value of the "context++" score, which is a metric of miRNA target prediction accuracy used by TargetScan.[15] For each miRNA, we removed all putative targets with a context++ score of 0. We also eliminated all mRNAs that were not present in our ancillary mRNA analysis. We then selected the top 200 genes (based on context++ score). This filtering ensured that only the highest quality relationships were validated. The list of gene targets from each filtering step is provided in Table S1.

Pathway Enrichment Analysis of miRNA Target Genes

We performed pathway enrichment analysis of the genes regulated by each cluster of miRNAs using the EnrichR R package (v2.1).[16] For this analysis, we included only the top 200 genes (based on context++ score) for each miRNA. Through Enrichr, we analyzed Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways (excluding KEGG "Human Disease" pathways). Pathways were considered statistically significant with an FDR-adjusted $P<0.05$. miRNA-gene pathway networks were visualized using cytoscape (version 3.7.2).[17]



All data were analyzed and visualized using R (v4.0.3; R Foundation for Statistical Computing).

Cytokine-Receptor Networks

A subnetwork of receptor-ligand networks was generated for target genes using the Fantom5 database,[18] which contained 708 ligands and 691 receptors. We performed a subanalysis of predefined cytokines, chemokines, and integrin/adhesion molecules from within this receptor ligand network, based on the class of molecules known to be mediators of inflammation.

Figure 1. Changes in microRNA (miRNA) expression during and after cardiopulmonary bypass (CPB).
A, The design and time course of the study. Neonates with different congenital heart diseases underwent CPB surgery that lasted 1.4 to 2.7 hours on average, followed by a short duration of modified ultrafiltration (MUF). Body temperature was cooled down to 18 to 30 °C during surgery and quickly rewarmed to 37 °C after MUF. Blood samples were collected at 7 time points; miRNA from isolated nucleated cells were submitted for sequencing (n=5 patients). The number of miRNAs were significantly downregulated or upregulated at different time points compared with the expression level before surgery (CPB-0h). Significance was defined as false discovery rate–adjusted $P<0.05$. **B**, A total of 54 miRNAs were divided into 3 clusters: cluster 1 with miRNAs upregulated in both phases, cluster 2 with miRNAs downregulated in both phases, and cluster 3 with miRNAs downregulated in the surgery phase but upregulated in the recovery phase. The heatmap shows the $\log_2$ fold changes in miRNAs in each cluster compared with before CPB. **C**, Bar plot showing the total number of messenger RNA (mRNA) targets within each cluster. A subset of the targets was previously associated with CPB.[4] Only the top 200 targets for each miRNA was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (**C**). The number of mRNA targets for each individual miRNA are presented in Table S1. **D**, Overlapping and distinct target genes within each cluster that were previously associated with CPB.[4] CPB indicates Cardiopulmonary bypass; DE, differentially expressed; MUF, modified ultrafiltration.

RESULTS

miRNA Sequencing Analysis of Total Patient Nucleated Cells Throughout CPB

To characterize the miRNA expression profiles of patient blood affected by CPB, miRNA sequencing analysis was performed on RNA-isolated miRNA from total nucleated cells of 5 neonate patients undergoing CPB surgery (Table 1). Neonates with varying congenital heart diseases (Table 1) underwent CPB surgery (bypass time range, 86–161 minutes; mean bypass time, 133 minutes [SD 37.33 minutes]), outlined in Figure 1A, with blood samples collected at 7 different time points before, during, and after CPB. miRNAs contributed to >60% of the small noncoding RNAs present in the blood collected during these times (Figure S1A). Detection of unique miRNAs were characterized as normalized expression in reads per million averaged over all 5 patient samples, within each CPB time point. The 4 most abundant miRNAs across all time points were hsa-miR-486-5p, hsa-miR-92a-3p, hsa-miR-451a, and hsa-miR-16-5p (Figure S1B).

Identification of Differentially Expressed miRNAs in Different Stages of CPB

To obtain a better understanding of how CPB affects miRNA expression, we compared the expression of miRNAs at surgery and recovery time points with the pre-CPB time point (before CPB). Differentially expressed miRNAs were identified through generalized linear regression models within DEseq2.[12] Within each time point, we observed that there were more differentially expressed miRNAs that were significantly downregulated compared with baseline than miRNAs that were upregulated compared with baseline (Figure 1A). Forty-two of these miRNAs had significantly altered expression at one time point during surgery compared with baseline, 54 miRNAs had significantly altered expression both during bypass and after bypass, and 13 miRNAs had altered expression compared with pre-CPB only in the recovery from CPB phase, using a cutoff for significance of an FDR-adjusted $P<0.05$ and a reads per million of >25.

To determine the effect of CPB on miRNA expression, the 54 miRNAs had significant modulation of expression at least one time point in both surgery and recovery were clustered into 3 groups (Table S2) based on their expression patterns throughout the course of CPB and recovery (Figure 1B). Cluster 2 had a total of 41 miRNAs with significantly decreased expression based upon our clustering threshold. The top 10 miRNAs based upon fold change are listed in Figure 1B. Cluster 1 (green) represents the miRNAs that remained significantly upregulated in both CPB and recovery phases (Figure 1B), cluster 2 (pink) represents the miRNAs that remained significantly downregulated in both CPB and recovery, and cluster 3 (purple) represents the miRNAs that were significantly downregulated in at least one time point during CPB and significantly upregulated in at least one time point in recovery. We used the TargetScan database to identify putative mRNA targets of the miRNAs within each of the 3 clusters. The number of genes associated with each miRNA and within each cluster is displayed in Table S1. We filtered out genes that were not present in a parallel mRNA sequencing analysis done by our group.[4] (Figure 1C).

In a previous study using a separate population, we identified 2688 genes that exhibited temporal dysregulation after CPB, which were categorized into 5 distinct groups.[4] We expanded on this analysis to investigate relationships between these differentially expressed genes (DEGs) and the miRNA target genes identified in this analysis. A total of 1046 of these 2688 DEGs (38.9%) were reported targets of differentially expressed miRNAs, as shown in Table 2 and Figure 1C. Seventy DEGs were targeted by miRNAs in all 3 clusters, 48 DEGs were targeted by miRNAs in clusters 1 and 3, 60 mRNAs were targeted by miRNAs in clusters 1 and 2, 208 DEGs were targeted by miRNAs in clusters 2 and 3, 75 DEGs were targeted by miRNAs in cluster 1 alone, 250 DEGs were targeted by miRNAs in cluster 3 alone, and 335 DEGs were targeted by miRNAs in cluster 2 alone (Figure 1D). Many of these DEGs were targeted by multiple miRNAs within the same cluster. miRNA target genes contained DEGs

Table 1. Patient Demographics

ID	Age, d	Weight, kg	Sex	Diagnosis	Lowest temp, °C	Surgery time, min	Bypass time, min
1	5	3.3	Male	TGA, IVS, ASD	27.4	270	161
2	5	3.3	Male	HLHS, PDA, ASD	18.2	234	158
3	19	2.7	Female	TAPVC	18.0	163	86
4	12	3.4	Female	TAPVC	21.4	173	99
5	4	3.9	Male	HLHS, PDA	17.6	295	161

ASD indicates atrial septal defect; HLHS, hypoplastic left heart syndrome; IVS, intact ventricular septum; PDA, patent ductus arteriosis; TAPVC, total anomalous pulmonary venous return; and TGA, transposition of the great artery.

across all 5 mRNA categories (Table 2), with most miRNAs across all clusters targeting genes in the "blue" category (indicating gene expression downregulated across all time points) or "red" category (ie, gene expression increased then decreased). Thus, these changes in miRNA expression may be attributable to some of the previously observed changes in gene expression after CPB.

KEGG Pathway and Target Gene Analysis

To identify biologic pathways that were modulated by miRNA clusters, we performed KEGG biological pathways analysis. For this analysis, we used the top 200 target genes for every miRNA based on context score. Overall, we observed that the target genes of these miRNAs were involved in the cell growth and death, cellular community, immune, and signal transduction KEGG subgroups (Figure 2). MiRNAs from cluster 1 targeted 512 genes that were enriched with the p53 signaling pathway and the cell cycle pathway (FDR-adjusted $P<0.05$), which are both involved in cellular growth and death (Figure 2). MiRNAs from cluster 2 targeted 1708 genes that were enriched within 32 different biological pathways (Figure 2). Several pathways related to immunity, such as phagocytosis, B-cell receptors, and T-cell receptors, were identified as being modulated by this cluster. The most significantly enriched pathway was for neurotrophin signaling. Nine of these pathways were signal transduction pathways, and 6 of these pathways were pathways related to the immune system (Figure 2). MiRNAs from cluster 3 targeted 1430 genes that were enriched with 5 different biological pathways. We also examined similarities and differences in the pathways targeted by these miRNAs. The p53 signaling pathway was enriched for genes, which were targets of miRNAs in both cluster 1 and cluster 2. There were no overlapping pathways between clusters 1 and 3. There were 4 pathways that overlapped between clusters 2 and 3. Overall, the most statistically significant pathway for each cluster were those related to the P53 signaling pathway (FDR-adjusted $P=4.77\times10^{-5}$, cluster l), the Fc gamma R-mediated phagocytosis pathway (FDR-adjusted 3.44×10^{-4}, cluster 2) (Figure 2), and the neurotrophin signaling pathway (FDR-adjusted 6.84×10^{-3}, cluster 3). From our previous research, we have demonstrated that MEK/ERK signaling is important for CPB-induced expression of interleukin (IL) 8 and tumor necrosis factor α (TNF-α).[4] miRNAs from cluster 2 were predicted targets of 38 genes in the mitogen-activated protein kinase (MAPK) pathway, and this pathway was significantly enriched for these genes ($P<0.05$). Some of the miRNA target genes included regulatory kinases of differentially expressed mRNAs found in our corresponding mRNA sequencing data (Figure 3).[4]

Cytokine-Receptor Networks

Since the many KEGG pathways enriched for the miRNA target genes involved immune signaling, including the chemokine signaling pathway, we generated a subnetwork of all receptor-ligand network reactions for our miRNA target genes using the FANTOM5 database.[17] Overall, cluster 1 was a potential regulator of 17 ligands and 11 receptors, cluster 3 was a potential

Table 2. Proportion of Target Genes in Each miRNA Cluster That Were Differentially Expressed in a Previous Analysis[4]

mRNA/miRNA cluster	Blue (n=775)		Orange (n=435)		Red (n=640)		Sky blue (n=422)		Yellow (n=407)	
	No. of mRNAs	%	No. of mRNAs	%	No. of mRNAs	%	No. of mRNAs	%	No. of mRNAs	%
Cluster 1	88	11.4	29	3.7	68	8.8	35	4.5	38	4.9
Cluster 2	263	33.9	157	20.3	281	36.3	143	18.5	146	18.8
Cluster 3	198	25.5	117	15.1	244	31.5	98	12.6	100	12.9
No cluster	511	65.9	270	34.8	325	41.9	269	34.7	258	33.3

miRNA indicates microRNA; and mRNA, messenger RNA.

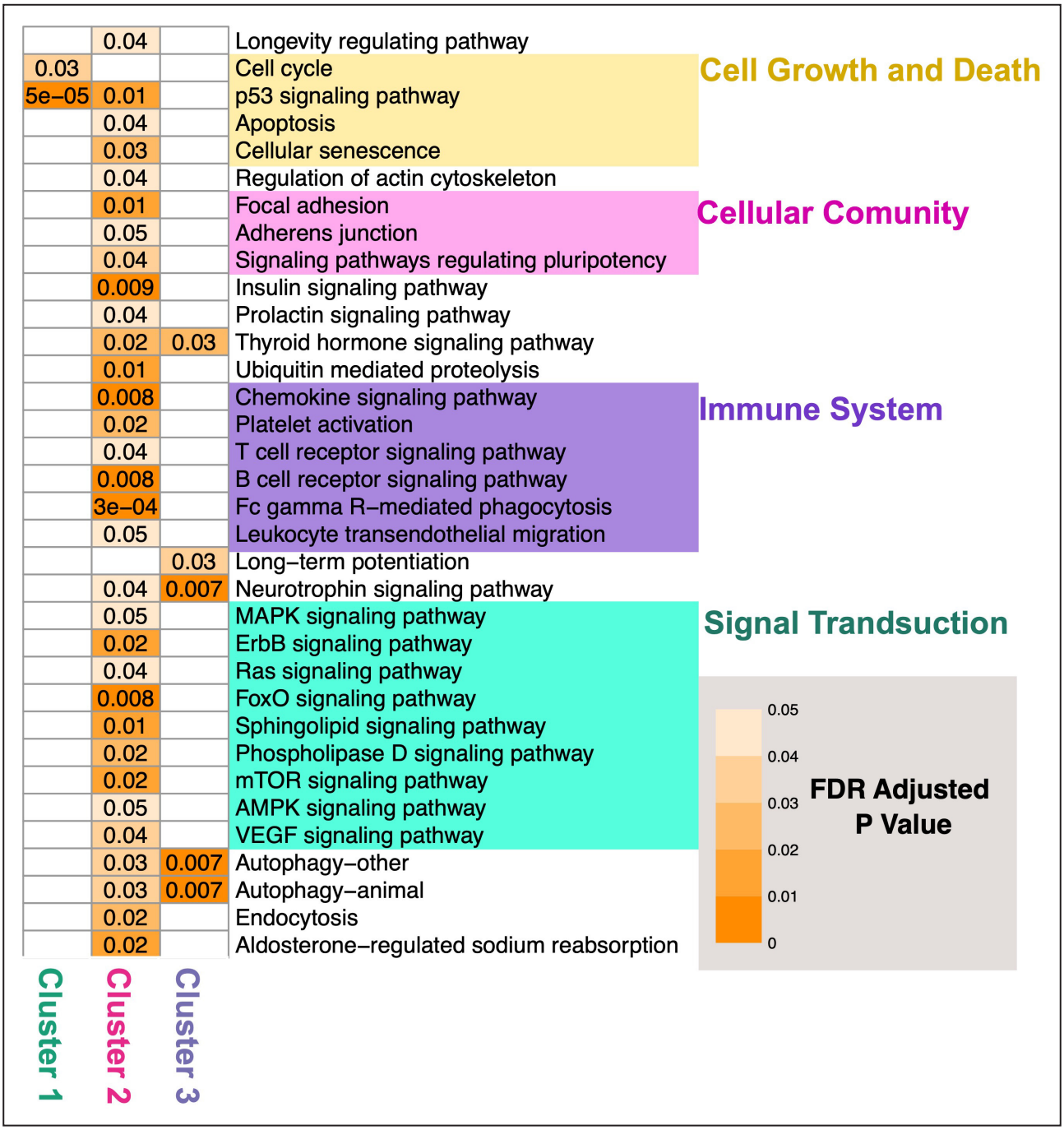


Figure 2. Pathway enrichment analysis of the microRNA (miRNA) target genes in each cluster.
The heatmap showing significant Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways enriched for the top genes regulated by the miRNAs in each cluster. The numbers indicate the adjusted *P*-values for the pathways. FDR indicates false discovery rate; MAPK, mitogen-activated protein kinase pathway; and VEGF, vascular endothelial growth factor.

regulator of 43 ligands and 31 receptors, and cluster 2 was a potential regulator of 47 receptors and 25 ligands (Table S1). We performed a targeted analysis by looking at different mediators of inflammation associated with each of the miRNA targets from each cluster. We filtered target genes of each miRNA by cytokines and their receptors (Figure 4A), chemokines and their receptors (Figure 4B), and integrin receptors and adhesion molecules (Figure 4C). The color code of each miRNA denotes which cluster each is derived from.

DISCUSSION

miRNAs play important roles in post-transcriptional regulation of target genes. Thus, understanding how miRNA levels in leukocytes fluctuate during and after

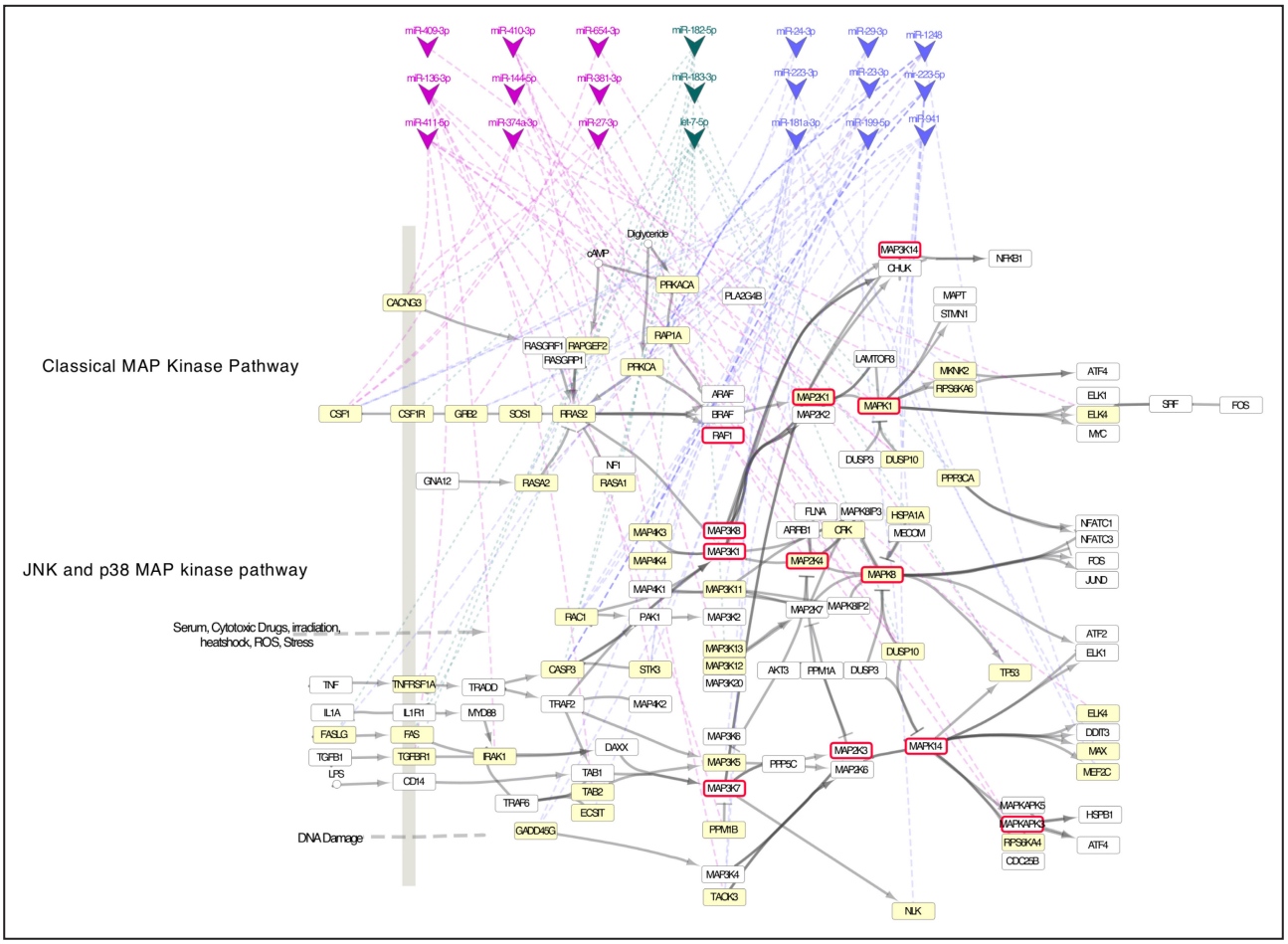


Figure 3. Targeted genes in the mitogen-activated protein kinase pathway (MAPK).
Genes in the MAPK pathway, which are targets of the differentially expressed microRNA (miRNA) are highlighted in yellow. Genes predicted as the regulatory kinases of differentially expressed mRNAs in the matched mRNA sequencing data set are highlighted with the red border.

CPB may significantly help to both advance the understanding of the mechanisms that underpin CPB-associated inflammation and develop biomarkers of CPB-related complications. To our knowledge, this study is the first to characterize the novel global miRNA changes that occur in circulating leukocytes of patients with CPB. The data set presented here significantly advanced the state of knowledge since the previously reported studies (Table S3) examined only a limited number of miRNAs change in patients with CPB using mainly serum or plasma samples. Our study specifically examined changes in circulating nucleated cells, a cell population that is an important driver of inflammation. In addition, the global profiling of all miRNA changes in an unbiased manner significantly advances the understanding of how circulating leukocytes are responding to CPB. The data presented in this study, especially regarding the miRNAs that are predicted to facilitate the resolution of inflammation, may form the basis of novel therapeutic and risk stratification strategies.

Given that miRNA typically reduce the protein levels of their target genes,[6] our data suggest that miRNAs are involved in the development and resolution of CPB-associated inflammation. During CPB, the changes in miRNA levels promote inflammation. miRNAs that are upregulated during CPB (cluster 1) target genes that are typically anti-inflammatory, such as IL-10 and IL-1R2 (Figure 4A). At the same time, miRNAs that are downregulated during CPB (clusters 2 and 3) target receptors for key inflammatory signaling pathways, such as TNF-α and IL-8 (Figure 4A and 4B). These changes have the net result of increasing the activity of inflammatory pathways.

The role of the members of cluster 2, miRNAs that are decreased during and after CPB, is especially interesting given that this cluster is predicted to uniquely modulate pathways and receptors involved with inflammation. As these miRNAs generally are thought to decrease gene expression, reduction of expression of these negative regulators during and after surgery may

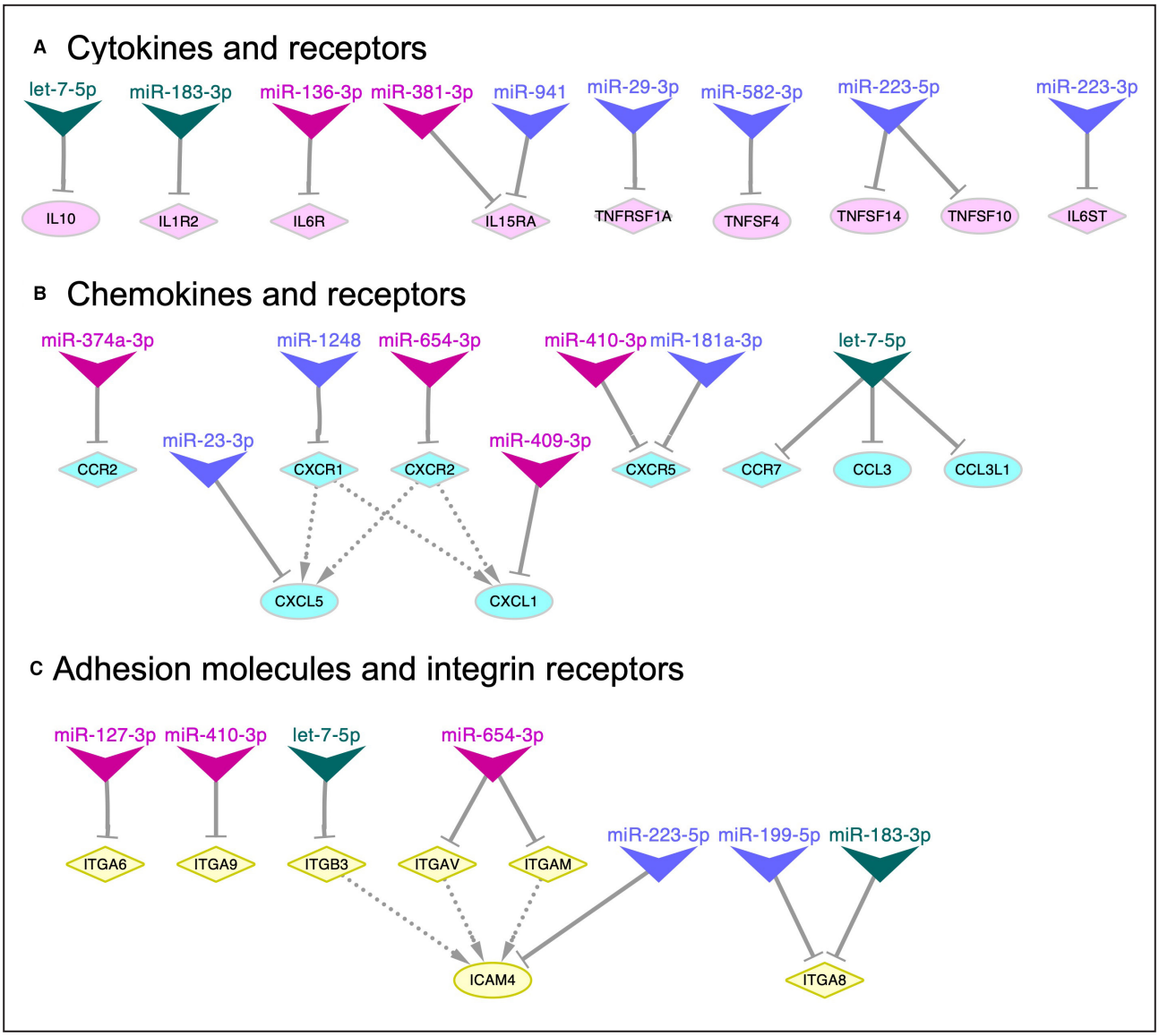


Figure 4. Receptor-ligand networks for the target genes in each cluster.
Analysis of receptor-ligand networks were performed for target genes using the Fantom5 database. The networks of cytokines and receptors (**A**), chemokines and receptors (**B**) and integrin receptors and adhesion molecules (**C**) targeted by the microRNA (miRNA) in each cluster were demonstrated. IL indicates interleukin.

lead to corresponding increased expression of their target genes involved in immune regulation. As shown in Figure 2, members of this cluster are predicted to repress pathways such as leukocyte migration, chemokine signaling, and MAPK signaling. Cytokines that are activated during CPB,[4,19–25] such as IL-6, IL-8, and TNF-α, or their receptors are targeted by members of this cluster of miRNAs.

Cluster 3, miRNAs that are decreased during CPB and rise after CPB, modulate genes related to cytokine signaling. For example, miR-223-3p (a member of cluster 3) has been shown to target IL-6.[26] miR-23a is an miRNA that targets and inhibits proinflammatory cytokine IL-8 via signal transducer and activator of transcription 3 inhibition.[27] The IL-8 receptor, CXCR1, is predicted to be targeted by miR-1248, while another of its receptors, *CXCR2* is targeted by miR-223.[26] miRNA-29-3p[28] and miR-223-5p[29] target *TNFRSF1*, which encodes a key receptor for TNF signaling. Based on these data, we believe that the members of miRNA cluster 3 play important roles in modulating CPB-associated inflammation in circulating leukocytes.

Our study is limited by its small sample size; however, there are clear patterns of specific miRNA differential expression among all patient samples, based on dense temporal mapping and comparisons within each

patient. We also based our analyses on the assumption that miRNAs negatively regulate gene expression, when in reality this relationship is more complex.[6] More research is necessary to validate and isolate specific functions of these miRNAs. The sample size precludes our ability to correlate the miRNA changes to clinical parameters—such as steroid treatment, patient age, cardiac lesion, or clinical outcomes. Some of the strengths of this study are the global miRNA profiling of circulating miRNAs at several time points during and after CPB. It is important to expand our understanding the role that miRNAs play in the context of CPB-induced inflammation to help us further uncover all of the mechanisms in which inflammation is induced and operates. Furthermore, miRNAs may also have the potential to be a noninvasive, nonpharmacologic treatment in moderating this inflammation.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Tables S1–S3
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SUPPLEMENTAL MATERIAL

Table S1. Ligands and receptors as target genes of differentially expressed miRNAs in each cluster

Cluster	miRNA	Receptors	Ligands
Cluster 1	let-7-5p	*TGFBR1, ADRB2, FAS, CCR7, IGF1R, PGRMC1, ITGB3, TGFBR3, FAS*	*EDN1, IL10, S100A8, CCL3L1, FASLG, CCL3, CCL3L3*
	miR-182-5p	*EDNRB, VLDLR*	*HBEGF*
	miR-183-3p	*IL1R2, LEPR, IFNGR1, ASGR2, KLRD1, ITGA8, CD109*	*BMP3, LPL, LYZ*
Cluster 3	miR-199-5p	*DDR1, BCAM, CELSR1, FZD6, ITGA8, ACVR1B, PLXNC1, ACVR1B, ACVR1B*	*LIN7C, HG*
	miR-582-3p	*TFRC, BMPR2, TFRC*	*TNFSF4*
	miR-223-3p	*IL6ST, ACVR2A, SCARB1, SDC2*	*HSP90B1, SORBS1*
	miR-223-5p	*DPP4, CD177, ERAP1, LRP8, DPP4, CD177, ERAP1, LRP8*	*TNFSF14, TIMP3, TNFSF10, OLAH, ICAM4, HSPA1A, EFNB2, FBN1*
	miR-29-3p	*ROBO1, TNFRSF1A, CNR1, F11R, CNR1*	*COL1A1, COL3A1, COL7A1, PDGFC, VEGFA, COL6A3*
	miR-23-3p	*ABCA1, NRXN3*	*PROK2, CXCL5*
	miR-181a-3p	*CD93, EDNRB, CXCR5, VANGL1, F2R*	*COL9A2, APP, ADAM10*
	miR-24-3p	*LMBR1L, SIRPA*	*FASLG, LAMB3*
	miR-1248	*KLRK1, CD81, EPHB6, CD28, CXCR1, ADCY7, LEPR*	*HBEGF, HMGB1, PDGFB, DUSP18*

	miR-941	*ORAI2, IFNAR1, GFRA2, MGRN1, TLR6, IL15RA*	*TIMP2*
Cluster 2	miR-127-3p	*ITGA6*	*DLK1*
	miR-136-3p	*ERAP1, IL6R, NPTN, CANX, NPTN*	*CLCF1*
	miR-144-5p	*TMEM67, F2R, TLR2, TFR2, CD3G*	*RELN*
	miR-27-3p	*ADORA2B, NRP2, CD28, ST14, BMPR2*	*HBEGF, COLQ, SEMA6A*
	miR-381-3p	*IL15RA, LRRC4, SLC18A2*	*PDAP1, PDGFC*
	miR-409-3p	*S1PR1, LRP6, PLXNC1, TGFBR3, MYLK, ACVR2B*	*CXCL1, CALM1, LIN7C, SEMA4F*
	miR-410-3p	*NRXN3, PLXNA2, ITGA9, CXCR5, MCAM, IFNAR2*	*HMGB1, ADM, RGMB*
	miR-411-5p	*TGFBR2*	*CLEC11A*
	miR-654-3p	*CXCR2, TFRC, DYSF, OLR1, ITGAM, ITGAV, SLC37A1, KCNJ15, KLRC1, CNR1, KLRC2, PLXNA4*	*MST1, FLT3LG, APP*
	miR-374a-3p	*KLRG1, LGR4, AMFR, CCR2*	*VEGFB, CALM3, LTBP3, EDIL3, B2M, LPL*

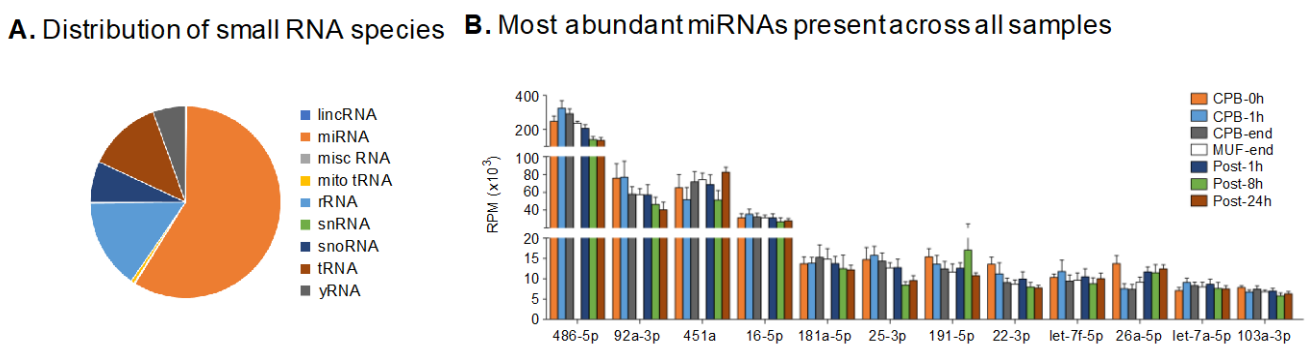
Table S2. The number of miRNA gene targets used in sub-analyses

miRNA	Cluster	Target Genes (Targetscan)	Target Genes with |Context Score|>0	Target Genes Present in mRNA Analysis*	Target Genes significantly DE*	Genes used for KEGG Pathway Enrichment
miR-183-3p	C1	2599	1722	1128	194	200
let-7-5p		332	332	237	41	200
miR-182-5p		172	172	119	23	119
miR-654-3p	C2	2523	1921	1264	232	200
miR-27-3p		1092	1055	751	135	200
miR-136-3p		1155	1137	726	128	200
miR-374a-3p		1355	737	487	73	200
miR-144-5p		938	715	485	79	200
miR-410-3p		604	558	395	68	200
miR-409-3p		316	286	199	21	199
miR-381-3p		128	128	93	13	93
miR-411-5p.1		123	118	89	7	89
miR-127-3p		25	25	17	1	17
miR-1248	C3	2843	2013	1347	223	200
miR-582-3p		1622	1622	1101	174	200
miR-29-3p		755	755	562	112	200
miR-23-3p		713	713	517	87	200
miR-199-5p		634	610	462	64	200
miR-223-5p		994	662	432	83	200
miR-223-3p		415	405	329	60	200
miR-941		387	362	222	41	200
miR-181a-3p		322	322	201	49	200
miR-24-3p		119	119	81	14	81

Table S3. Plasma and serum miRNAs reported in the literature as biomarkers for complications in CPB surgery

miRNA	Age	Elevation time point	Associated complications	Reference
miR-208a/b miR-499	Pediatric	6-24h post CPB	Myocardial injury Length of hospital stay	[30,31]
miR-223	Adult	2-6h after start of CPB	Inflammation	[32]
miR-21	Pediatric	6-24h post CPB	Acute kidney injury	[33]
miR-1	Adult	1-24h post CPB	Myocardial injury	[34]
miR-210 miR-16	Adult	CPB & 4h post CPB	Myocardial injury	[35]
miR-320 miR-200c miR-205	Adult	8-16h post CPB	Acute lung injury	[36]
miR-133a miR-499	Adult	1-6h post CPB	Myocardial injury	[37]

Figure S1. miRNA sequencing analysis of total nucleated cells from pediatric patients undergoing CPB



(A) Pie chart showing the mean distribution of different small RNA species in all sequenced samples. **(B)** Normalized expression in Reads per millions (RPM) of the most abundant miRNAs detected in patient samples at different time points.