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MicroRNA dysregulation in the heart and lung of infants with bronchopulmonary dysplasia

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Abstract

Background and Objectives: Bronchopulmonary dysplasia (BPD) is a serious complication of preterm birth, resulting in significant morbidity and mortality. Recent studies have suggested that microRNA (miRNA) dysregulation is involved in the pathogenesis of BPD and may serve as biomarkers for early detection. We conducted a directed search for dysregulated miRNAs in lung and heart autopsy samples of infants with histologic BPD.

Methods: We used archived lung and heart samples from BPD (13 lung, 6 heart) and control (24 lung, 5 heart) subjects. To measure miRNA expression, RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue specimens then reverse-transcribed, labeled, and hybridized to miRNA microarrays. The microarrays were scanned, and data were quantile normalized. Statistical analysis with a moderated t-test and control of the false discovery rate (5%) was used to compare normalized miRNA expression values between clinical categories.

Results: With our set of 48 samples, 43 miRNAs had a significant difference in expression comparing BPD to non-BPD controls. Among the most statistically significant miRNAs, miR-378b, miRNA-184, miRNA-3667-5p, miRNA-3976, miRNA-4646-5p, and miRNA-7846-3p were all consistently upregulated in both the heart and lung tissues of BPD subjects. The cellular pathway predicted to be most affected by these miRNAs is the Hippo signaling pathway.

Conclusions: This study identifies miRNAs that are similarly dysregulated in postmortem lung and heart samples in subjects with histologic BPD. These miRNAs may contribute to the pathogenesis of BPD, have potential as biomarkers, and may provide insight to novel approaches for diagnosis and treatment.

KEYWORDS

autopsy, BPD, bronchopulmonary dysplasia, extremely preterm, microRNA, miR, miRNA, neonate, neonatology, postmortem, preterm, pulmonology

Sara Koussa and Alan Dombkowski contributed equally as co-first authors.

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1 | INTRODUCTION

Bronchopulmonary dysplasia (BPD), defined as repetitive injury to developing lungs of the newborn and a subsequent aberrant reparative response, is the most common complication of extreme preterm birth.[1] In an attempt to mitigate irreversible lung damage, alveogenesis, and vasculogenesis are arrested in the developing lung, leading to fewer, larger alveoli.[2,3] Hyperoxia and barotrauma associated with neonatal mechanical ventilation have been linked to long-lasting complications that persist in the adult lung, such as pulmonary or systemic hypertension and pulmonary vascular disease.[1,4–6] While the commonly accepted clinical definition of moderate-to-severe BPD requires supplemental oxygen for a minimum of 28 days and respiratory support at 36 weeks postmenstrual age (PMA), this definition has been challenged.[1,2,4,7–10] In addition, histological evidence of BPD has been detected in postmortem lung samples of infants as early as 6 days of life,[11] suggesting that the inception of BPD begins earlier than the clinical definition allows. Because of the imminent risk of irreversibly damaging the infant lung in the first few weeks of life, there is a need to understand the molecular underpinnings of BPD to identify biomarkers that may screen premature infants for BPD before irreversible damage occurs.

While there is already a myriad of molecular biomarkers including various cytokines and brain natriuretic peptide that have been associated with BPD, current literature fails to identify one single biomarker or combination of biomarkers that has a good predictive value for the disease.[5,12–15] Some studies estimate that the ideal point to determine risk for BPD is when an infant is 7 days old, as the lung will still have healing capability before fibrotic stages of the disease evolve.[16,17] Because of the clinical treatment window being so narrow for this disease, assessing risk factors for BPD and initiating therapy within the first 2 weeks of life is crucial. The multifactorial and complex etiology of BPD leads to an impartial preference for molecular biomarkers, as they may be more reliable than clinical phenotypes for predictive accuracy.[12,13]

Among some of the most promising molecular biomarkers, microRNAs (miRNAs/miRs) have recently been utilized to investigate disease pathogenesis in pulmonary diseases such as BPD. miRNAs are highly stable noncoding RNAs that posttranscriptionally regulate gene expression.[18–20] miRNAs are involved in the pathogenesis of most diseases and offer exceptional potential as biomarkers for early detection and treatment.[6,18,20–22] There are several miRNAs that have been proven to be dysregulated in different pulmonary diseases,[19,20,23–25] and recent studies have suggested that miRNA dysregulation is also intimately involved in the pathogenesis of BPD in preterm infants.[20,26,27]

While previous studies have implicated miRNA dysregulation in the etiology and pathogenesis of BPD, a shortcoming of current literature is in identifying if there is a consistent dysregulation in miRNA(s) across multiple tissue types. The development of reproducible detection methods of miRNAs is of paramount importance to utilize them as diagnostic, predictive, and prognostic biomarkers.[28] Identifying consistencies in dysregulation across tissue types will not only allow for a more holistic insight into the miRNA's mechanism, but it may also enhance the detection reproducibility and, therefore, the clinical application as potential biomarkers. By conducting a directed analysis to identify similar expression patterns and alterations, our study attempts to identify reliable and clinically relevant biomarkers for BPD, as well as to extrapolate useful information about the pathogenic mechanism of BPD. The objective of the current study is to investigate miRNA expression levels in postmortem lung and heart samples in a cohort of infants previously reported by our group to determine if there is consistent dysregulation in miRNA expression in subjects with histologic BPD compared to those without histologic BPD across multiple tissue types.[11]

2 | METHODS

This study included all live-born preterm infants born <37 weeks gestational age (GA) who received an autopsy between 2010 and 2017 at Children's Hospital of Michigan or Hutzel Women's Hospital previously reported by our group.[11] The study was approved by the Institutional Review Board. Of 43 subjects identified by review of autopsy records, one infant was excluded because the autopsy specimens could not be located, leaving 42 infants as subjects of this study. Infants with major congenital defects were excluded, including congenital cardiac defects other than atrial septal defect, ventricular septal defect, and patent ductus arteriosus. Of 42 deceased infants with available autopsy specimens, 33 (79%) were <32 weeks GA at birth.[11] Of these 33 infants, lung and/or heart tissue curls for miRNA analysis were available for 19 subjects. Autopsy specimens of lung and heart tissue were examined by a single pathologist, and classified according to presence or absence of defining features of BPD.

The features used to classify histological BPD were (1) the presence of alveolar simplification, defined as enlargement and consolidation of alveolar spaces which become rounded or elongate and lack septation,[29] (2) low radial alveolar count, defined as the number of alveoli transected by a perpendicular line drawn from the center of a respiratory bronchiole to the next septal division,[30] (3) type 2 pneumocyte hyperplasia, defined as a relative increase from baseline in the number of type 2 pneumocytes lining the alveolar epithelium,[31] and (4) alveolar fibrosis, defined as thickening and stiffening of the alveolar septae.[32] These features were subjectively defined as either present or absent in our samples by a single experienced pathologist based on the above-defined criteria.

2.1 | RNA isolation from formalin-fixed, paraffin-embedded (FFPE) samples

Total RNA was extracted from FFPE tissue sections of heart, right and left lung samples from BPD (7 right lung, 6 left lung, and 6 heart) and control (12 right lung, 12 left lung, and 5 heart) subjects using deparaffinization solution and the miRNeasy FFPE Kit (Qiagen). Two freshly cut 10 μm sections of each FFPE tissue were placed in

1.5 mL tube with 160 µL deparaffinization solution and incubated at 56°C for 3 min. Samples were then cooled to room temperature, and lysis was performed by adding 150 µL of PKD buffer and 10 µL of proteinase K, incubation at 56°C for 15 min, then heat treatment at 80°C to inactivate proteinase K. The lower uncolored phase of each sample was transferred into a new tube, then incubated on ice for 3 min. and centrifuged at 20,000*g* for 15 min. Supernatant was transferred into a new tube and 16 µL of DNase Booster Buffer was added with 10 µL of DNase I stock solution (15 min at RT). Then 320 µL of RBC buffer was added and mixed with 720 µL of 100% ethanol. Samples were loaded on RNeasy MinElute columns, and during centrifugation (9000*g* for 30 s) total RNA was bound to the membrane of the columns. Two rounds of washing the columns with 500 µL of RPE buffer were completed with drying columns and an additional full-speed centrifugation. Total RNA was eluted from the column with 25 µL of RNase-free water. Concentrations of RNA were measured with NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific).

2.2 | miRNA microarrays

miRNA expression in each RNA sample was measured using Agilent G3 Human v21 8X60K miRNA arrays as described previously.[33]

2.3 | Microarray data analysis

Microarray data were quantile normalized and statistical analysis was performed in GeneSpring 14.9.1. Samples were categorized by tissue and as either BPD or non-BPD samples. Identification of statistically significant miRNAs was performed using a moderated t-test and the Storey with bootstrapping multiple-test correction to control the false discovery rate (*q* value). The false discovery rate (FDR) cutoff was set at q = 0.05 (5%) along with a minimum fold change of 1.25. Principal component analysis (PCA) was performed in GeneSpring.

2.4 | miRNA target prediction

Predicted miRNA/target gene interactions were identified using the microRNA data integration portal (mirDIP)[34] and a filter to select those in the top 1% ("very high") of all predictions. Functional analysis of cellular pathways associated with predicted target genes was performed using DAVID (Database for Annotation, Visualization, and Integrated Discovery) and KEGG pathways.[35,36]

3 | RESULTS

All infants included in this study were <32 weeks GA at birth and majority were of African American race. The demographic, clinical and histological characteristics for the infants included in this study are summarized in Table 1. Premature prolonged rupture of membranes was more likely to be observed in the obstetric history of mothers of infants with histologic BPD compared to those without. Compared to infants without histologic BPD, infants with histologic BPD were significantly more likely to be of lower GA at birth, have a clinical diagnosis of BPD, and receive an echocardiogram in the neonatal intensive care unit. None of the infants without histologic BPD had a clinical diagnosis of BPD, whereas, 3 (43%) of infants with histologic BPD had a clinical diagnosis of BPD; all three infants had severe BPD as defined in the NICHD consensus statement.[2] None of the infants in either group were diagnosed clinically with pulmonary hypertension; however, histologic pulmonary hypertension was observed in 7 (58%) of infants without histologic BPD and 5 (71%) of infants with histologic BPD ($p = 0.656$). The mean ± SD age at death in infants with histologic BPD was 31 ± 23 days as compared to 10 ± 8 days in infants without histologic BPD ($p < 0.008$). The corrected GA at death was not statistically significant between the two groups (Table 1).

3.1 | Identification of differentially expressed miRNAs

For potential biomarkers we sought to identify miRNAs having consistent changes in expression in both heart and lung of BPD subjects. Statistical analysis was performed to compare miRNA expression values between BPD and non-BPD groups. A multiple-test correction was applied to limit the false discovery rate (*q* value) to 5%, and a minimum fold change of 1.25 was specified. This resulted in 43 significant miRNAs (Supporting Information: Table 1). The top six miRNAs having the greatest statistical significance were each upregulated in BPD subjects (Figure 1) and consistently altered in both lung and heart (Table 2).

3.2 | Identification of predicted target genes

We investigated potential functional effects of these miRNAs using computational prediction of miRNA target genes. To ensure robustness, we used mirDIP, a miRNA target prediction tool that integrates multiple prediction algorithms.[34] For each of the top six miRNAs, we identified predicted target genes having a score in the top 1% of all predicted interactions. The cumulative prediction for the six miRNAs was 1065 miRNA/target interactions, representing 1009 unique genes. This list of target genes was analyzed for enrichment of signaling pathways using DAVID[36] and KEGG pathways.[35] The signaling pathway having the greatest statistical significance was the Hippo pathway ($p = 2.0e^{-6}$, $FDR = 6.1e^{-4}$), with 25 predicted target genes (Supporting Information: Table 2), representing a 3-fold increase over what is expected by chance. Figure 2 shows the 25 target genes in the Hippo pathway. Recent work has strongly implicated the Hippo pathway in lung diseases, including BPD.[37–40]

TABLE 1 Perinatal, clinical, and pulmonary histological characteristics for deceased infants with miRNA analysis.

	Histological BPD		
	No	Yes	*p* Value
N (%)	12 (63)	7 (37)	
Birth weight (g) (mean ± SD)	903 ± 348	684 ± 161	0.138
GA (weeks) (mean ± SD)	27.0 ± 2.3	24.8 ± 1.1	0.028
African American race	10 (83)	5 (71)	0.603
Male gender	7 (58)	4 (57)	1.000
SGA	4 (33)	2 (29)	0.568
Multiple gestation	2 (17)	2 (29)	0.603
PPROM	0 (0)	3 (43)	0.036
ANCS—Any	7 (58)	7 (100)	1.000
ANCS—two doses	2 (17)	5 (71)	0.680
Cesarean section	9 (75)	6 (86)	1.000
Delivery Room intubation	10 (83)	6 (86)	1.000
High-frequency ventilation	10 (83)	5 (71)	0.603
Surfactant	11 (92)	7 (100)	1.000
Inhaled nitric oxide	1 (8)	0 (0)	1.000
Pulmonary hemorrhage	3 (25)	2 (33)	1.000
Xanthines	4 (33)	4 (67)	0.321
Steroids	9 (75)	2 (29)	0.074
Vasopressors	12 (100)	7 (100)	
BPD—clinical diagnosis	0 (0)	3 (43)	0.036
BPD—clinical diagnosis—severity			
No BPD	12 (100)	4 (57)	0.036
Severe BPD	0 (0)	3 (43)	
BPD—histologic	5 (25)	12 (92)	0.000
Histologic BPD type			
Old	0 (0)	3 (43)	<0.001
New	0 (0)	3 (43)	
Both	0 (0)	1 (14)	
Echocardiogram obtained	4 (33)	7 (100)	0.013
Pulmonary hypertension—clinical diagnosis	0 (0)	0 (0)	
Pulmonary hypertension—histologic	7 (58)	5 (71)	0.656
Age in days at death (mean ± SD)	10 ± 8	31 ± 23	0.008
Corrected GA at death (mean ± SD)	28.4 ± 2.8	29.3 ± 3.2	0.551

Note: Numbers represent N (%) or (mean ± SD).

Abbreviations: ANCS, antenatal corticosteroids; BPD, bronchopulmonary dysplasia; GA, gestational age; PPROM, preterm premature rupture of membranes; SD, standard deviation; SGA, small for gestational age.

We used PCA to investigate if the six miRNAs can distinguish BPD specimens from non-BPD. PCA is a dimensional reduction technique that allowed us to plot the 48 samples in a three-dimensional space having axes derived from linear combinations of the expression levels of the six miRNAs.[41] The results show that the samples in each clinical category (BPD and non-BPD) are clustered and there is clear distinction between the two groups (Figure 3).

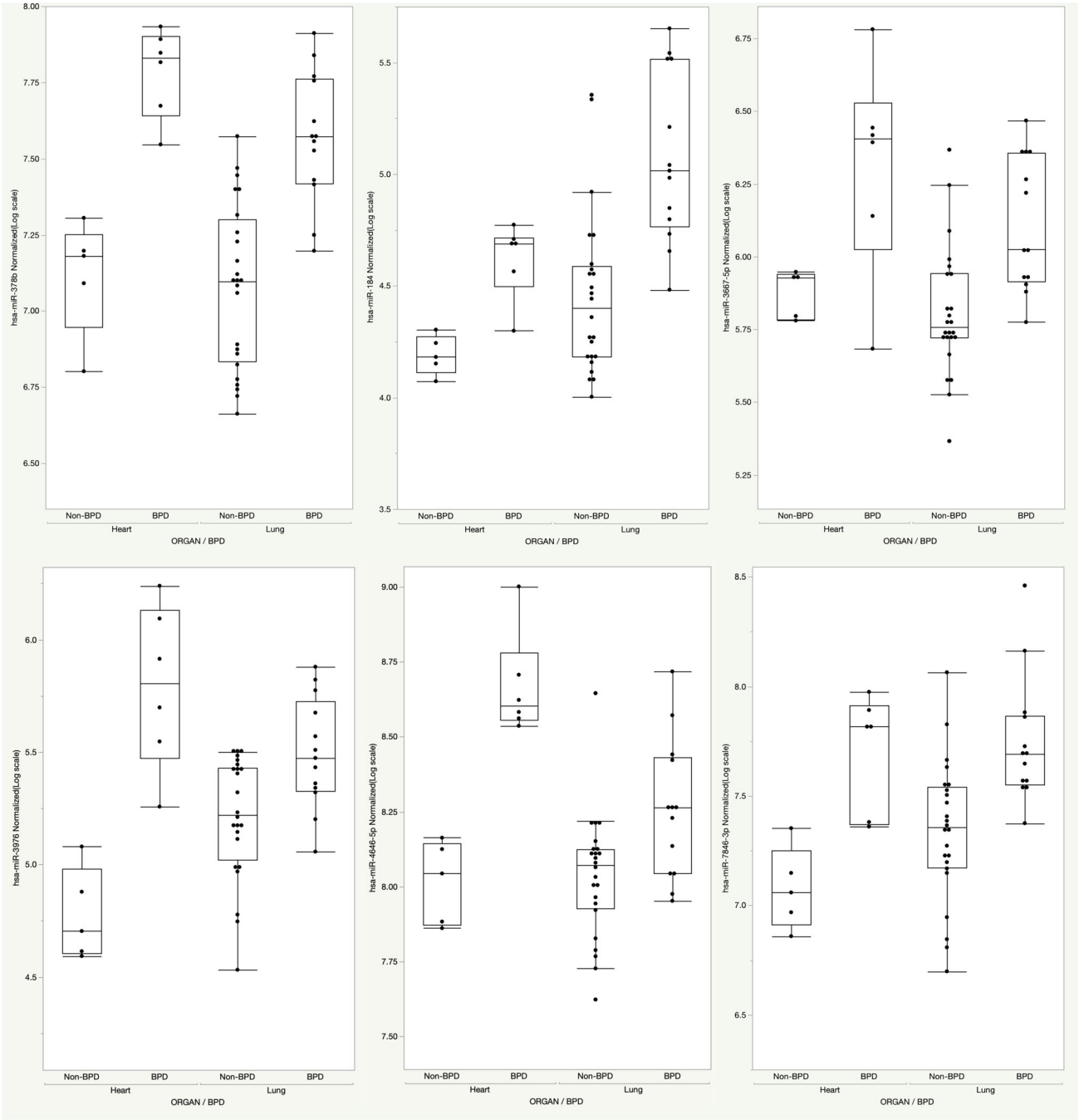

FIGURE 1 The top six microRNAs having the greatest statistical significance were each upregulated in the heart and lung tissue of bronchopulmonary dysplasia (BPD) subjects.

4 | DISCUSSION

Research and clinical advancements in the field of neonatology have eliminated a wide variety of complications previously common in premature infants. However, BPD maintains a relatively high incidence in preterm newborns, with the global incidence being between 17% and 75% among infants requiring supplemental oxygen at 36 weeks PMA.[42] The early onset of long-term complications associated with BPD indicates a critical need for timely biomarkers that may accurately indicate predisposal, as well as prognosis as the disease progresses. MiRNA analysis has proven to be useful in identifying biomarkers in the pathogenesis of many respiratory diseases including BPD, but the literature falls short in proving consistency in miRNA dysregulation patterns as related to the disease.[20,26,27] Identifying consistencies in dysregulation of a miRNA across tissue types allows for reproducibility and precision in detection, as well as an opportunity to delineate more subtle nuances in the pathogenesis of BPD. Our study demonstrates statistically

TABLE 2 Cause of death in infants with and without histologic BPD.

	Histological BPD	
	No	Yes
1	Cardiorespiratory failure, hypotension, severe metabolic acidosis	Acute renal failure, systemic candida infection
2	Extreme prematurity	Multiorgan system failure
3	Hypoxic respiratory failure, metabolic acidosis, pulmonary hemorrhage	Reaccumulation of pneumothorax
4	Large PDA, severe metabolic acidosis, septic shock, hypotension	Severe IVH, meningitis, ARF, metabolic acidosis, septic shock, DIC, hypotension
5	Meningitis	Significant metabolic acidosis. high vent support, desaturations
6	Pneumothorax (right), pulmonary hemorrhage	VLBW, PDA ASD, suspected NEC, hypoxic respiratory failure
7	Severe capillary leak syndrome, progressive multiorgan failure	
8	Severe prematurity	

Note: Cause of death not listed in autopsy record for four infants without histologic BPD and one infant with histologic BPD.

Abbreviations: ARF, acute renal failure; ASD, atrial septal defect; BPD, bronchopulmonary dysplasia; DIC, disseminated intravascular coagulation; IVH, intraventricular hemorrhage; NEC, necrotizing enterocolitis; PDA, patent ductus arteriosus; VLWB, very low birth weight.

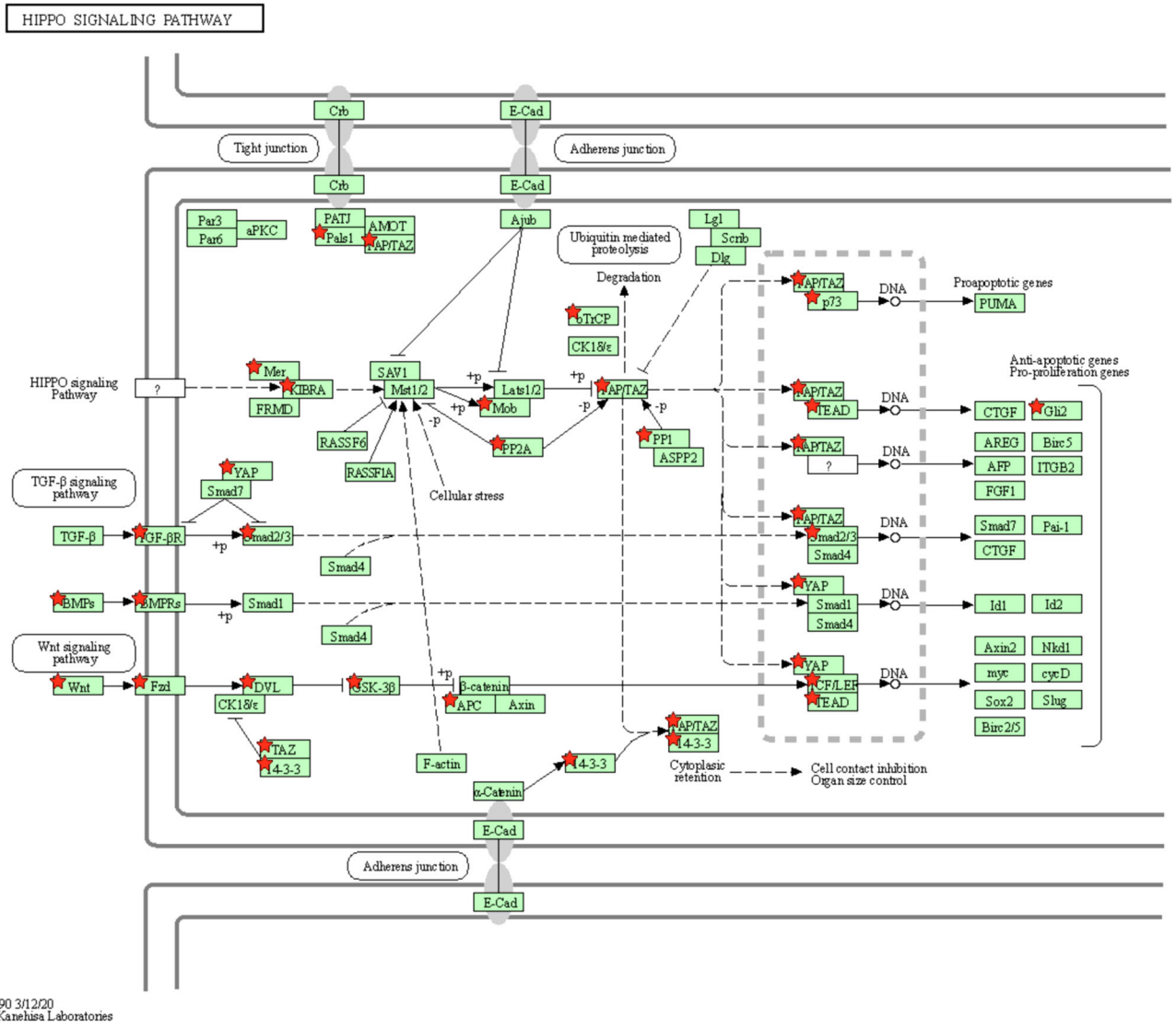

FIGURE 2 Predicted target genes of the top six overexpressed microRNAs in bronchopulmonary dysplasia (BPD) specimens were analyzed for overrepresentation in cellular pathways. The most significant pathway targeted was the Hippo signaling pathway (P = 2.0e-6, FDR = 6.1e-4). Target genes are shown with a red star and are listed in Supporting Information: Table 2.

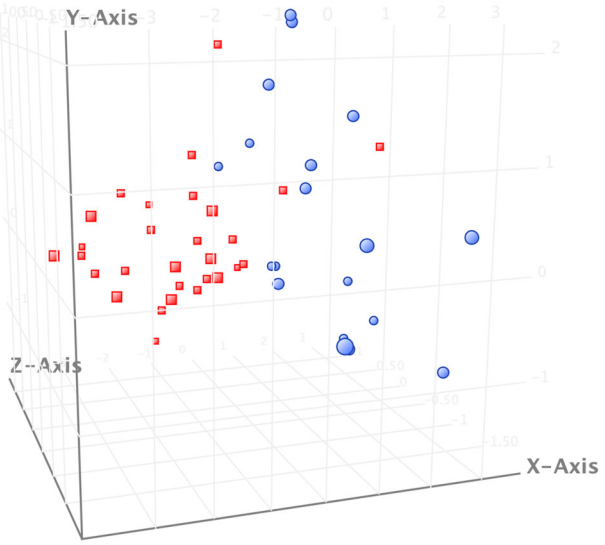

FIGURE 3 Principal component analysis (PCA) was used to investigate clustering of the samples with respect to microRNA expression levels. Each axis represents a linear combination of the expression levels of the top six microRNAs in Supporting Information: Table 1. Bronchopulmonary dysplasia (BPD) specimens are represented by blue circles, non-BPD specimens are represented by red squares.

significant upregulation of six miRNAs when comparing heart and lung samples from BPD versus control subjects: miRNA-378b, miRNA-184, miRNA-3667-5p, miRNA-3976, miRNA-4646-5p, and miRNA-7846-3p. These trends are consistent across both heart and lung tissues, demonstrating that these miRNAs may be excellent candidates as biomarkers for early, reproducible detection in a clinical setting.

The comprehensive mechanistic role of these miRNAs in BPD is not clear, but the literature offers some insight. Expression of miR-378b in human dermal fibroblasts has been shown to target SIRT6, which represses the expression levels of α-1-type 1 collagen (COL1A1), the gene that encodes the alpha1 chains of Type 1 collagen.[22] Dysregulation in collagen deposition and elastin fiber organization are known hallmarks in the pathogenesis of BPD.[43,44] Dermal fibroblasts essentially differ from pulmonary fibroblasts in that they have different responses to growth factors; in relation to Type III collagen formation, dermal fibroblasts were shown to respond to PDGF stimulation, whereas pulmonary fibroblasts respond to transforming growth factor beta (TGF-β) stimulation.[45] Previous studies have demonstrated that BPD-associated hyperoxia potentiates TGF-β signaling, which subsequently induces COL1A1 and leads to increased collagen deposition in developing septa and blunted alveolar development.[43,44] Another gene that has been associated with miR-378b is the COL6A2 gene, which encodes for a part of the Type VI collagen chain. This gene has been implicated in stabilizing the elongating airway branches in lung development as well as contributing to extracellular matrix (ECM) organization; dysregulation of this gene has been associated with chronic lung diseases such as BPD.[46] In light of these findings, we postulate that the elevation of miR-378b in BPD tissues may indicate a compensatory mechanism of the infant lung tissue to repress collagen deposition. This deposition is normally induced by factors such as SIRT6, and repression may prevent development or worsening of BPD progression. MiR-378b may therefore play a role in controlling pulmonary ECM collagen deposition as it is related to BPD, further elucidating details of the disease pathogenesis.

MiR-378b has also been implicated in prohibiting the proliferation, migration, and differentiation of keratinocytes.[47] Keratinocyte growth factor (KGF) has been shown to stimulate proliferation of keratinocytes and is often upregulated in chronically injured tissue, functioning to prevent epithelial injury and enhance repair.[48] A high concentration of KGF has been demonstrated to predict absence of BPD in premature neonates.[48,49] By prohibiting the differentiation and proliferation of keratinocytes, miR-378b may be further implicated in the pathogenesis of BPD. Finally, a related miRNA within the same family, miR-378, has been isolated in the tracheal aspirate of severe BPD infants, and has been shown to participate in the oxidative stress response in a variety of tissues as well as found in invasive lung cancer cell lines.[50]

Li et al. demonstrated that miR-184 may inhibit TGF-β1-induced proliferation and epithelial-mesenchymal transition (EMT) in pulmonary fibrosis.[51] Promotion of EMT in alveolar epithelial type II cells is known to alter normal alveolar development processes, thus contributing to BPD phenotypes.[50] Madden et al. also demonstrates increased and localized miR-184 expression in the lungs of mice with induced pulmonary fibrosis.[52] Taken together, this may suggest that a reparative process was active in the lungs of infants with BPD in our study. The upregulation of miR-184 could be a compensatory reaction in an attempt to inhibit the EMT process and therefore suppress further progression of BPD. Other pulmonary pathologies have also been implicated with miR-184; expression level has been demonstrated as significantly increased in nonsmall cell lung cancer (NSCLC) patients in a dose-dependent manner, suggesting a direct effect on the prognosis of patients.[53] In line with our hypothesis and much of the available literature, both miR-378b and miR-184 may play a significant role in the pathogenesis of BPD.

The remaining four miRNAs found significantly overexpressed in the lung and heart tissue of severe BPD infants in our study were miR-4646-5p, miR-3667-5p, miR-3976, and miR-7846-3p. To the best of our knowledge, these miRNAs have not yet been implicated in BPD or lung diseases, although several of them have been found to impact disease progression in other organs.[54–56] While the impact of upregulation of these miRNAs is unclear, our study identifies them as possible mediators in the pathogenesis of BPD. Future studies are necessary to determine if these miRNAs play a role in aberrant lung development.

For each of the top six miRNAs described above, we also aimed to identify predicted target genes that may further our understanding of the pathogenesis of BPD in preterm infants. We analyzed cellular signaling pathways associated with predicted target genes. The Hippo signaling pathway had the greatest statistical significance, with

25 of the predicted target genes being associated with this pathway. This pathway is highly evolutionarily conserved, and plays an important role in regulating major developmental processes such as cell proliferation, determination of organ size, tissue development, and regeneration.[37] Recent research has provided valuable insight into the role of the Hippo pathway in a number of lung diseases, as well as in cardiac development and disease. Upon activation of the Hippo signaling pathway cascade effector Yes-associated protein (YAP), pulmonary arterial vascular smooth muscle cells have been shown to proliferate, leading to pulmonary vascular remodeling and subsequent development of pulmonary arterial hypertension.[37,57] Loss of YAP as also been demonstrated to encourage expression of inflammatory genes and the accumulation of pulmonary inflammatory cells, which is involved in most barotrauma—and infection-associated lung pathologies.[37,58] In addition, the Hippo signaling pathway has been implicated in promoting the differentiation and proliferation of lung epithelial progenitor cells as well as intervening in the repair of pulmonary capillary endothelium, which could lead to a number of adverse lung sequelae.[37,59] The Hippo pathway is an essential regulator of prenatal and postnatal cardiac development, as well as in cardiac stress response.[60,61] Substantial evidence shows that the Hippo pathway plays an important role in cardiomyocyte proliferation, myocardial infarction, coronary heart disease, and cardiomyopathies.[62] Recent studies have demonstrated that miRNAs are key regulators of the Hippo pathway in heart tissue.[63,64] Therefore, the miRNA dysregulation that we observed in both heart and lung samples with evidence of BPD may reflect the importance of these miRNAs in regulating the Hippo pathway in both organs.

The literature also strongly implicates the Hippo pathway in the pathogenesis of BPD specifically.[38] Several genes of the dysregulated Hippo pathway were shown to be predictive of BPD with a moderately high degree of accuracy. YAP has also been demonstrated to promote nuclear β-catenin expression, leading to improvement in the self-renewal of type II alveolar epithelial cells, as well as their differentiation into type I alveolar epithelial cells in the setting of lung injury.[39] Additionally, decreased YAP activity has been associated with alveolar simplification, which is a known intrinsic pathogenic process in the development of BPD.[40] Activation of YAP lead to partial reversal of alveolar simplification, regenerating the gas-exchange surface area necessary for proper gas transfusion. The Hippo pathway is also known to be regulated in part through mechanical stress and has downstream effects on cell proliferation.[65] Our results indicate that miRNA dysregulation in both heart and lung tissue targeting the Hippo pathway may be feedback response to disease progression, particularly in diseases such as BPD that involve significant pressure differentials and changes in mechanical plasticity of both organs.[66] While the exact role of the Hippo cellular signaling pathway in BPD is yet to be fully understood, our research adds to the consensus of literature establishing a strong relationship between the Hippo pathway and BPD pathogenesis.

Interestingly, the observed changes in expression for the top six miRNAs are generally more robust in heart than in lung. The underlying cause is unclear; however, a study of tissue-specific miRNA expression during mouse development provides additional insight. The study reported that of all organs studied, the heart and brain had the highest number of detected miRNAs, and expression in these tissues was markedly dynamic during development.[67] Thus, the larger changes in miRNA expression that we observed in heart compared to lung may reflect a tissue-dependent propensity for dynamic expression during development that may also be triggered by pathogenic events. There are several theories that may explain the consistent dysregulation of miRNAs in lung and heart samples that we have demonstrated. One notable theory is the vascular hypothesis, which asserts that the alteration in pulmonary vascular biomechanics in BPD may lead to increased arterial wall stiffness, which then negatively impacts the normal pulmonary alveolarization process.[68] This interplay between vascular physics and pulmonary development may lend insight to the consistency in dysregulation of the miRNAs in the heart and lung and supports our study's approach of using multiple tissue types to identify miRNA alterations that may serve as biomarkers.

Limitations of our study have been previously described and include its observational nature with no control over the interventions given to the subjects.[11] Despite its observational nature, infants in the two groups were similar in GA at birth and corrected GA at the time of death because of the well-defined inclusion criteria. It is likely that infants that were not as severely affected survived, and therefore were not included in this autopsy series. This study is also limited by the small, declining number of infants receiving autopsies[69] as well as by the number of parents that consented for their infant's autopsy results to be used for research purposes. Another limitation of the study is the inability to address the unique miRNA dysregulation specific to pulmonary hypertension, an important co-morbidity in preterm infants with BPD, because of the small sample size and the co-occurrence of histologic pulmonary hypertension in more than half the subjects in infants with and without histologic BPD.

Our study was strengthened by very stringent statistical parameters when analyzing significant miRNAs in our samples. We used a stringent multiple-test correction to control the false discovery rate so that fewer than 5% of the identified miRNAs are expected to be false positives. Furthermore, we only analyzed miRNAs with consistency in dysregulation across multiple tissue types, providing additional confidence. Regarding the sample tissue quality, miRNA profiling in FFPE tissue has been demonstrated to be a reliable means for molecular characterization.[70,71] While miRNA stability in postmortem tissues versus live, fresh frozen tissues has not been investigated explicitly in BPD, numerous studies have utilized miRNAs collected from postmortem samples and proven their stability in FFPE cardiac and brain tissues at both early and late postmortem intervals.[72–74] MiRNA expression analysis in FFPE tissue is well correlated to matched fresh frozen tissues in other tissues and diseases, so we may infer that FFPE is a reliable method of tissue preparation in BPD tissues.[71,75]

5 | CONCLUSIONS

This study identifies miRNAs that are similarly dysregulated in archived postmortem lung and heart samples in subjects with histologic BPD. Our results indicate a consistent upregulation in miR-378b, miR-184, miR-3667-5p, miR-3976, miR-4646-5p, and miR-7846-3p across both tissue types. Our results further reveal potential mechanistic details of the pathogenesis of BPD, as miR-378b has been implicated in collagen and keratinocyte regulation, miR-184 has several associations with lung pathologies, and all significant miRNAs were determined to have downstream genes associated with the Hippo cell signaling pathway. These miRNAs may contribute to the pathogenesis of BPD, have potential as biomarkers, and may provide insight to novel approaches for treatment. Future research should aim to identify dysregulation patterns of these miRNAs using plasma, urine, or other noninvasive assays of BPD infants to confirm the results of our study.

AUTHOR CONTRIBUTIONS

Sara Koussa: Writing—review and editing; writing—original draft; data curation; investigation; formal analysis. **Alan Dombkowski**: Conceptualization; writing—review and editing; data curation; investigation; software; formal analysis. **Daniela Cukovic**: Writing—review and editing; investigation; methodology; data curation. **Janet Poulik**: Writing—review and editing; conceptualization; formal analysis; data curation. **Beena G. Sood**: Conceptualization; writing—review and editing; supervision; project administration; funding acquisition; formal analysis; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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